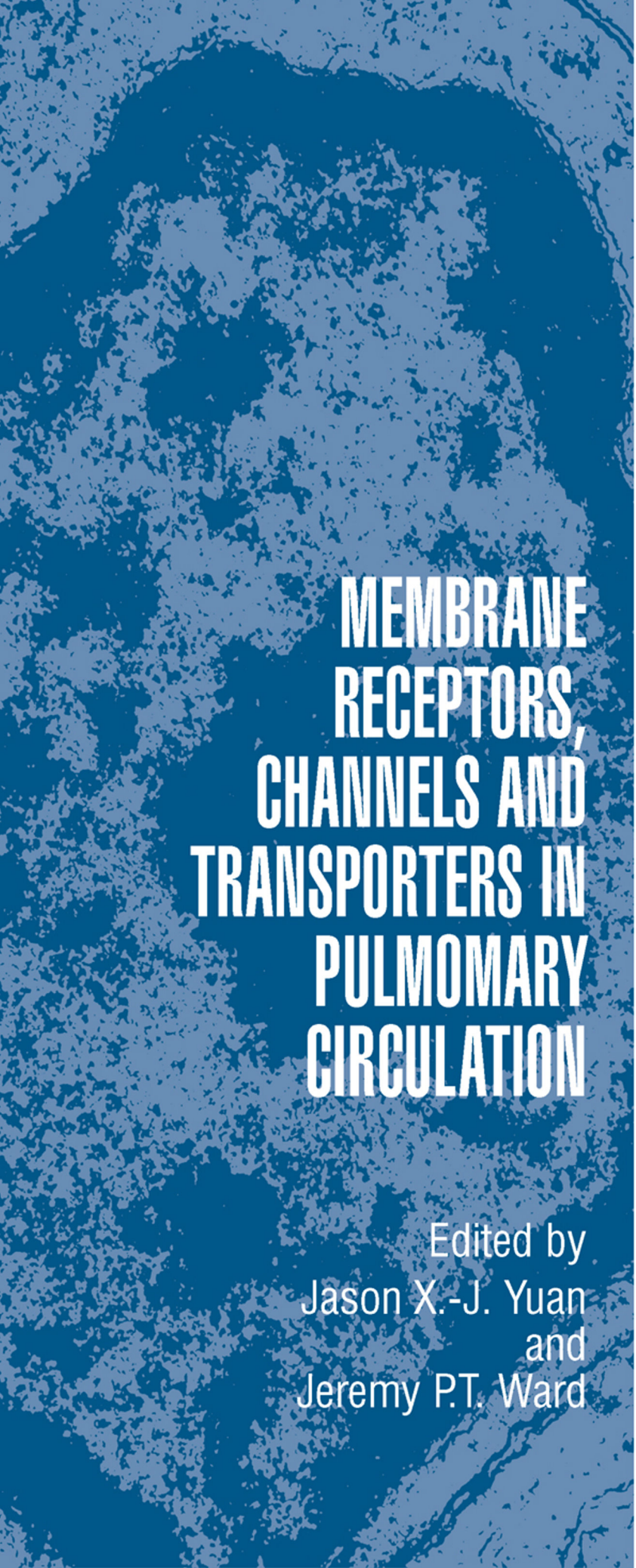

ADVANCES IN EXPERIMENTAL MEDICINE AND BIOLOGY

Volume 661

MEMBRANE RECEPTORS, CHANNELS AND TRANSPORTERS IN PULMOMARY CIRCULATION

Edited by
Jason X.-J. Yuan
and
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Editors

Membrane Receptors, Channels and Transporters in Pulmonary Circulation

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Preface

Receptors, channels, and transporters play a critical role in vascular signal transduction and are key elements in the pathogenesis of pulmonary vascular disease. They provide front-line mechanisms for regulation of normal smooth muscle, for endothelial and inflammatory cellular homeostasis, and for responding to the extracellular environment and external mediators. Pathophysiological perturbations in their function and expression are associated with profound alterations in cellular function and make significant contributions to the development and progression of disease. As they are in the main situated in the plasma membrane and their molecular nature is often conducive to modulation of function by relatively highly specific agents, receptors, channels, and transporters are potentially key targets for novel therapeutics. Indeed, a high proportion of currently available therapeutic agents function as channel or transporter modulators or receptor antagonists and agonists.

Over the last few years, there have been significant advances in our understanding concerning the expression and function of novel channels, receptors, and transporters in the pulmonary circulation. Since the last Grover conference on the role of ion flux in pulmonary vascular control in 1992, several entirely new molecular classes of ion channels (e.g., transient receptor potential [TRP] channels and two-pore domain K^+ channels) have been identified and have more recently been shown to play key roles in pulmonary vascular function and the development of pulmonary arterial hypertension. In addition, the advent of enhanced molecular techniques, gene knockout models, and the Human Genome Project has provided new insight into the molecular identity and role of K^+ and Cl^- channels, water channels (aquaporins), and intracellular Ca^{2+} channels in pulmonary vascular function and disease.

One of the major advances in the research field of pulmonary arterial hypertension has been the identification of an association between disease development and genetic mutations in the bone morphogenetic protein receptor II (BMPR-II) and related pathways. New evidence suggests that there are significant interactions between bone morphogenetic protein and serotonin signaling and indeed modulation of ion channel expression and function. These and other similar interrelationships are likely to define a significant proportion of the altered vascular function and modeling of the pulmonary circulation in disease.

We therefore saw a need for a forum where these mechanisms and pathways can be discussed together, so identifying and highlighting the potential therapeutic

opportunities that this apparent convergence of pathways may reveal. This book presents the proceedings of the 2008 Grover Conference (Lost Valley Ranch and Conference Center, Sedalia, CO; September 3–7, 2008), which provided a forum for experts in the fields of those receptors, channels, and transporters that have been identified as playing key roles in the physiology and pathophysiology of the pulmonary circulation. The book rigorously addresses (1) recent advances in our knowledge of receptors, channels, and transporters and their role in regulation of pulmonary vascular function; (2) how modulation of expression and function of receptors, channels, and transporters and their interrelationships contribute to the pathogenesis of pulmonary vascular disease; and (3) the therapeutic opportunities that may be revealed by enhancing our understanding of this area.

The overall goal was to explore the mechanisms by which specific receptors, channels, and transporters contribute to pulmonary vascular function in both health and disease and how this knowledge may lead to novel interventions in lung dysplasia, pulmonary edema, lung injury, and pulmonary and systemic hypertension to reduce and prevent death from lung disease.

The book is divided into six parts. Part I ("Ion Channels in the Pulmonary Vasculature: Basics and New Findings") is designated for basic knowledge and recent findings in the research field of ion channels in pulmonary circulation. There are five chapters in Part I discussing the function, expression, distribution, and regulation of various ion channels present in pulmonary vascular smooth muscle cells and how these channels are integrated to regulate intracellular Ca^{2+} and cell functions. Part II ("TRP Channels in the Pulmonary Vasculature: Basics and New Findings") is composed of five chapters that are exclusively designed to discuss the role of a recently identified family of cation channels, transient receptor potential (TRP) channels, in the regulation of pulmonary vascular tone and arterial structure. Part III ("Pathogenic Role of Ion Channels in Pulmonary Vascular Disease") presents four chapters that discuss how abnormal function and expression of various ion channels contribute to changes in cell functions and the development of pulmonary hypertension. Part IV ("Receptors and Signaling Cascades in Pulmonary Arterial Hypertension") consists of five chapters devoted to the role of bone morphogenetic protein receptors, Notch receptors, serotonin receptors, Rho kinase, and vascular endothelial growth factor receptors in the development of pulmonary arterial hypertension. Part V ("Receptors and Transporters: Role in Cell Function and Hypoxic Pulmonary Vasoconstriction") has four chapters designed to illustrate the potential mechanisms involved in oxygen sensing and hypoxia-induced pulmonary vasoconstriction and hypertension. Part VI ("Targeting Ion Channels and Membrane Receptors in Developing Novel Therapeutic Approaches for Pulmonary Vascular Disease") consists of five chapters that discuss the translational research involving membrane receptors, channels, and transporters, including their potential as novel drug targets.

We hope that this book will allow readers to foster new concepts and new collaborations and cooperation among investigators to further understand the role of receptors, channels, and transporters in lung pathophysiology. The ultimate goal is to identify new mechanisms of disease as well as new therapeutic targets for pulmonary vascular diseases. An additional outcome should be enhanced understanding of the

role of these entities in systemic vascular pathophysiology since the conference included researchers and clinicians with interests in both pulmonary and systemic circulations.

The book could not have been completed without the support and encouragement of our families (Ayako Makino, Dolores) as well as our mentors, colleagues, and students at the University of California, San Diego (La Jolla, CA) and the King's College (London, UK). We are especially grateful to Ms. Mindy Okura-Marszycki for her instruction for compiling the book, to Dr. Carmelle V. Remillard for her diligence in preparing the figures and editing the text, and to all the contributors and speakers for their patience and conscientiousness in writing the manuscripts and presenting at the conference (Fig. 1). In addition, we thank the staff of the American Thoracic Society for their excellent help in running the conference and all our sponsors for supporting it. Finally, we would like to take this opportunity to thank Dr. Robert F. Grover and his wife, to whom this book is dedicated.

Fig. 1 Grover Conference 2008. *Front row* (*left* to *right*): Andrea Olshewski, Chandran Nagaraj, Angel Cogolludo, Tom Resta, Michelle Connoly, Donna Cioffi, Robert Grover, Karen Fagan, Ming-Yuan Jian, Konstantin Birukov, and Phillip Aaronson. *Middle rows* (*second* and *third row*) (*left* to *right*): Liliana Moreno-Vinasco, James Sham, Lih Chyan Ng, Usha Raj, Christina Barry, Charles Hales, Carmelle Remillard, Nikki Jernigan, Claudie Hecquete, Sabine Lange, Michael Sanderson, Larissa Shimoda, Francisco Perez-Vizcaino, Gaurav Choudhary, Normand Leblanc (inside the car), Ken Weir, Liz Weir, Alison Gurney (inside the car), Elise Grover, Mary Townsley, Mark Evans, Patricia Thistlethwaite, Gregory Knock, Eloa Adams, Navdeep Chandel, Rich Minshall, Norbert Weissmann, Zhigang Hong, Troy Stevens. *On the car* (*left* to *right*): John Westwick, Marlene Rabinovitch, Jeremy Ward, Jason Yuan, David Cornfield, Jens Lindert, Jessica Snow, Ralph Schermuly. *Absent from picture*: Steve Abman, Stephen Archer, David Clapham, Mark Gillespie, Brian Hanna, Joseph Hume, Landon King, Margaret MacLean, Asrar Malik, Ivan McMurtry, Dolly Mehta, Nicholas Morrell, Kurt Stenmark, Matt Thomas, and James West

History of the Grover Conference

The Grover Conferences on the Pulmonary Circulation were initiated in 1984 by Drs. John T. Reeves and E. Kenneth Weir in recognition of the many contributions of Dr. Robert F. Grover (Fig. 2) to our understanding of the physiology and pathophysiology of the pulmonary vasculature. His studies of brisket disease in cattle at high altitudes were among the first (1960) and were certainly the most complete descriptions of chronic pulmonary hypertension. He initiated or participated in investigations into factors influencing acute and chronic hypoxic pulmonary hypertension, including species differences, sympathetic activity, prostaglandins, endotoxins, calcium antagonists, mast cells and histamine, acetylcholine, unilateral pulmonary arterial ligation, ethyl alcohol, platelets, genetic factors, and cold exposure.

Dr. Grover conducted the initial studies showing a reversible component in pulmonary hypertension in congenital heart disease (1961) and the presence and reversibility of pulmonary hypertension in normal North American residents at high altitude (1963, 1966). He was involved in the first measurements of pulmonary vascular reactivity in pregnant women and in persons susceptible to high-altitude pulmonary edema and in investigations of high-altitude pulmonary edema in children of Leadville, Colorado.

These conferences provide North America's only ongoing forum dedicated to the pulmonary circulation. The conferences have been held in Deckers, Colorado, every second year (with one extra interpolated conference in 1991). The first in 1984 focused on pulmonary reactivity; the second on lipid mediators in the pulmonary circulation; the third on the control of cellular proliferation in the pulmonary vascular wall; the fourth on the diagnosis and treatment of pulmonary hypertension; and the fifth on the pathophysiology of the pulmonary circulation and gas exchange. The sixth in 1992 applied the knowledge of ion channels and transporters to the

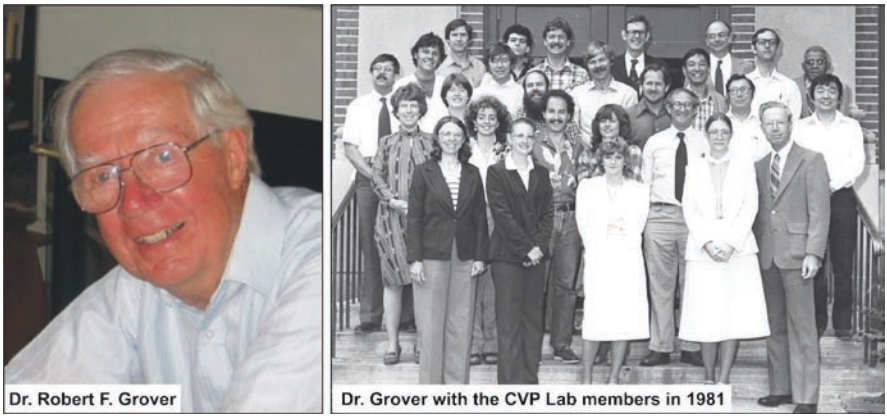

Fig. 2 Robert F. Grover, MD, PhD (*left*), and members of the Cardiovascular Pulmonary (CVP) Research Laboratory (in 1981, *right*)

Table 1 The Grover Conferences held in 1984–2008

Year	Title	References
1984	Pulmonary vascular reactivity	Chest 88:199S–272S, 1985 Clin Resp Physiol 21:583–590, 1985
1986	Lipid mediators in the pulmonary circulation	Am Rev Respir Dis 136:196–224, 455–491, 762–788, 1987
1988	The control of cellular proliferation in the pulmonary circulation	Am Rev Respir Dis 140:1092–1135, 1446–1493, 1989
1990	The diagnosis and treatment of pulmonary hypertension	Weir EK, Archer SL, Reeves JT (eds) The diagnosis and treatment of pulmonary hypertension. Futura, New York, 1992
1991	The pulmonary circulation and gas exchange	Wagner WW Jr, Weir EK (eds) The pulmonary circulation and gas exchange. Futura, New York, 1994
1992	The role of ion flux in pulmonary vascular control	Weir EK, Hume JR, Reeves JT (eds) Ion flux in pulmonary vascular control. Plenum, New York, 1993
1994	Nitric oxide and oxygen radicals in the pulmonary vasculature	Weir EK, Archer SL, Reeves JT (eds) Nitric oxide and radicals in the pulmonary vasculature. Futura, New York, 1996
1996	Pathogenesis and treatment of pulmonary edema	Weir EK, Reeves JT (eds) Pulmonary edema, Futura, New York, 1998
1998	Control mechanisms in the fetal and neonatal pulmonary circulations	Weir EK, Archer SL, Reeves JT (eds) The fetal and neonatal pulmonary circulations. Futura, New York, 2000
2000	Interactions of blood and the pulmonary circulation	Weir EK, Reeve HL, Reeves JT (eds) Interactions of blood and the pulmonary circulation. Futura, New York, 2002
2002	Pro-inflammatory signaling mechanisms in the pulmonary circulation	Bhattacharya J (ed) Cell signaling in vascular inflammation. Humana, Totowa, New Jersey, 2005
2004	Genetic and environmental determinants of pulmonary endothelial cell function	
2006	Rho family GTPases in pulmonary vascular pathophysiology	
2008	Membrane receptors, channels and transporters in pulmonary circulation: role in the development of pulmonary vascular disease	Yuan JX-J, Ward JPT (eds) Membrane receptors, channels and transporters in pulmonary circulation. Humana Press-Springer, Totowa, New Jersey, 2009

pulmonary vasculature; the seventh studied the role of radicals; the eighth examined the pathogenesis and treatment of pulmonary edema; the ninth discussed the fetal and neonatal pulmonary circulations; the tenth explored the interactions of the blood and the pulmonary circulation; the eleventh looked at proinflammatory signaling mechanisms; the twelfth covered genetic and environmental determinants of

pulmonary endothelial cell function; the thirteenth was devoted to Rho family guanosine triphosphatases (GTPases); and the last in 2008 returned again to the theme of ion channels and transporters (Table 1).

The proceedings of the first conference was published in *Chest* and the second and third in the *American Review of Respiratory Disease*, and the subsequent conferences have been published as books by Futura Publishing Company, Plenum Press, Humana Press, or Humana Press-Springer (Table 1). Thirteen eminent investigators in the field have been selected by the Grover Conference Committee to give the Estelle Grover Lecture (established in 1992), Terry Wagner Lecture (established in 2004), and John Reeves Lecture (established in 2006) (Table 2). In 2008, the Grover Conference Committee and the Program Committee of the American Thoracic Society Pulmonary Circulation Assembly established the Young Investigator Award to recognize outstanding young and new investigators in the research fields of pulmonary circulation, pulmonary vascular biology, and pulmonary vascular disease (Table 2). In addition to invited speakers in the conference, the committee also selected the authors of five abstracts to give oral presentations on their research findings.

In Grover Conference 2008, Dr. Margaret R. MacLean from the University of Glasgow (Glasgow, UK) presented the Estelle Grover Lecture, Dr. Ivan F. McMurtry from the University of South Alabama (Mobile, AL) presented the Terry Wagner Lecture, and Dr. Nicholas W. Morrell from the University of Cambridge (Cambridge, UK) presented the John Reeves Lecture (Fig. 3). Dr. Navdeep S. Chandel from Northwestern University (Chicago, IL) and Dr. Larissa A. Shimoda from Johns Hopkins University (Baltimore, MD) were Young Investigator Awardees in Grover Conference 2008 (Fig. 3).

We encourage you to participate in future Grover Conferences by coming to the meetings as well as by taking part in planning and development of new subjects for the meetings.

Table 2 The named lectures and awards given at the Grover Conferences during 1984–2008

Year	Estelle Grover Lecture	Terry Wagner Lecture	John Reeves Lecture
1992	John A. Bevan, MD		
1994	Edward R. Block, MD		
1996	Timothy W. Evans, MD, PhD		
1998	John T. Reeves, MD		
2000	Wiltz W. Wagner Jr, PhD		
2002	E. Kenneth Weir, MD		
2004	Norbert F. Voelkel, MD	Mark N. Gillespie, PhD	
2006	Barry L. Fanburg, MD	Jeremy P.T. Ward, PhD	Avril V. Somlyo, PhD
2008	Margaret R. MacLean, PhD	Ivan F. McMurtry, PhD	Nicholas W. Morrell, MD

Year	Young Investigator Award
2008	Navdeep S. Chandel, PhD (Northwestern University Feinberg School of Medicine, Chicago, IL) Larissa A. Shimoda, PhD (Johns Hopkins University School of Medicine, Baltimore, MD)

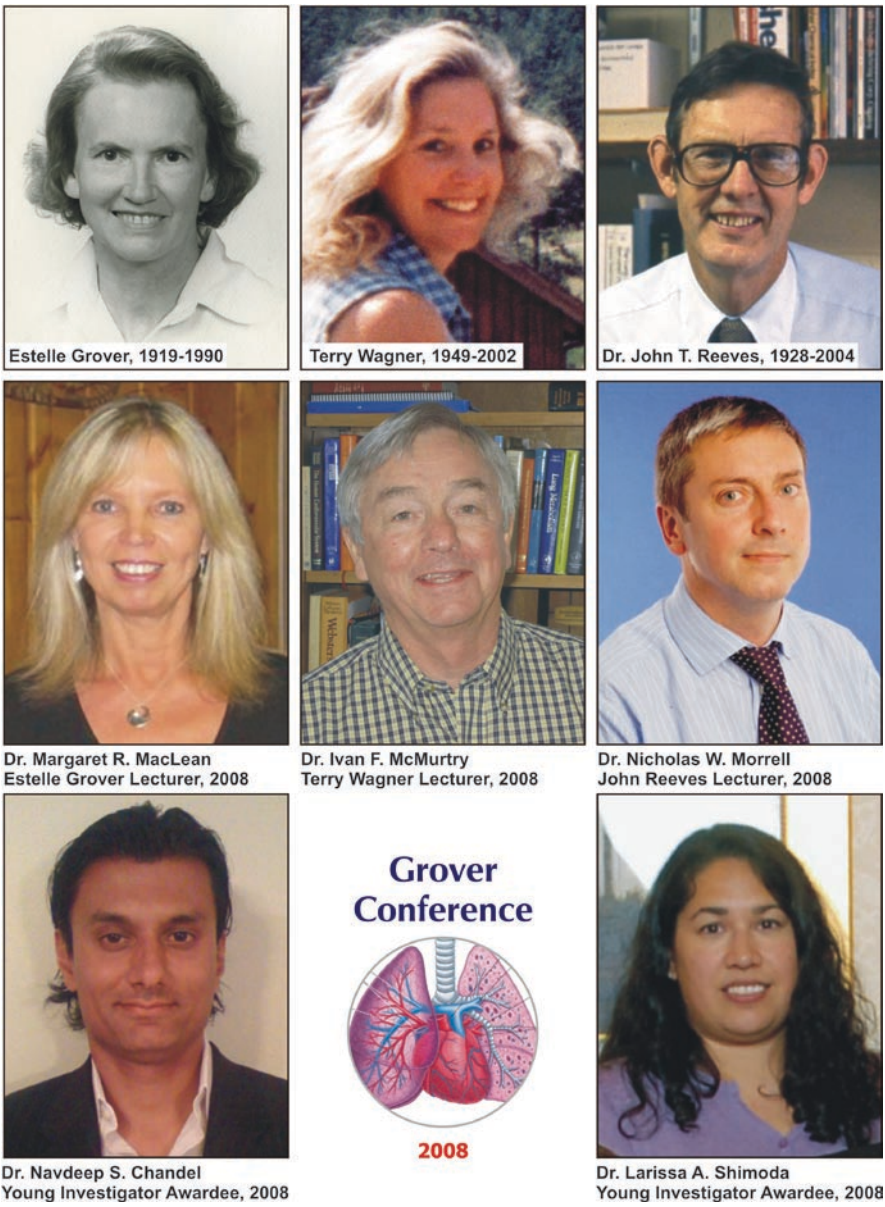

Fig. 3 Estelle Grover (1919–1990), Terry Wagner (1949–2002), and John T. Reeves (1928–2004), as well as the named lecturers and young investigator awardees at Grover Conference 2008

Contents

Part I
Ion Channels in the Pulmonary Vasculature: Basics and New Findings

The Role of Ion Channels in Hypoxic Pulmonary Vasoconstriction

E. Kenneth Weir, Jésus A. Cabrera, Saswati Mahapatra, Douglas A. Peterson, and Zhigang Hong

Abstract Hypoxic pulmonary vasoconstriction (HPV) is an important mechanism by which localized flow of blood in small resistance pulmonary arteries is matched to alveolar ventilation. This chapter discusses the role of several potassium and calcium channels in HPV, both in enhancing calcium influx into smooth muscle cells (SMCs) and in stimulating the release of calcium from the sarcoplasmic reticulum, thus increasing cytosolic calcium. The increase in calcium sensitivity caused by hypoxia is reviewed in Chapter 19. Particular attention is paid to the activity of the L-type calcium channels which increase calcium influx as a result of membrane depolarization and also increase calcium influx at any given membrane potential in response to hypoxia. In addition, activation of the L-type calcium channel may, in the absence of any calcium influx, cause calcium release from the sarcoplasmic reticulum. Many of these mechanisms have been reported to be involved in both HPV and in normoxic contraction of the ductus arteriosus.

Keywords Hypoxia • resistance pulmonary arteries • ductus arteriosus • voltage-gated potassium channels • L-type calcium channels • store-operated channels • sarcoplasmic reticulum

1 Introduction

Hypoxic pulmonary vasoconstriction (HPV) is one example of how the body senses a change in oxygen and, speaking teleologically, puts the information to good use. In the fetus, HPV helps to reduce blood flow through the unventilated lungs and consequently to direct blood through the ductus arteriosus (DA), which is open during the hypoxic, fetal stage of life. As a result of the onset of ventilation at birth and the associated increase in oxygen, the small, resistance pulmonary arteries (PAs) dilate, while

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the DA constricts. While the two diametrically opposite responses are both modulated by endothelial factors, the basic mechanisms of HPV and normoxic contraction of the DA reside in the smooth muscle cells (SMCs) of these vessels. In both cases, the "executive" mechanisms have two major components: an increase in cytosolic calcium and an increase in calcium sensitization. The former depends on ion channels and the sarcoplasmic reticulum and the latter on Rho/Rho kinase signaling.

2 Oxygen Sensing and Ion Channels in the Carotid Body

The role of ion channels in sensing hypoxia was first described in the carotid body, and this is still the best-understood model. Twenty years ago, López-Barneo et al. reported that hypoxia inhibited the outward potassium current (I_K) passing through voltage-gated potassium (K_v) channels in the plasmalemma of type 1 or glomus cells of the carotid body.[1] This in turn leads to membrane depolarization and calcium entry through L-type (voltage-gated) calcium channels down the calcium concentration gradient (2 m*M* extracellular, 100 n*M* intracellular). Subsequently, it has been demonstrated that other K^+ channels, calcium-sensitive (K_{Ca}) and Two-pore Domain Acid-Sensitive (TASK)-like, are also inhibited by hypoxia[2,3] and contribute to the depolarization. The essential part played by membrane depolarization in oxygen sensing is shown in an elegant experiment (Fig. 1.1),[4] in which membrane potential and cytosolic calcium are measured simultaneously in a type 1 cell. When the cell is made

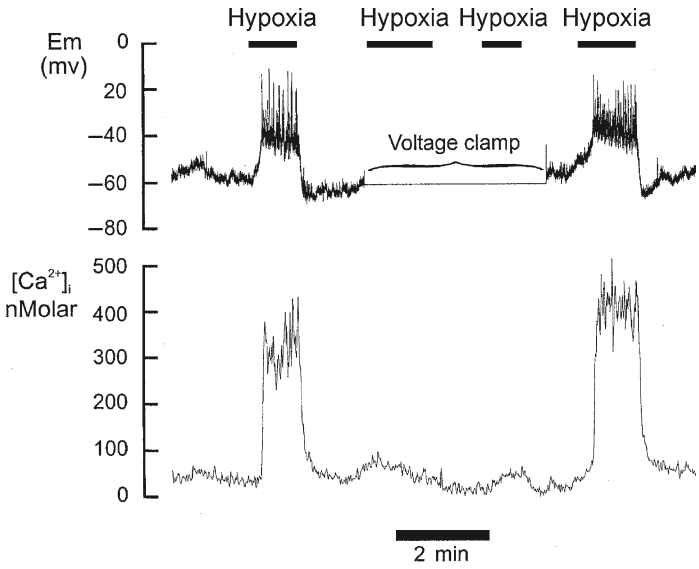

Fig. 1.1 The depolarization caused by hypoxia in the type 1 cell of the carotid body is associated with an increase in cytosolic calcium. If the membrane potential E_m is clamped at –60 mV, hypoxia does not increase the level of calcium. Adapted with permission[53]

hypoxic, the membrane depolarizes, and the calcium level increases. If, however, the membrane potential is artificially "clamped" at the approximate resting level of −60 mV, then another identical hypoxic challenge causes no increase in calcium. When the clamp is taken off, the next hypoxic challenge again stimulates an increase in cytosolic calcium. In the absence of external calcium or in the presence of an L-type calcium channel blocker, hypoxia causes little increase in cytosolic calcium.[4,5]

3 Hypoxic Pulmonary Vasoconstriction

In the adult animal, as first described in detail by von Euler and Liljestrand in 1946, acute alveolar hypoxia causes pulmonary vasoconstriction, which redistributes desaturated, mixed venous blood away from poorly ventilated areas of lung. Inhibition of HPV leads to a decrease in systemic arterial oxygen tension both in normal subjects and in patients with small airway disease or lung injury[6] because of a failure to match ventilation and perfusion. The executive mechanisms involving ion channels, which are responsible for hypoxic contraction of pulmonary artery smooth muscle cells (PASMCs), include inhibition of K^+ channels, membrane depolarization, and calcium entry through voltage-gated, L-type Ca^{2+} channels; an increase in Ca^{2+} influx through the L-type Ca^{2+} channels unrelated to depolarization; and the release of Ca^{2+} from the sarcoplasmic reticulum (SR), associated with repletion by Ca^{2+} influx through store-operated channels (SOCs).

4 K^+ Channels and HPV

In 1976, McMurtry et al. demonstrated that inhibitors of L-type calcium channels, such as verapamil, could ablate HPV in the isolated, perfused rat lung, while only moderately reducing the pressor response to angiotensin II or prostaglandin $F_{2\alpha}$.[7] This raised the possibility that hypoxia causes depolarization of the PASMCs. Hypoxia-induced depolarization in small PAs was subsequently reported by Harder et al. in 1985.[8] In the pancreatic islet cells, an increase in glucose metabolism yields adenosine triphosphate (ATP), which inhibits K_{ATP} channels, causing membrane depolarization, calcium entry, and insulin secretion.[9,10] Based on this observation, it was suggested that hypoxia might, through a change in redox status, reduce K^+ current I_K in PASMCs, again leading to calcium entry through L-type calcium channels.[11] The hypothesis was validated in part when it was shown that hypoxia inhibits I_K in PASMCs but not in SMCs from renal or mesenteric arteries.[12,13] Subsequently, a number of voltage-gated K^+ channels (K_v channels: $K_v1.2$, $K_v1.5$, $K_v2.1$, $K_v9.3$) have been found to be oxygen sensitive.[14,15] The evidence associating $K_v1.5$ with HPV is the best documented. Hypoxia inhibits $K_v1.5$ cloned from human PAs,[16] and HPV is diminished in mice in which $K_v1.5$ has been deleted.[17] The effect of hypoxia is apparently not directly on the channel protein

because, when the human $K_v1.5$ is overexpressed in mesenteric artery SMCs, hypoxia does not reduce I_K, but when it is overexpressed in PASMCs, hypoxia reduces I_K by 40%.[18] In the PASMC, studies using single-cell reverse-transcription polymerase chain reaction (PCR), have demonstrated that the level of expression of $K_v1.5$ correlates with the sensitivity of I_K, as a whole, in the individual SMCs to hypoxia.[19] This observation that different PASMCs have varying responses to hypoxia gave rise to the concept of pacemaker cells, which may initiate contraction and pass the signal to other PASMCs through gap junctions. This would be analogous to the calcium waves that have been described in pulmonary endothelial cells.[20]

In addition to the inhibition of I_K by acute hypoxia, the expression of K_v channels is reduced quite rapidly in chronic hypoxia. Thus, single PASMCs dispersed from the small PAs of rats maintained in 10% oxygen for 4 weeks had a resting membrane potential of −43.5 ± 2 mV compared to –54.3 ± 2 mV in cells from normoxic animals.[21] Subsequent work has shown that there is decreased messenger RNA (mRNA) and protein for several oxygen-sensitive K^+ channels in the chronically hypoxic cells.[22] In the case of $K_v1.2$, $K_v1.5$, and $K_v2.1$, the decrease in mRNA can occur within 6 h of the start of hypoxia.[23] In concordance with this observation, acute HPV is reduced in the isolated perfused lungs of rats that have previously been exposed to chronic hypoxia[24] and can be restored by the transfection of human $K_v1.5$ using an aerosol.[25] This experiment illustrated the importance of $K_v1.5$ in the mechanism of HPV, which is further reinforced by the finding that $K_v1.5$ protein is most abundant in the SMCs of resistance PAs, even though the mRNA for many K_v channels is present.[16] These experiments demonstrated that if oxygen-sensitive K_v channels are not expressed, there is membrane depolarization in PASMCs and loss of acute HPV, which can be restored by transfection of specific K_v channels.

It will be clear from the subsequent discussion that oxygen-sensitive K_v channels are not the sole executive mechanism of HPV but they play an important part. One question that arises concerning the role of K_v channels is the membrane potential at which they are active under normoxic conditions. As K_v channels are inactivated by depolarization, it may be that patch-clamp protocols that involve depolarization to positive membrane potentials (greater than 0 mV) inactivate some K_v channels that would otherwise be active at −60 mV.[16] Other K^+ channels that are known to be open at physiologic resting membrane potentials are KCNQ[26] (K_v 7) and the two-pore domain acid-sensitive potassium channel, TASK-1.[27] Consequently, it is possible that hypoxic inhibition of these channels might help to depolarize membrane potentials of PASMCs into the range where K_v channels are more active.[27,28]

5 L-Type Calcium Channels and HPV

Much attention has been paid to the effect of hypoxia on K^+ channels and the subsequent membrane depolarization. However, it is also important to note the effect of hypoxia on calcium influx through L-type channels into PASMCs at a specific fixed membrane potential. Just as there is a difference in the K^+ channels expressed in SMCs from conduit and resistance PAs,[29] so there is a difference in the

expression and behavior of L-type/Ca^{2+} channels.[30] In the PASMCs from resistance arteries, hypoxia increases $I_{Ca}{}^{2+}$ at membrane potentials below 0 mV, while inhibiting the calcium entry at more positive potentials. Interestingly, in the PASMCs from proximal or conduit arteries, hypoxia causes only inhibition. The differences in K^+ and Ca^{2+} channel expression and gating in response to hypoxia may help to explain HPV in the resistance arteries and hypoxic relaxation in the conduit arteries. The importance of L-type calcium channels in HPV is emphasized by the marked reduction of HPV by verapamil in the isolated rat lung mentioned earlier and by nifedipine and nisoldipine in the intact dog.[31]

6 Intracellular Calcium Release and HPV

More than 20 years ago, Hoshino et al. reported that a switch from hyperoxia to hypoxia caused contraction of human PA strips, in the presence of either histamine or KCL and the calcium ionophore A23187.[32] They considered that this hypoxic condition involved both calcium entry and calcium release from intracellular stores. However, the inhibitor of calcium release, HA 1004, also reduced the histamine preconstriction by half, so it is not clear whether the decrease in HPV was really an effect on the mechanism triggered by hypoxia or on the preconstriction. The same caveat applies to all interventions on HPV conducted in the setting of preconstriction.

A subsequent study demonstrated that release of calcium from the SR by caffeine or thapsigargin would reduce the increase in cytosolic calcium in cultured PASMCs caused by "hypoxia."[33] In this instance, concern arises because the cells were cultured from conduit PA, not resistance PA, and sodium dithionite was used to induce hypoxia. Sodium dithionite leads to hypoxia by generating reactive oxygen species (ROS).[34] Authentic hypoxia was used in an experiment looking at the effect of hypoxia on cytosolic calcium in PASMCs from resistance and conduit arteries and in SMCs from cerebral arteries.[35] Calcium went up with hypoxia in resistance PASMCs but down in SMCs from the other two arteries. Pretreatment of the resistance PASMCs with ryanodine, to release calcium from the SR, diminished the hypoxic increase in calcium. The importance of extracellular calcium influx was shown by the observation that chelation of extracellular calcium with EGTA rapidly ablated the elevated cytosolic calcium induced by hypoxia. A potential role for a hypoxic increase in calcium sensitivity was also raised in this article.[35]

In subsequent work using rings of canine resistance PA pretreated with phenylephrine, it was found that switching from hyperoxia to hypoxia caused contraction that could be reduced by ryanodine and caffeine or enhanced by cyclopiazonic acid (CPA).[36] These data suggest that hypoxia may release calcium from a ryanodine- and caffeine-sensitive store, and that the contraction may be modulated by calcium uptake into a distinct $InsP_3$ (inositol 1,4,5-trisphosphate) store, which can be blocked by CPA. HPV in this ring model did not require the presence of endothelium. The authors raised the possibility that some of the calcium entered the PASMC through a capacitative calcium entry (CCE) pathway activated by store depletion.[36]

7 Store-Operated Calcium Channels

HPV does not occur in the absence of external calcium in the isolated PA ring[37] or in the isolated perfused rat lung.[38] In the ring model, much of the calcium entry stimulated by hypoxia occurs through store-operated channels (SOCs).[37] More recent studies confirmed, in cultured rat PASMCs, that CCE induced by CPA is increased by hypoxia and inhibited by the SOC blockers SKF-96365, nickel, and lanthanum.[39] Incidentally, these workers reported that nifedipine (5 μ*M*) inhibited about 50% of the increase in calcium caused by hypoxia. Similar results have been described in canine PASMCs[36,40] in which the component of the hypoxia-induced increase in cytosolic calcium that was insensitive to nisoldipine (10 μ*M*) could be inhibited by SKF-96365 or nickel. In the isolated perfused rat lung, the same two SOC blockers prevented HPV at concentrations that did not alter the pressor response to KCl but did inhibit the pressor responses to angiotensin II that was used to "prime" the lungs prior to HPV.[38] Nifedipine (5 μ*M*) completely reversed HPV in this model. These observations reinforce the view that HPV requires calcium entry through both the SOC and voltage-gated calcium channels.

A recent article provided evidence that may help to link both elements.[41] Using mice with homozygous and heterozygous gene deletion of the ryanodine receptor 1, it was observed that in PASMCs from these mice there was a marked decrease in the ability of hypoxia to cause a rise in cytosolic calcium. Hypoxic vasoconstriction was diminished in the heterozygous mouse PAs (Fig. 1.2). This finding, along with similar data on the effects of homozygous RyR3 gene deletion,[42] confirmed the importance of calcium release from the ryanodine receptor in HPV. Interestingly, the increase in PASMC cytosolic calcium and in PA contraction stimulated by the addition of KCl is also less in the RyR1 heterozygous gene deletion mice.[41] This might point to the effect of calcium entering through the L-type voltage-dependent calcium channels, leading to calcium-induced calcium release (CICR). However, a reduction in KCl-induced calcium in $RyR1^{-/-}$ PASMCs and in contraction in the $RyR1^{+/-}$ PA rings was also reported in the absence of calcium in the perfusing solution. This observation suggests that membrane depolarization can lead to release of calcium from the ryanodine receptor even in the absence of calcium entry. Such a mechanism has been demonstrated.

8 Depolarization and Calcium Release

In addition to CICR, there is another sequence (described in basilar artery SMCs) in which the L-type calcium channels sense membrane depolarization and activate G proteins and the phospholipase C-$InsP_3$ pathway.[43] Subsequently, $InsP_3$-induced release of calcium leads to further release from the ryanodine-sensitive compartment of the SR. This occurs without influx of calcium through the L-type channel, for instance, in the absence of external calcium. However, the calcium release induced by calcium channel activation can be blocked by diltiazem or

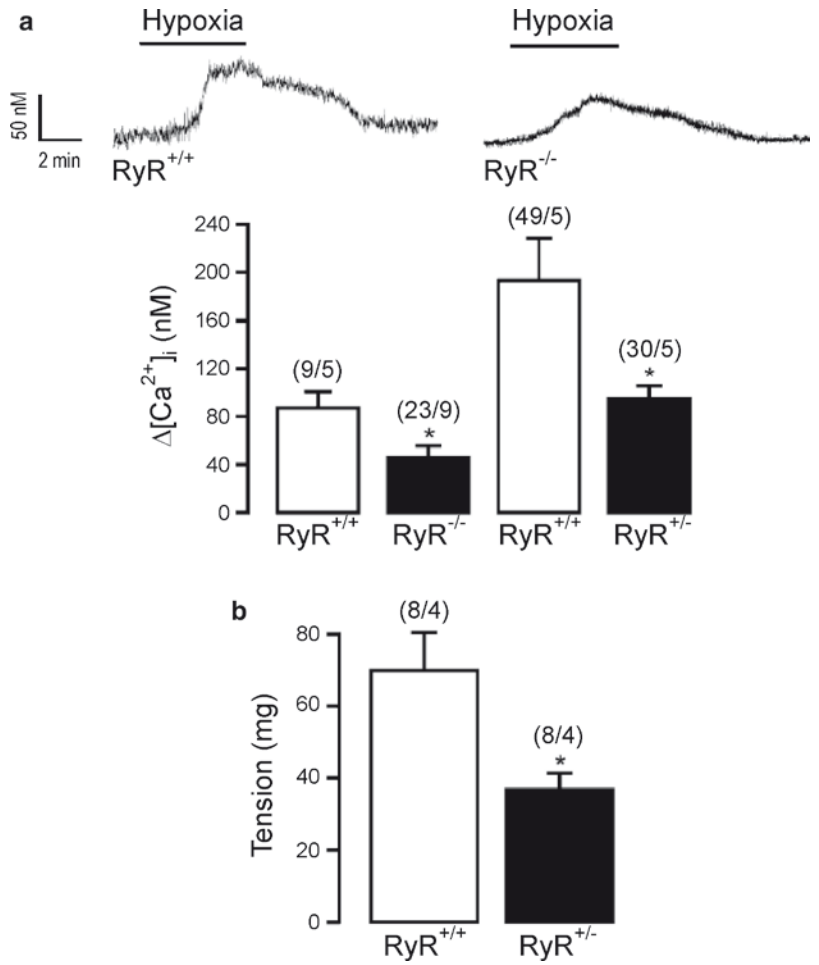

Fig. 1.2 The hypoxic increase in cytosolic calcium in PASMCs (**a**) and contraction in pulmonary artery rings (**b**) is reduced in mice that have homozygous ($RyR1^{-/-}$) or heterozygous ($RyR1^{+/-}$) gene deletion of the ryanodine receptor 1. Reproduced with permission[41]

D600. This phenomenon has been observed in PASMCs[44] and might explain the many references mentioned here that described the efficacy of calcium channel blockers in inhibiting HPV. If the blockers prevent activation of the L-type channels, they may not only inhibit calcium entry through these channels but also reduce the release of calcium from the SR that is initiated by the activation but unrelated to the calcium influx. Interestingly, in coronary artery SMCs, hypoxia inhibits the calcium release initiated by L-type calcium channel activation.[45] This mechanism may help us to understand the interaction of depolarization induced by K^+ channel inhibition and the increase in calcium observed in HPV as well as the action of calcium channel blockers.

9 TRPCs and HPV

How could the signal of calcium depletion in the SR be translated into calcium entry? SOC may be heterotetrameric assemblies of canonical transient receptor potential (TRPC) proteins. Many TRPC isoforms have been identified in the PA smooth muscle. By both mRNA and protein measurements, the expression of TRPC1, TRPC4, and TRPC6 is greater in distal resistance PAs than in more proximal conduit arteries.[46] This corresponds with evidence for greater calcium entry through the SOC, stimulated by hypoxia, in PASMCs from resistance arteries. Stromal interacting molecule 1 (STIM1) plays a role in translating the signal of calcium depletion in the SR so that calcium entry through the SOC is increased. Interestingly, the expression of STIM1 is also greater in the resistance PAs.[46]

Additional evidence for the involvement of TRPC-related channels comes from the study of mice lacking TRPC6. The isolated lungs of wild-type mice had an acute pressor response to hypoxia of 1.2 mmHg. This relatively small degree of HPV was absent in the TRPC6$^{-/-}$ mice, although the vasoconstrictor response to U46619 was the same in both groups.[47] HPV occurring later, after 60 min of continued hypoxia, was also the same in both groups. The increase in calcium stimulated by hypoxia, after "priming" with endothelin 1 or AII, seen in the PASMCs of the wild-type mice, was not present in TRPC6$^{-/-}$ PASMCs. Thus, SOCs/TRPCs are likely to be important in the CCE component of HPV. The authors of this paper also reported that nicardipine almost completely inhibited acute HPV in the isolated lungs and the calcium influx caused by hypoxia in the wild-type PASMCs.[47] They speculated that Na^+ influx through the TRPC6 leads to membrane depolarization and activation of voltage-gated calcium channels.

10 Normoxic Contraction of the Ductus Arteriosus

The mechanisms relating normoxic contraction of the DA to ion channels and calcium flux are a mirror image of those discussed in HPV and may help to provide insight into the signaling of changes in oxygen tension. Instead of acute hypoxia that inhibits K^+ channels in PASMCs, it is the transition from hypoxia to normoxia that inhibits K^+ channels in ductus arteriosus smooth muscle cells (DASMCs).[48] Normoxia causes the same depolarization in DASMCs as the K_v channel blocker 4-aminopyridine (4-AP) (1 m*M*). As 4-AP also causes PA depolarization and contraction, the difference in oxygen sensing must be proximal to the channel protein. Normoxic contraction of the DA can be markedly reduced by nisoldipine (0.5 μ*M*), indicating an important role for the L-type calcium channel.[48] As described in the PA, calcium entry through the L-type channel occurs not only because of membrane depolarization but also because the change in oxygen tension (in the case of DASMCs, the shift to normoxia) increases the influx of calcium at any given membrane potential.[49] This mechanism only appears in DASMCs near to term and thus may prevent premature contraction of the DA.

11 Store-Operated Channels and Normoxic Contraction of the DA

Nifedipine (1 μ*M*) is sufficient to completely inhibit the contraction of a DA ring caused by KCl (80 m*M*). However, approximately half of the normoxic contraction remains after nifedipine addition, suggesting that an alternative mechanism exists.[50] Much of the normoxic contraction can be blocked by the SOC inhibitors 2-APB (2-Aminoethyl Diphenylborinate) (30 μ*M*) or SKF-96365 (10 μ*M*), indicating a role for SOC. Similarly, in the presence of nifedipine, switching from 0 to 2 m*M* calcium in the bath solution causes a much stronger contraction of the DA ring in normoxia than in hypoxia (Fig. 1.3). These observations and the identification of TRPC1 and TRPC4 protein in the DA make it likely that SOCs play a significant part in the normoxic contraction of the DA.

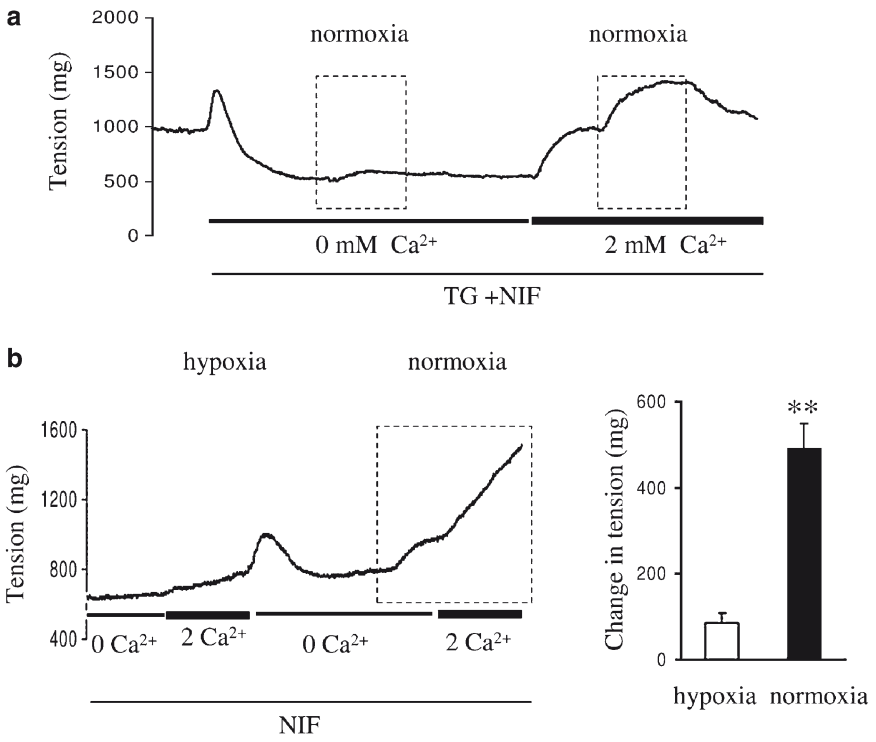

Fig. 1.3 Ductus arteriosus ring tension traces. (**a**) In the presence of thapsigargin (TG) and nifedipine (NIF), normoxia causes only a small contraction with no calcium in the bath solution but significant contraction in the presence of 2 m*M* calcium. (**b**) Switching the bath calcium from 0 to 2 m*M* causes greater contraction under normoxic than under hypoxic conditions. $^{**}p < 0.01$ versus hypoxia ($n = 14$). Reproduced with permission[50]

12 Conclusion

Much of the work reviewed in this chapter illustrates an expanded role for the L-type calcium channel in the response to hypoxia in the PA or to normoxia in the DA. In addition to responding to membrane depolarization by increased calcium influx in the traditional manner, hypoxia in the PA or normoxia in the DA increases calcium influx at any given membrane potential. Depolarization may also stimulate the L-type channel to signal the release of calcium from the SR. Clearly, hypoxia in the PA and normoxia in the DA also increase calcium entry through SOCs and enhance calcium sensitivity. Our knowledge of the role of ion channels in HPV and in normoxic contraction of the ductus is increasing, but we still do not have a good understanding of why these vessels behave in an opposite manner to a decrease in oxygen tension, and this continues to be debated.[51,52]

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References

1. López-Barneo J, López-López J, Ureña J, González C (1988) Chemotransduction in the carotid body: K^+ current modulated by PO_2 in type I chemoreceptor cells. Science 242:580–582
2. Wyatt CN, Wright C, Bee D, Peers C (1995) O_2-sensitive K^+ currents in carotid body chemoreceptor cells from normoxic and chronically hypoxic rats and their role in hypoxic chemotransduction. Proc Natl Acad Sci U S A 92:295–299
3. Buckler KJ (1997) A novel oxygen-sensitive potassium current in rat carotid body type I cells. J Physiol 498:649–662
4. Buckler KJ, Vaughan-Jones RD (1994) Effects of hypoxia on membrane potential and intracellular calcium in rat neonatal carotid body type I cells. J Physiol 476:423–428
5. Ureña J, Fernández-Chacón R, Benot A, Alvarez de Toledo G, López-Barneo J (1994) Hypoxia induces voltage-dependent Ca^{2+} entry and quantal dopamine secretion in carotid body glomus cells. Proc Natl Acad Sci U S A 91:10208–10211
6. Hales CA, Westphal D (1978) Hypoxemia following the administration of sublingual nitroglycerin. Am J Med 65:911–918
7. McMurtry I, Davidson AB, Reeves JT, Grover RF (1976) Inhibition of hypoxic pulmonary vasoconstriction by calcium antagonists in isolated rat lungs. Circ Res 38:99–104
8. Harder DR, Madden JA, Dawson C (1985) A membrane electrical mechanism for hypoxic vasoconstriction of small pulmonary arteries from cat. Chest 88:233S–245S
9. Ashcroft FM, Harrison DE, Ashcroft SJ (1984) Glucose induces closure of single potassium channels in isolated rat pancreatic β-cells. Nature 312:446–448
10. Ammon HP, Hägele R, Youssif N, Eugen R, El-Amri N (1983) A possible role of intracellular and membrane thiols of rat pancreatic islets in calcium uptake and insulin release. Endocrinology 112:720–726
11. Archer SL, Will JA, Weir EK (1986) Redox status in the control of pulmonary vascular tone. Hertz 11:127–141
12. Post JM, Hume JR, Archer SL, Weir EK (1992) Direct role for potassium channel inhibition in hypoxic pulmonary vasoconstriction. Am J Physiol 262:C882–C890
13. Yuan X-J, Goldman WF, Tod ML, Rubin LJ, Blaustein MP (1993) Hypoxia reduces potassium currents in cultured rat pulmonary but not mesenteric arterial myocytes. Am J Physiol 264:L116–L123

14. Coppock EA, Tamkun MM (2001) Differential expression of K_v channel α-and β-subunits in the bovine pulmonary arterial circulation. Am J Physiol Lung Cell Mol Physiol 281:L1350–L1360
15. Moudgil R, Michelakis ED, Archer SL (2005) Hypoxic pulmonary vasoconstriction. J Appl Physiol 98:390–403
16. Archer SL, Wu XC, Thébaud B et al (2004) Preferential expression and function of voltage-gated, O_2-sensitive channels in resistance pulmonary arteries explains regional heterogeneity in hypoxic pulmonary vasoconstriction: ionic diversity in smooth muscle cells. Circ Res 95:308–318
17. Archer SL, London B, Hampl V et al (2001) Impairment of hypoxic pulmonary vasoconstriction in mice lacking the voltage-gated potassium channel $K_v1.5$. FASEB J 15:1801–1803
18. Platoshyn O, Brevnova EE, Burg ED, Yu Y, Remillard CV, Yuan JX-J (2006) Acute hypoxia selectively inhibits KCNA5 channels in pulmonary artery smooth muscle cells. Am J Physiol Cell Physiol 290:C907–C916
19. Platoshyn O, Yu Y, Ko EA, Remillard CV, Yuan JX-J (2007) Heterogeneity of hypoxia-mediated decrease in $I_{K(V)}$ and increase in $[Ca^{2+}]_{cyt}$ in pulmonary artery smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 293:L402–L416
20. Kuebler WM, Ying X, Bhattacharya J (2002) Pressure-induced endothelial Ca^{2+} oscillations in lung capillaries. Am J Physiol Lung Cell Mol Physiol 282:L917–L923
21. Smirnov SC, Robertson TP, Ward JPT, Aaronson PI (1994) Chronic hypoxia is associated with reduced delayed rectifier K^+ current in rat pulmonary artery muscle cells. Am J Physiol 266:H365–H370
22. Osipenko ON, Alexander D, Maclean MR, Gurney AM (1998) Influence of chronic hypoxia on the contributions of non-inactivating and delayed rectifier K currents to the resting potential and tone of rat pulmonary artery smooth muscle. Br J Pharmacol 124:1335–1337
23. Hong Z, Weir EK, Nelson DP, Olschewski A (2004) Subacute hypoxia decrease voltage-activated potassium channel expression and function in pulmonary artery myocytes. Am J Resp Cell Mol Biol 31:1–7
24. McMurtry IF, Petrun MD, Reeves JT (1978) Lungs from chronically hypoxic rats have decreased pressor response to acute hypoxia. Am J Physiol 235:H104–H109
25. Pozeg Z, ED M, McMurtry M et al (2003) In vivo gene transfer of the O_2-sensitive potassium channel $K_v1.5$ reduces pulmonary hypertension and restores hypoxic pulmonary vasoconstriction in chronically hypoxic rats. Circulation 107:2037–2044
26. Joshi S, Balan P, Gurney AM (2006) Pulmonary vasoconstrictor action of KCNQ potassium channel blockers. Respir Res 7:31–41
27. Gurney A, Manoury B (2009) Two-pore potassium channels in the cardiovascular system. Eur Biophys J 38:305–318
28. Olschewski A, Li Y, Tang B et al. (2006) Impact of TASK-1 in human pulmonary artery smooth muscle cells. Circ Res 98:1072–1080
29. Archer SL, Huang JMC, Reeve HL, Hampl V, Tolorová S, Michelakis E, Weir EK (1996) Differential distribution of electrophysiologically distinct myocytes in conduit and resistance arteries determines their response to nitric oxide and hypoxia. Circ Res 78:431–442
30. Franco-Obregon A, Lopez-Barneo J (1996) Differential oxygen sensitivity of calcium channels in rabbit smooth muscle cells of conduit and resistance pulmonary arteries. J Physiol 491:511–518
31. Archer SL, Yankovich RD, Chesler E, Weir EK (1985) Comparative effects of nisoldipine, nifedipine and bepridil on experimental pulmonary hypertension. J Pharmacol Exp Ther 233:12–17
32. Hoshino Y, Obara H, Kusunoki M, Fujii Y, Iwai S (1988) Hypoxic contractile response in isolated human pulmonary artery role of calcium ion. J Appl Physiol 65:2468–2474
33. Salvaterra CG, Goldman WF (1993) Acute hypoxia increases cytosolic calcium in cultured pulmonary arterial myocytes. Am J Physiol 264:L323–L328
34. Archer SL, Hampl V, Nelson DP, Sidney E, Peterson DA, Weir EK (1995) Dithionite increases radical formation and decreases vasoconstriction in the lung. Circ Res 77:174–181
35. Vadula MS, Kleinman JG, Madden JA (1993) Effect of hypoxia and norepinephrine on cytoplasmic free Ca^{2+} in pulmonary and cerebral arterial myocytes. Am J Physiol 265:L591–L597

36. Jabr RI, Toland H, Gelband CH, Wang XX, Hume JR (1997) Prominent role of intracellular Ca^{2+} release in hypoxic vasoconstriction of canine pulmonary artery. Br J Pharmacol 122:21–30
37. Robertson TP, Hague D, Aaronson PI, Ward JPT (2000) Voltage-independent calcium entry in hypoxic pulmonary vasoconstriction of intrapulmonary arteries of the rat. J Physiol 525:669–680
38. Weigand L, Foxson J, Wang J, Shimoda LA, Sylvester JT (2005) Inhibition of hypoxic pulmonary vasoconstriction by store-operated Ca^{2+} and nonselective cation channel antagonists. Am J Physiol Lung Cell Mol Physiol 289:L5–L13
39. Wang J, Shimoda LA, Weigand L, Wang W, Sun D, Sylvester JT (2005) Acute hypoxia increases intracellular [Ca^{2+}] entry. Am J Physiol Lung Cell Mol Physiol 288:L1059–L1069
40. Ng LC, Wilson SM, Hume JR (2005) Mobilization of sarcoplasmic reticulum stores by hypoxia leads to consequent activation of capacitative Ca^{2+} entry in isolated canine pulmonary arterial smooth muscle cells. J Physiol 263:409–419
41. Li X-Q, Zheng Y-M, Rathore R, Ma J, Takeshima H, Wang Y-X (2009) Genetic evidence for functional role of ryanodine receptor 1 in pulmonary artery smooth muscle cells. Eur J Physiol 457:771–783
42. Zheng Y-M, Wang Q-S, Rathore R et al (2005) Type-3 ryanodine receptors mediate hypoxia-, but not neurotransmitter-induced calcium release and contraction in pulmonary artery smooth muscle cells. J Gen Physiol 125:427–440
43. del Valle-Rodríguez A, López-Barneo J, Ureña J (2003) Ca^{2+} channel-sarcoplasmic reticulum coupling: a mechanism of arterial myocyte contraction without Ca^{2+} influx. EMBO J 22:4337–4345
44. del Valle-Rodríguez A, Calderón E, Ruiz M, Ordoñez A, López-Barneo J, Ureña J (2006) Metabotropic Ca^{2+} channel-induced Ca^{2+} release and ATP-dependent facilitation of arterial myocyte contraction. Proc Natl Acad Sci U S A 103:4316–4321
45. Calderón-Sanchez E, Fernández-Tenorio M, Ordóñez A, López-Barneo J, Ureña J (2009) Hypoxia inhibits vasoconstriction induced by metabotropic Ca^{2+} channel-induced Ca^{2+} release in mammalian coronary arteries. Cardiovasc Res 82(1):115–124
46. Lu W, Wang J, Shimoda LA, Sylvester JT (2008) Differences in STIM1 and TRPC expression in proximal and distal pulmonary arterial smooth muscle are associated with differences in Ca^{2+} responses to hypoxia. Am J Physiol Lung Cell Mol Physiol 295:L104–L113
47. Weissmann N, Zeller S, Schäfer R et al (2006) Impact of mitochondria and NADPH oxidases on acute and sustained hypoxic pulmonary vasoconstriction. Am J Respir Cell Mol Biol 34:505–513
48. Tristani-Firouzi M, Reeve HL, Tolarova S, Weir EK, Archer SL (1996) Oxygen-induced constriction of rabbit ductus arteriosus occurs via inhibition of a 4-aminopyridine-, voltage-sensitive potassium channel. J Clin Invest 98:1959–1965
49. Thébaud B, Wu X-C, Kajimoto H et al (2008) Development absence of the O_2 sensitivity of L-type calcium channels in preterm ductus arteriosus smooth cells impairs O_2 constriction contributing to patent ductus arteriosus. Pediatr Res 63:176–181
50. Hong Z, Hong F, Olschewski A et al (2006) Role of store-operated calcium channels and calcium sensitization in normoxic contraction of the ductus arteriosus. Circulation 114:1372–1379
51. Ward JPT (2006) Hypoxic pulmonary vasoconstriction is mediated by increased production of reactive oxygen species. J Appl Physiol 101:993–995
52. Weir EK, Archer SL (2006) Hypoxic pulmonary vasoconstriction is/is not mediated by increased production of reactive oxygen species. J Appl Physiol 101:995–998
53. Weir EK, López-Barneo J, Buckler KJ, Archer SL (2005) Acute oxygen-sensing mechanisms. N Engl J Med 353:2042–2055

Two-Pore Domain K^+ Channels and Their Role in Chemoreception

Keith J. Buckler

Abstract A number of tandem P-domain K^+- channels (K_2P) generate background K^+-currents similar to those found in enteroreceptors that sense a diverse range of physiological stimuli including blood pH, carbon dioxide, oxygen, potassium and glucose. This review presents an overview of the properties of both cloned K_2P tandem-P-domain K-channels and the endogenous chemosensitive background K-currents found in central chemoreceptors, peripheral chemoreceptors, the adrenal gland and the hypothalamus. Although the identity of many of these endogenous channels has yet to be confirmed they show striking similarities to a number of K_2P channels especially those of the TASK subgroup. Moreover these channels seem often (albeit not exclusively) to be involved in pH and nutrient/metabolic sensing.

Keywords Tandem-P-domain K-channels • TASK channels • acid sensing • CO_2 sensing • oxygen sensing • glucose sensing • potassium sensing • central chemoreceptors • peripheral chemoreceptors

1 Chemosensing and Background/Leak K^+ Channels

Many sensory modalities are mediated via electrical signalling pathways in which the presence of a stimulus is signalled by a change in resting membrane potential, a receptor potential, which leads to electrical activity, calcium signalling and neurosecretion (or some other response). This receptor potential may be generated by the activation of a cation conductance or by the inhibition of a background or leak potassium conductance. The principle focus of this review is the role of background or leak potassium channels in chemosensing.

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The term *leakage current/conductance* was introduced by Hodgkin and Huxley to describe a voltage- and time-independent conductance pathway in squid axon.[1] The properties and cause of this conductance were not investigated in any detail; indeed, the authors commented that "So many processes may contribute towards a leakage current that measurements of its properties is unlikely to give useful information about the nature of the charged particles on which it depends." It was perhaps the view that leakage currents were somehow unspecific and unregulated, coupled with the experimental practice of deliberately subtracting leakage currents in most voltage clamp studies, that led to leak currents/channels remaining relatively unexplored for many years. This is somewhat surprising given that resting membrane potential and conductance determine cell excitability.

The importance and nature of these background/resting/leak conductances is, however, becoming increasingly better appreciated. First, specific channels, the two-pore-domain K^+ channels, have been identified as prime candidates for mediating a potassium leak conductance; second, it is clear that these currents are not simply invariant determinants of a constant resting potential but are subject to modulation by a multitude of physical factors, signalling molecules and chemical stimuli. In this review, I first describe some of the properties of cloned two-pore-domain K^+ (K_{2P}) channels and then look at the role played by endogenous K_{2P}-like potassium channels in chemosensing.

2 Two-Pore-Domain Potassium Channels

2.1 Structure

The four transmembrane domain (4-TM) K_{2P} channels are the most recently discovered potassium channel family. First identified in the *Caenorhabditis elegans* genome[2] (in which they are particularly numerous[3]), there are now known to be 15 genes encoding K_{2P} channels in mammalians; the first discovered was TWIK (tandem of P-domains in a weakly inwardly rectifying K; KCNK1).[4] The term *two pore domain* relates to the fact that each channel subunit contains two pore-forming domains. Pore domains line the inner vestibule of many cation channels to form the selectivity filter that determines which ions may pass through the channel. Other potassium channels (e.g., delayed rectifier, inward rectifier and Ca^{2+}-activated K^+ channels) have only one pore-forming domain per subunit, with functional channels formed from a tetramer of subunits. In contrast, K_{2P} channels are thought to comprise of a dimer of subunits.[5] There are relatively few data on the propensity of K_{2P} channels to form heterodimers, but it has been reported for TASK (TWIK-related acid-sensitive K channel)-1 and TASK-3.[6–8] Consequently, the number of different channels that could be formed from K_{2P} subunits could be greater than the number of subunits alone. Many of the K_{2P} channels also contain another structural feature that is not seen in other K^+ channels. This is a large extracellular loop between the first transmembrane domain and the P-domain (pore domain) (the M1P1 loop). The function of this is largely unknown,

although it may play a role in channel assembly[5] and has also been implicated in pH sensing and regulation of channel gating in TASK channels.[9]

2.2 Classification and Nomenclature

The first channels of this type to be identified were given simple acronyms such as TWIK (tandem of P-domains in a weakly inwardly rectifying K channel). This approach has been followed for almost all of the channels discovered, with the acronyms either reflecting some biophysical or pharmacological characteristic or sequence similarity to another known channel. The Human Genome Organisation (HGO) classification is KCNK followed by a number representing the chronological order in which channels were discovered. The International Union of Basic and Clinical Pharmacology (IUPHAR) adopted a similar convention naming the proteins formed from these genes K_{2P} followed by the same number as used in the HGO classification. Thus, TWIK-1 is also known as KCNK1 and K_{2P} 1.1 (see Ref. [10]). Whilst this nomenclature is unambiguous, it is not particularly memorable and does nothing to inform regarding the properties of any of these channels or their structural or phylogenetic relationship to each other. Through comparison of sequence homology, the K_{2P} channels can be divided into six broad groups that relate closely to the original acronyms given when they were first discovered,[11–13] although those originally named TASK are now split into two groups. These groupings together with channel names are as follows:

1. *TWIK* channels, including
   TWIK-1 (KCNK1, K_{2P}1.1)
   TWIK-2 (KCNK6, K_{2P}6.1)
   KCNK7 (KCNK7, KCNK*8*, K_{2P}7.1)
2. *TREK* channels (TWIK-1 related K channel), including
   TREK-1 (KCNK2, K_{2P}2.1)
   TREK-2 (KCNK10*,* K_{2P}10.1)
   TRAAK (TWIK related arachidonic acid stimulated K channel) (KCNK4, K_{2P}4.1)
3. *TASK* channels, including
   TASK-1 (KCNK3, K_{2P}3.1, tBAK-1, OAT-1)
   TASK-3 (KCNK9, K_{2P}9.1)
   TASK-5 (KCNK15, K_{2P}15.1)
4. *TALK* channels, including
   TASK-2 (KCNK5, K_{2P}5.1)
   TALK-1 (KCNK16, K_{2P}16.1)
   TALK-2 (KCNK17, K_{2P}17.1, TASK-4)
5. *THIK* channels (Tandem pore domain halothane inhibited K channel), including
   THIK-1 (KCNK13, K_{2P}13.1)
   THIK-2. (KCNK12, K_{2P}12.1)
6. *TRESK* channel (TWIK-related spinal chord K channel) (only one)
   TRESK-1 (KCNK18, K_{2P}18.1, TRESK-2)

2.3 Biophysical Characteristics

Of the 12 subunits that have shown functional activity in homologous expression systems, none shows particularly strong voltage sensitivity. Consequently, most of these channels are active over a wide range of potentials and would therefore be expected to contribute to the resting, background or leak K^+ conductance of any cell in which they are expressed. In a physiological extracellular [K^+], the current voltage relationship of TWIK currents is probably weakly inwardly rectifying (although TWIK-1 expresses only weakly and KCNK-7 not at all), whereas all other K_{2P} channels show weak outward rectification that closely resembles rectification of the Goldman Hodgkin Katz type. In a symmetrical K^+ gradient, TASK-1, TASK-3, TASK-2, TRAAK, TREK-2, THIK-1, TALK-1, TALK-2 and TRESK all display either linear current voltage relationships or only show weak rectification. The only channel that seems to show significant voltage sensitivity is TREK-1.[14,15]

2.4 Regulation and Pharmacology of K_{2P} Channels

There are a number of distinctive regulatory and pharmacological profiles relating to some of the different groups of K_{2P} channels. Those outlined either suggest specific physiological or pharmacological roles for these channels or have proved to be of use in trying to identify endogenous K_{2P} channels.

2.4.1 Classical K^+ Channel Inhibitors

The K_{2P} channels are all largely or wholly resistant to the effects of TEA and 4-aminopyridine (4-AP). This feature, coupled with weak voltage sensitivity (see Section 2.3), is now a common means of categorising endogenous currents as background or leak channels likely to belong to the K_{2P} family. In contrast, quinidine inhibits most K_{2P} channels (with the possible exception of TRAAK).

2.4.2 Extracellular pH

Changes in extracellular pH have a significant influence over the activity of all channels in the TASK and TALK subgroups. Acidosis (relative to a normal pH_o of 7.4) inhibits TASK-1 (pK = 7.2–7.3),[16–18] TASK-3 (pK = 6.0–6.7),[19–22] TALK-1 (pK = 7.2),[23] and TASK-2 (pK = 7.8).[24] Alkalosis (pH > 7.4) activates TASK-1,[16–18] TALK-1 & TALK-2 (TASK-4).[25,26] In addition to the TASK and TALK channels, acidosis is reported to inhibit TWIK-1[27] and weakly inhibits TRESK[28] and TWIK-2.[29] These channels may therefore play an important role in pH sensing in various regions of the body and thus contribute to whole-body pH homeostasis.

2.4.3 Intracellular pH

An interesting feature of both TREK-1 and TREK-2 is that they are strongly *activated* by intracellular acidosis.[30,31] In contrast, TRAAK is activated by intracellular alkalosis. Weak inhibition by intracellular acidosis has been reported for TRESK[28] and TWIK-2.[32]

2.4.4 Temperature

Although the activity of a great many channels is affected by temperature, TREK-1, TREK-2 and TRAAK have an extraordinarily strong temperature dependence (TREK-1 Q_{10} = 7; TREK-2 Q_{10} = 14).[33,34] These channels would therefore be well suited to temperature (perhaps cold) sensing, although as yet no such role has been identified. Intriguingly, temperature sensitivity is lost from these channels on patch excision, which suggests that it must be dependent on some unknown intracellular signalling pathway.[33]

2.4.5 Polyunsaturated Fatty Acids and Lysophospholipids

Polyunsaturated fatty acids (PUFAs) are powerful activators of TREK-1, TREK-2 and TRAAK, with a threshold for activation of around 100 n*M* and no evidence of saturation at higher concentrations.[35–37] Extracellular lysophospholipids have a similar effect.[38] The actions of PUFAs are relatively slow (minutes), are retained in excised patch and are thought to be mediated through changes in membrane structure (e.g., an increase in membrane curvature or crenation[39]). The effects of lysophospholipids, however, require cell integrity and so are presumed to depend on an unknown intracellular signalling pathway.[38] PUFAs also weakly activate THIK-1 and TWIK-2,[29,40] but neither has an effect on other K_{2P} channels or weakly inhibits them (TASK-3, TASK-1, TRESK).[28,41]

2.4.6 Membrane Stretch and Stress

TREK-1, TREK-2 and TRAAK are all stretch-activated channels.[30,35,36,42–44] They are strongly activated by a decrease in pressure acting at the external side of the membrane both in the intact cell and in excised patches. This suggests that the effects of pressure may be linked to an increase in outward curvature of the membrane. The effects of stretch in TREK-1 and TREK-2 are dependent on internal pH. Acidosis shifts the pressure activation curve to less-negative outside pressures until, at very low intracellular pH, the channel becomes constitutively active, and stretch sensitivity is lost.[30] At the whole-cell level, these channels are likely to be activated by cell swelling or hypotonic solutions, so they have the capacity to play a role in cell volume regulation and perhaps as osmoreceptors.

2.4.7 Gaseous General Anaesthetics

TREK-1 is strongly activated by a wide range of gaseous general anaesthetics, including halothane, isoflurane, chloroform, diethyl ether, cyclopropane, nitrous oxide and xenon.[45,46] TREK-1 is therefore now thought to be a key target for these agents in inducing anaesthesia.[47–49] Comparable data are not yet available for TREK-2, but it is similarly activated by halothane, isoflurane and chloroform.[43] In addition to the TREK channels, a number of other K_{2P} channels are activated by some general anaesthetics but not all. TASK-3, for example, is activated by halothane, halogenated ethers and chloroform but not by nitrous oxide, cyclopropane or xenon.[45,46] TASK-1 can also be activated by halothane and halogenated ethers but is resistant, or may be inhibited, by chloroform and diethyl ether.[45,50,51] Halothane and halogenated ethers also activate TRESK.[52] In contrast, TALK-1, TALK-2 and THIK-1 are all weakly inhibited by halothane.[26,40]

2.4.8 Other Pharmacology

TASK-1 and TASK-3 differ slightly in their pH sensitivity and in their single-channel conductance but are otherwise difficult to distinguish. TASK-1 is, however, directly inhibited by anandamide[53], whereas TASK-3 is inhibited by external Mg^{2+},[19] Zn^{2+} and ruthenium red.[54,55] Hydroxy-α-sanshool, the active ingredient of Szechwan pepper, has also been reported to be a selective inhibitor of TASK-1, TASK-3 and TRESK. It has greatest affinity for TASK-1 > TRESK >> TASK-3.[56] TRESK is also strongly inhibited by low concentrations (3 μ*M*) of Hg^{3+}.[57]

2.5 Identifying Endogenous Channels

Many of the pharmacological properties described, together with single-channel biophysics, may be used to help identify endogenous K_{2P} channel activity, but care needs to be exercised. Not all of the regulatory features described have been tested on all K_{2P} channels or indeed members of other K^+ channel families that can generate leak-like K^+ conductance. It is also a strong possibility that properties of endogenous K_{2P} channels may well be different from those of heterologously expressed channels. First, the extent to which these channels can form heterodimers or associate with other protein subunits is unknown. Second, three members of this family have never been seen to form functional channels in heterologous expressions systems (KCNK7, TASK5 and THIK2). As will be discussed, there are currently a number of endogenous K_{2P}-like channels that cannot as yet be definitively ascribed to any specific K_{2P} channel, although they display many of the hallmarks of these channels.

3 Endogenous Background Potassium Channels and Their Role in Chemoreception

3.1 Central Chemoreceptors and Acid or CO_2 Sensing

Breathing is powerfully regulated by both peripheral and central chemoreceptors that monitor blood pH, pCO_2 and oxygen levels. The ventilatory response to even a small change in CO_2 levels is particularly marked. A large part, about 60–70%, of the steady-state ventilatory response to CO_2 derives from the central chemoreceptors.[58,59] It is still not altogether certain whether the central chemoreceptors detect CO_2 directly or, more likely, respond to a consequential change in brain extracellular fluid pH or neuronal intracellular pH. To some extent, this reflects not only the difficulty in experimentally discriminating between these two potential stimuli in intact brain stem preparations but also uncertainties over the precise locations and identities of the chemosensitive neurons themselves. A number of sites have been found within the brain stem at which there appears to be CO_2- or pH-sensitive neuronal activity and at which focal acidification evokes a ventilatory response. These include locations in and around the ventral surface of the medulla, including the medullary raphe, the retrotrapezoid nucleus (RTN) and the ventral respiratory group as well as dorsal locations in the nucleus tractus solitarii (NTS) and locus coeruleus (LC).[60] Discussion of the relative importance of these various sites is beyond the scope of this review, but there seems to be growing evidence that the retrotrapezoid nucleus plays a particularly prominent role.[61]

The effects of CO_2 or acidosis on neurons within some of these regions can be either depolarizing/excitatory or hyperpolarizing/inhibitory. There is generally little information on how acidosis inhibits electrical activity in brain stem neurons, but inhibition of I_H (hyperpolarisation-activated channels) has been implicated in some.[62] In the case of acid-excited neurons, however, there seems to be growing evidence for the widespread involvement of background potassium channels.

The first example to be described was in the LC, which expresses high levels of TASK-1.[63] Increasing CO_2 causes membrane depolarization and increased discharge frequency in LC neurons.[64] In contrast, halothane hyperpolarizes and inhibits electrical activity. The effects of both acidosis and halothane were mediated by the same background potassium current, with halothane activating this current and acidosis inhibiting it.[63,65] Inhibition by acidosis and activation by halothane are characteristic properties of TASK channels.

In the medullary raphe, serotonergic neurons are also excited by acidosis.[66] These neurons express both TASK-1 and TASK-3 at the messenger RNA (mRNA) level and display a pH- and halothane-sensitive background K^+ conductance.[67] Genetic ablation of either TASK-1 or TASK-3 results in a marked decrease in the pH sensitivity of serotonergic raphe neurons, confirming the role of TASK channels in acid sensing in these cells.[68]

In CO_2-stimulated glutamatergic neurons of the RTN, there is again evidence for the presence of an acid-sensitive background/leak potassium conductance.[69] This current, however, is unaffected by halothane, and the pH sensitivity of RTN neurons is retained in TASK-1/TASK-3 double-knockout mice.[68] The identity of the pH-sensitive background K^+ current in RTN neurons is at present unknown, but there are clearly other K_{2P} candidates besides TASK1 and TASK-3, for example, TALK-1 and TASK-2, which are pH sensitive and halothane resistant.

3.2 Peripheral Chemoreceptors: Acid and Oxygen Sensing

Peripheral chemoreceptors play a vital role in initiating a number of protective responses to hypoxemia, including an increase in ventilation and modulation of regional blood flow, cardiac output and catecholamine secretion. The principal arterial chemoreceptor is the carotid body, which is comprised of primary receptive cells, type 1 cells, which synapse with afferent neurons that project to the brain stem. Type 1 cells respond within seconds to both hypoxia and acidosis with a depolarising receptor potential.[70–72] This depolarisation then initiates a sequence of events, including electrical activity, voltage-gated calcium entry[70,71,73] and neurosecretion[74,75], which culminates in excitation of afferent nerve endings.

The receptor potential generated in response to both hypoxic and acidic stimuli results primarily from the inhibition of a background potassium conductance.[71,76,77] This current is resistant to the classical K^+ channel inhibitors TEA and 4-AP[76] but can be weakly inhibited by millimolar levels of barium, by quinidine and by the local anaesthetic bupivacaine.[77] It is also strongly activated by the general anaesthetic halothane[77], which may explain the well-known ability of this class of general anaesthetics to suppress ventilatory responses to hypoxia.[78–81] It is weakly outwardly rectifying in normal physiological saline[76], but in a symmetrical K^+ gradient the current voltage relationship is linear.[77]

The channels responsible for this current have a relatively low conductance (approximately 16 pS), are highly potassium selective and are active over a wide range of membrane potentials.[77,82] The biophysical and pharmacological properties of these background K^+ channels are similar to those of TASK channels but do not conform precisely to either TASK-1 or TASK-3.[77,82] Preliminary studies utilising in situ hybridisation, reverse-transcription polymerase chain reaction (RT-PCR) or immunohistochemistry indicated that a number of closely related tandem-p-domain K^+ channel subunits are expressed in the carotid body or type 1 cell, including TASK-1, TASK-2, TASK-3, TASK-5, TREK-1, TREK-2 and TRAAK.[77,83–85]

An interesting feature of the carotid body is that it is strongly excited by all inhibitors of oxidative phosphorylation, including uncouplers, electron transport inhibitors and inhibitors of adenosine triphosphate (ATP) synthase.[86–94] These same agents also inhibit background K^+ channel activity in type 1 cells. The mechanism of excitation by mitochondrial inhibitors is thus identical to that of other chemostimuli; that is, inhibition of background K^+ channel activity causes membrane depolarisation,

voltage-gated calcium entry[95,96] and neurosecretion.[94] Although the nature of the link between metabolism and channel activity has not yet been definitively established, studies have identified two candidate pathways. The first is direct regulation by cytosolic MgATP, which strongly activates channels in the excised patch with a $K_{½}$ of 2.3 m*M*.[97] The second possibility is that channel activity may be regulated by the energy sensor adenosine monophosphate (AMP)-kinase.[98]

There are many hypotheses regarding how oxygen is sensed within arterial chemoreceptors.[99] One of the oldest and most enduring is that oxygen sensing is linked to mitochondrial function.[87] Since background K^+ channel activity is strongly dependent on oxidative phosphorylation, oxygen sensing via this mechanism is clearly possible. Moreover, evidence suggests that it may be the main mechanism by which oxygen levels influence background K^+ channel activity. Blockade of oxidative phosphorylation does not fully inhibit background K^+ channel activity but does occlude further effects of hypoxia on residual channel activity.[77,95,96] Thus, oxygen sensitivity is dependent on metabolism.

3.3 Adrenal Gland: Potassium Sensing

The zona glomerulosa cells of the adrenal cortex synthesise and secrete the mineralocorticoid aldosterone. Aldosterone is a major regulator of salt and extracellular fluid balance. It primarily acts on the distal tubule of the kidney to enhance Na^+ reabsorption and K^+ secretion. The secretion of aldosterone is influenced by a number of factors, the most important of which are probably plasma K^+, angiotensin II and adrenocorticotropic hormone (ACTH). Angiotensin II appears to work through two mechanisms; first, AT1 receptor-coupled mobilization of Ca^{2+} from intracellular stores causes an immediate increase in aldosterone release, which is then followed by a more sustained release mediated via membrane depolarization and Ca^{2+} entry through voltage-gated Ca^{2+} channels. High plasma potassium also evokes sustained aldosterone release through membrane depolarization and Ca^{2+} influx through T-type or L-type calcium channels.[100,101]

Zona glomerulosa cells have a prominent leak potassium current that maintains the negative resting membrane potential of the cells.[102] This current is inhibited by angiotensin II to lead to membrane depolarization and voltage-gated Ca^{2+} entry. The high resting membrane conductance to potassium ions generated by these channels also sets resting membrane potential close to the potassium equilibrium potential such that small changes in extracellular potassium have marked effects on membrane potential. This, in combination with the presence of T-type Ca^{2+} channels that may be activated at relatively negative membrane potentials, probably forms the molecular basis for extracellular (plasma) potassium sensing in these cells.[103] The biophysical and pharmacological properties of the background potassium channels in zona glomerulosa cells are similar to those of cloned TASK channels, especially TASK-3.[104] Both TASK-1 and TASK-3 channels are known to be expressed in glomerulosa cells (at the mRNA level).[105,106] TASK-1 knockout in mice has been

shown to lead to marked disruption of mineralocorticoid homeostasis with hyperaldosteronism independent of salt intake.[107] Similar studies using a double knockout of TASK-1 and TASK-3 resulted in depolarization of the resting membrane potential and loss of acid- and halothane-sensitive background K^+ currents in zona glomerulosa cells and a marked primary hyperaldosteronism phenotype.[108]

Although there is compelling evidence for the involvement of TASK channels in potassium sensing in zona glomerulosa cells of rodents, this may not be the case for all species. In bovine zona glomerulosa cells (and zona fasiculata cells), the predominant background potassium conductance is mediated by a TREK-like potassium channel.[109,110] In addition to regulation by angiotensin II, this channel is activated by PUFAs[111] and is dependent on intracellular ATP,[109] although the significance of these potential regulatory features is as yet unclear. The relative importance of TASK versus TREK channels in human zona glomerulosa cells is unknown.

3.4 Hypothalamus: Glucose Sensing

Orexinergic neurons of the lateral hypothalamus promote wakefulness and may also be involved in regulating appetite and metabolism. These neurons are exquisitely sensitive to glucose concentration. Unlike the classical glucose sensor the pancreatic beta cell, the activity of orexinergic neurons is suppressed by glucose. The mechanism by which glucose inhibits electrical activity in some neurons has long been a mystery. Studies utilising electrophysiological studies in green fluorescent protein (GFP)-expressing orexinergic neurons of mice has identified a key role for background potassium channels. In these neurons, 4.5 m*M* glucose was found to strongly activate a potassium current with only weak rectification, resulting in membrane hyperpolarisation and cessation of spontaneous electrical activity.[112] Even relatively small, physiological changes in extracellular glucose concentration (from 1 to 2.5 m*M*) were sufficient to cause significant changes in neuronal firing. The same background K^+ current was also inhibited by acidosis and activated by halothane, characteristics of a TASK-like channel. The combination of a relatively high single-channel conductance (40 pS) and resistance to ruthenium red do not, however, permit these endogenous channels to be ascribed to either TASK-1 or TASK-3, although the presence of TASK-3 protein in these cells led the authors to speculate that the endogenous channels may contain TASK-3 subunits.[112] Modulation of background K^+ current in these neurons appears to be selectively dependent on extracellular, not intracellular, glucose.[112] Moreover, studies indicated that it is independent of glucose metabolism and can be mimicked by the glucose analog 2-deoxyglucose.[113] These observations suggest that glucose may regulate channel activity via an unknown membrane receptor.

The discovery that these neurons are also sensitive to small changes in pH, a property conferred by the TASK-like background potassium current,[114] is also consistent with a role in the vigilance state-dependent control of ventilation. It has long been known that the regulation of ventilation differs quite markedly between sleep and

awake states. Orexin-deficient mice are reported to have a reduced ventilatory response to hypercapnia in the awake state but not during sleep.[115] These mice also suffer from more frequent sleep apnoea. The ability of orexinergic neurons to detect changes in PCO_2 or pH may therefore provide an additional mechanism for promoting ventilatory responses to hypercapnia whilst awake and opposing apnoea during sleep.

4 Summary

In summary, K_{2P}-like background K^+ channels play an important role in a number of internal chemoreceptors involved in homeostatic regulation. In some instances, the channels responsible have been identified through the use of genetic knockout strategies; in others, the molecular identity of the channel concerned remains to be confirmed. A pattern seems to be emerging, however, in that a number of these channels are involved in pH sensing and in nutrient or metabolic sensing.

In this review, I adopted a rather strict definition for chemosensing in which the sensor signals to, and thus controls, a homeostatic process. There are numerous other examples of K_{2P}-like potassium channels playing a role in regulating tissue function in response to some stimulus or environmental challenge. For example, TASK-1 has been implicated in oxygen sensing and hypoxic pulmonary vasoconstriction[116]; TASK-2 is activated by, and is important in sustaining, renal bicarbonate reabsorbtion[117]; and activation of TREK by intracellular acidosis and PUFAs is thought to be important in ischemic neuroprotection.[38,48,49] Moreover, I have not even touched on the role of K_{2P} channels and endogenous leak or background K^+ channels as targets for the neuromodulation of cell excitability. Although these are also important areas of research, reviewing them would require another full chapter.

It seems likely that our appreciation of the importance of the K_{2P} family of channels and of background K^+ currents in general (whether mediated by K_{2P} channels or not) is now set to grow at an increasing pace. This is due not only to the availability of knockout mice with which to investigate the physiological roles of K_{2P} channels, but also from the long overdue realisation that although seemingly small and dull, leak currents are every bit as important as their larger voltage-activated cousins in controlling cell activity.

References

1. Hodgkin AL, Huxley AF (1952) The components of membrane conductance in the giant axon of Loligo. J Physiol 116:473–496
2. Ketchum KA, Joiner WJ, Sellers AJ, Kaczmarek LK, Goldstein SA (1995) A new family of outwardly rectifying potassium channel proteins with two pore domains in tandem. Nature 376:690–695
3. Wei A, Jegla T, Salkoff L (1996) Eight potassium channel families revealed by the *C. elegans* genome project. Neuropharmacology 35:805–829

4. Lesage F, Guillemare E, Fink M et al (1996) TWIK-1, a ubiquitous human weakly inward rectifying K^+ channel with a novel structure. EMBO J 15:1004–1011
5. Lesage F, Reyes R, Fink M, Duprat F, Guillemare E, Lazdunski M (1996) Dimerization of TWIK-1 K^+ channel subunits via a disulfide bridge. EMBO J 15:6400–6407
6. Berg AP, Talley EM, Manger JP, Bayliss DA (2004) Motoneurons express heteromeric TWIK-related acid-sensitive K^+ (TASK) channels containing TASK-1 (KCNK3) and TASK-3 (KCNK9) subunits. J Neurosci 24:6693–6702
7. Czirjak G, Enyedi P (2002) Formation of functional heterodimers between the TASK-1 and TASK-3 two-pore domain potassium channel subunits. J Biol Chem 277:5426–5432
8. Kang D, Han J, Talley EM, Bayliss DA, Kim D (2004) Functional expression of TASK-1/TASK-3 heteromers in cerebellar granule cells. J Physiol 554:64–77
9. Clarke CE, Veale EL, Wyse K, Vandenberg JI, Mathie A (2008) The M1P1 loop of TASK3 K2P channels apposes the selectivity filter and influences channel function. J Biol Chem 283:16985–16992
10. Goldstein SA, Bayliss DA, Kim D, Lesage F, Plant LD, Rajan S (2005) International Union of Pharmacology. LV. Nomenclature and molecular relationships of two-P potassium channels. Pharmacol Rev 57:527–540
11. Lesage F (2003) Pharmacology of neuronal background potassium channels. Neuropharmacology 44:1–7
12. Bayliss DA, Barrett PQ (2008) Emerging roles for two-pore-domain potassium channels and their potential therapeutic impact. Trends Pharmacol Sci 29:566–575
13. Talley EM, Sirois JE, Lei Q, Bayliss DA (2003) Two-pore-Domain (KCNK) potassium channels: dynamic roles in neuronal function. Neuroscientist 9:46–56
14. Maingret F, Honore E, Lazdunski M, Patel AJ (2002) Molecular basis of the voltage-dependent gating of TREK-1, a mechano-sensitive K^+ channel. Biochem Biophys Res Commun 292:339–346
15. Bockenhauer D, Zilberberg N, Goldstein SA (2001) KCNK2: reversible conversion of a hippocampal potassium leak into a voltage-dependent channel. Nat Neurosci 4:486–491
16. Duprat F, Lesage F, Fink M, Reyes R, Heurteaux C, Lazdunski M (1997) TASK, a human background K^+ channel to sense external pH variations near physiological pH. EMBO J 16:5464–5471
17. Lopes CM, Gallagher PG, Buck ME, Butler MH, Goldstein SA (2000) Proton block and voltage gating are potassium-dependent in the cardiac leak channel Kcnk3. J Biol Chem 275:16969–16978
18. Kim Y, Bang H, Kim D (1999) TBAK-1 and TASK-1, two-pore K^+ channel subunits: kinetic properties and expression in rat heart. Am J Physiol 277:H1669–H1678
19. Rajan S, Wischmeyer E, Xin Liu G et al (2000) TASK-3, a novel tandem pore domain acid-sensitive K^+ channel. An extracellular histidine as pH sensor. J Biol Chem 275:16650–16657
20. Kim Y, Bang H, Kim D (2000) TASK-3, a new member of the tandem pore K^+ channel family. J Biol Chem 275:9340–9347
21. Chapman CG, Meadows HJ, Godden RJ et al (2000) Cloning, localisation and functional expression of a novel human, cerebellum specific, two pore domain potassium channel. Brain Res Mol Brain Res 82:74–83
22. Meadows HJ, Randall AD (2001) Functional characterisation of human TASK-3, an acid-sensitive two-pore domain potassium channel. Neuropharmacology 40:551–559
23. Han J, Kang D, Kim D (2003) Functional properties of four splice variants of a human pancreatic tandem-pore K^+ channel, TALK-1. Am J Physiol Cell Physiol 285:C529–C538
24. Reyes R, Duprat F, Lesage F et al (1998) Cloning and expression of a novel pH-sensitive two pore domain K^+ channel from human kidney. J Biol Chem 273:30863–30869
25. Decher N, Maier M, Dittrich W et al (2001) Characterization of TASK-4, a novel member of the pH-sensitive, two-pore domain potassium channel family. FEBS Lett 492:84–89
26. Girard C, Duprat F, Terrenoire C et al (2001) Genomic and functional characteristics of novel human pancreatic 2P domain K^+ channels. Biochem Biophys Res Commun 282:249–256

27. Rajan S, Plant LD, Rabin ML, Butler MH, Goldstein SA (2005) Sumoylation silences the plasma membrane leak K^+ channel K2P1. Cell 121:37–47
28. Sano Y, Inamura K, Miyake A et al (2003) A novel two-pore domain K^+ channel, TRESK, is localized in the spinal cord. J Biol Chem 278:27406–27412
29. Patel AJ, Maingret F, Magnone V, Fosset M, Lazdunski M, Honore E (2000) TWIK-2, an inactivating 2P domain K^+ channel. J Biol Chem 275:28722–28730
30. Maingret F, Patel AJ, Lesage F, Lazdunski M, Honore E (1999) Mechano- or acid stimulation, two interactive modes of activation of the TREK-1 potassium channel. J Biol Chem 274:26691–26696
31. Honore E, Maingret F, Lazdunski M, Patel AJ (2002) An intracellular proton sensor commands lipid- and mechano-gating of the K^+ channel TREK-1. EMBO J 21:2968–2976
32. Chavez RA, Gray AT, Zhao BB et al (1999) TWIK-2, a new weak inward rectifying member of the tandem pore domain potassium channel family. J Biol Chem 274:7887–7892
33. Maingret F, Lauritzen I, Patel AJ et al (2000) TREK-1 is a heat-activated background K^+ channel. EMBO J 19:2483–2491
34. Kang D, Choe C, Kim D (2005) Thermosensitivity of the two-pore domain K^+ channels TREK-2 and TRAAK. J Physiol 564:103–116
35. Patel AJ, Honore E, Maingret F et al (1998) A mammalian two pore domain mechano-gated S-like K^+ channel. EMBO J 17:4283–4290
36. Bang H, Kim Y, Kim D (2000) TREK-2, a new member of the mechanosensitive tandem-pore K^+ channel family. J Biol Chem 275:17412–17419
37. Fink M, Lesage F, Duprat F et al (1998) A neuronal two P domain K^+ channel stimulated by arachidonic acid and polyunsaturated fatty acids. EMBO J 17:3297–3308
38. Maingret F, Patel AJ, Lesage F, Lazdunski M, Honore E (2000) Lysophospholipids open the two-pore domain mechano-gated K^+ channels TREK-1 and TRAAK. J Biol Chem 275:10128–10133
39. Patel AJ, Lazdunski M, Honore E (2001) Lipid and mechano-gated 2P domain K^+ channels. Curr Opin Cell Biol 13:422–428
40. Rajan S, Wischmeyer E, Karschin C et al (2001) THIK-1 and THIK-2, a novel subfamily of tandem pore domain K^+ channels. J Biol Chem 276:7302–7311
41. Patel AJ, Honore E (2001) Properties and modulation of mammalian 2P domain K^+ channels. Trends Neurosci 24:339–346
42. Maingret F, Fosset M, Lesage F, Lazdunski M, Honore E (1999) TRAAK is a mammalian neuronal mechano-gated K^+ channel. J Biol Chem 274:1381–1387
43. Lesage F, Terrenoire C, Romey G, Lazdunski M (2000) Human TREK2, a 2P domain mechano-sensitive K^+ channel with multiple regulations by polyunsaturated fatty acids, lysophospholipids, and Gs, Gi, and Gq protein-coupled receptors. J Biol Chem 275:28398–28405
44. Lesage F, Maingret F, Lazdunski M (2000) Cloning and expression of human TRAAK, a polyunsaturated fatty acids-activated and mechano-sensitive K^+ channel. FEBS Lett 471:137–140
45. Patel AJ, Honore E, Lesage F, Fink M, Romey G, Lazdunski M (1999) Inhalational anesthetics activate two-pore-domain background K^+ channels. Nat Neurosci 2:422–426
46. Gruss M, Bushell TJ, Bright DP, Lieb WR, Mathie A, Franks NP (2004) Two-pore-domain K^+ channels are a novel target for the anesthetic gases xenon, nitrous oxide, and cyclopropane. Mol Pharmacol 65:443–452
47. Patel AJ, Honore E (2001) Anesthetic-sensitive 2P domain K^+ channels. Anesthesiology 95:1013–1021
48. Franks NP, Honore E (2004) The TREK K2P channels and their role in general anaesthesia and neuroprotection. Trends Pharmacol Sci 25:601–608
49. Heurteaux C, Guy N, Laigle C et al (2004) TREK-1, a K^+ channel involved in neuroprotection and general anesthesia. EMBO J 23:2684–2695
50. Andres-Enguix I, Caley A, Yustos R et al (2007) Determinants of the anesthetic sensitivity of two-pore domain acid-sensitive potassium channels: molecular cloning of an anesthetic-activated potassium channel from *Lymnaea stagnalis*. J Biol Chem 282:20977–20990

51. Putzke C, Hanley PJ, Schlichthorl G et al (2007) Differential effects of volatile and intravenous anesthetics on the activity of human TASK-1. Am J Physiol Cell Physiol 293: C1319–C1326
52. Liu C, Au JD, Zou HL, Cotten JF, Yost CS (2004) Potent activation of the human tandem pore domain K channel TRESK with clinical concentrations of volatile anesthetics. Anesth Analg 99:1715–1722
53. Maingret F, Patel AJ, Lazdunski M, Honore E (2001) The endocannabinoid anandamide is a direct and selective blocker of the background K^+ channel TASK-1. EMBO J 20:47–54
54. Czirjak G, Enyedi P (2003) Ruthenium red inhibits TASK-3 potassium channel by interconnecting glutamate 70 of the two subunits. Mol Pharmacol 63:646–652
55. Clarke CE, Veale EL, Green PJ, Meadows HJ, Mathie A (2004) Selective block of the human 2-P domain potassium channel, TASK-3, and the native leak potassium current, IK_{SO}, by zinc. J Physiol 560:51–62
56. Bautista DM, Sigal YM, Milstein AD et al (2008) Pungent agents from Szechuan peppers excite sensory neurons by inhibiting two-pore potassium channels. Nat Neurosci 11:772–779
57. Czirjak G, Enyedi P (2006) Zinc and mercuric ions distinguish TRESK from the other two-pore-domain K^+ channels. Mol Pharmacol 69:1024–1032
58. Nattie E (2006) Why do we have both peripheral and central chemoreceptors? J Appl Physiol 100:9–10
59. Smith CA, Rodman JR, Chenuel BJ, Henderson KS, Dempsey JA (2006) Response time and sensitivity of the ventilatory response to CO_2 in unanesthetized intact dogs: central vs. peripheral chemoreceptors. J Appl Physiol 100:13–19
60. Nattie E, Li A (2006) Central chemoreception 2005: a brief review. Auton Neurosci 126–127:332–338
61. Guyenet PG, Bayliss DA, Mulkey DK, Stornetta RL, Moreira TS, Takakura AT (2008) The retrotrapezoid nucleus and central chemoreception. Adv Exp Med Biol 605:327–332
62. Wellner-Kienitz MC, Shams H (1998) Hyperpolarization-activated inward currents contribute to spontaneous electrical activity and CO_2/H^+ sensitivity of cultivated neurons of fetal rat medulla. Neuroscience 87:109–121
63. Sirois JE, Lei Q, Talley EM, Lynch C 3rd, Bayliss DA (2000) The TASK-1 two-pore domain K^+ channel is a molecular substrate for neuronal effects of inhalation anesthetics. J Neurosci 20:6347–6354
64. Oyamada Y, Ballantyne D, Muckenhoff K, Scheid P (1998) Respiration-modulated membrane potential and chemosensitivity of locus coeruleus neurones in the in vitro brainstem-spinal cord of the neonatal rat. J Physiol 513:381–398
65. Bayliss DA, Talley EM, Sirois JE, Lei Q (2001) TASK-1 is a highly modulated pH-sensitive 'leak' K^+ channel expressed in brainstem respiratory neurons. Respir Physiol 129:159–174
66. Wang W, Tiwari JK, Bradley SR, Zaykin RV, Richerson GB (2001) Acidosis-stimulated neurons of the medullary raphe are serotonergic. J Neurophysiol 85:2224–2235
67. Washburn CP, Sirois JE, Talley EM, Guyenet PG, Bayliss DA (2002) Serotonergic raphe neurons express TASK channel transcripts and a TASK-like pH- and halothane-sensitive K^+ conductance. J Neurosci 22:1256–1265
68. Mulkey DK, Talley EM, Stornetta RL et al (2007) TASK channels determine pH sensitivity in select respiratory neurons but do not contribute to central respiratory chemosensitivity. J Neurosci 27:14049–14058
69. Mulkey DK, Stornetta RL, Weston MC et al (2004) Respiratory control by ventral surface chemoreceptor neurons in rats. Nat Neurosci 7:1360–1369
70. Buckler KJ, Vaughan Jones RD (1994) Effects of hypoxia on membrane potential and intracellular calcium in rat neonatal carotid body type I cells. J Physiol 476:423–428
71. Buckler KJ, Vaughan Jones RD (1994) Effects of hypercapnia on membrane potential and intracellular calcium in rat carotid body type I cells. J Physiol 478:157–171
72. Rocher A, Geijo Barrientos E, Caceres AI, Rigual R, Gonzalez C, Almaraz L (2005) Role of voltage-dependent calcium channels in stimulus-secretion coupling in rabbit carotid body chemoreceptor cells. J Physiol 562:407–420

73. Weir EK, Lopez-Barneo J, Buckler KJ, Archer SL (2005) Acute oxygen-sensing mechanisms. N Engl J Med 353:2042–2055
74. Gonzalez C, Almaraz L, Obeso A, Rigual R (1992) Oxygen and acid chemoreception in the carotid body chemoreceptors. Trends Neurosci 15:146–153
75. Montoro RJ, Urena J, Fernandez Chacon R, Alvarez de Toledo G, Lopez Barneo J (1996) Oxygen sensing by ion channels and chemotransduction in single glomus cells. J Gen Physiol 107:133–143
76. Buckler KJ (1997) A novel oxygen-sensitive potassium current in rat carotid body type I cells. J Physiol 498:649–662
77. Buckler KJ, Williams BA, Honore E (2000) An oxygen-, acid- and anaesthetic-sensitive TASK-like background potassium channel in rat arterial chemoreceptor cells. J Physiol 525: 135–142
78. Ponte J, Sadler CL (1989) Effect of halothane, enflurane and isoflurane on carotid body chemoreceptor activity in the rabbit and the cat. Br J Anaesth 62:33–40
79. Davies RO, Edwards MW Jr, Lahiri S (1982) Halothane depresses the response of carotid body chemoreceptors to hypoxia and hypercapnia in the cat. Anesthesiology 57:153–159
80. Knill RL, Gelb AW (1978) Ventilatory responses to hypoxia and hypercapnia during halothane sedation and anesthesia in man. Anesthesiology 49:244–251
81. Pandit JJ (2002) The variable effect of low-dose volatile anaesthetics on the acute ventilatory response to hypoxia in humans: a quantitative review. Anaesthesia 57:632–643
82. Williams BA, Buckler KJ (2004) Biophysical properties and metabolic regulation of a TASK-like potassium channel in rat carotid body type 1 cells. Am J Physiol Lung Cell Mol Physiol 286:L221–L230
83. Yamamoto K, Kummer W, Atoji Y, Suzuki Y (2002) TASK-1, TASK-2, TASK-3 and TRAAK immunoreactivities in the rat carotid body. Brain Res 950:304–307
84. Kim I, Kim JH, Carroll JL (2006) Postnatal changes in gene expression of subfamilies of TASK K+ channels in rat carotid body. Adv Exp Med Biol 580:43–47
85. Yamamoto Y, Taniguchi K (2006) Immunolocalization of tandem pore domain K+ channels in the rat carotid body. Adv Exp Med Biol 580:9–14
86. Wilson DF, Mokashi A, Chugh D, Vinogradov S, Osanai S, Lahiri S (1994) The primary oxygen sensor of the cat carotid body is cytochrome a3 of the mitochondrial respiratory chain. FEBS Lett 351:370–374
87. Anichkov S, Belen'kii M (1963) Pharmacology of the carotid body chemoreceptors. Pergamon, Oxford, UK
88. Mulligan E, Lahiri S, Storey BT (1981) Carotid body O2 chemoreception and mitochondrial oxidative phosphorylation. J Appl Physiol 51:438–446
89. Shen TCR, Hauss WH (1939) Influence of dinitrophenol, dinitroortocresol and paranitrophenol upon the carotid sinus chemoreceptors of the dog. Arch. Int Pharmacodyn Ther 63:251–258
90. Biscoe TJ, Duchen MR (1990) Responses of type I cells dissociated from the rabbit carotid body to hypoxia. J Physiol 428:39–59
91. Mosqueria M, Iturriaga R (2002) Carotid body chemosensory excitation induced by nitric oxide: involvement of oxidative metabolism. Respir Physiol Neurobiol 131:175–187
92. Mulligan E, Lahiri S (1981) Dependence of carotid chemoreceptor stimulation by metabolic agents on PaO2 and PaCO2. J Appl Physiol 50:884–891
93. Obeso A, Almaraz L, Gonzalez C (1989) Effects of cyanide and uncouplers on chemoreceptor activity and ATP content of the cat carotid body. Brain Res 481:250–257
94. Ortega-Sáenz P, Pardal R, Garcáa Fernández M, López Barneo J (2003) Rotenone selectively occludes sensitivity to hypoxia in rat carotid body glomus cells. J Physiol 548:789–800
95. Buckler KJ, Vaughan Jones RD (1998) Effects of mitochondrial uncouplers on intracellular calcium, pH and membrane potential in rat carotid body type I cells. J Physiol 513: 819–833
96. Wyatt CN, Buckler KJ (2004) The effect of mitochondrial inhibitors on membrane currents in isolated neonatal rat carotid body type I cells. J Physiol 556:175–191

97. Varas R, Wyatt CN, Buckler KJ (2007) Modulation of TASK-like background potassium channels in rat arterial chemoreceptor cells by intracellular ATP and other nucleotides. J Physiol 583:521–536
98. Wyatt CN, Kumar P, Aley P, Peers C, Hardie DG, Evans AM (2006) Does AMP-activated protein kinase couple hypoxic inhibition of oxidative phosphorylation to carotid body excitation? Adv Exp Med Biol 580:191–196
99. Prabhakar NR (2006) O_2 sensing at the mammalian carotid body: why multiple O_2 sensors and multiple transmitters? Exp Physiol 91:17–23
100. Lotshaw DP (2001) Role of membrane depolarization and T-type Ca^{2+} channels in angiotensin II and K^+ stimulated aldosterone secretion. Mol Cell Endocrinol 175:157–171
101. Balla T, Varnai P, Hollo Z, Spat A (1990) Effects of high potassium concentration and dihydropyridine Ca^{2+}-channel agonists on cytoplasmic Ca^{2+} and aldosterone production in rat adrenal glomerulosa cells. Endocrinology 127:815–822
102. Lotshaw DP (1997) Characterization of angiotensin II-regulated K+ conductance in rat adrenal glomerulosa cells. J Membr Biol 156:261–277
103. Spat A (2004) Glomerulosa cell — a unique sensor of extracellular K^+ concentration. Mol Cell Endocrinol 217:23–26
104. Lotshaw DP (2006) Biophysical and pharmacological characteristics of native two-pore domain TASK channels in rat adrenal glomerulosa cells. J Membr Biol 210:51–70
105. Czirjak G, Fischer T, Spat A, Lesage F, Enyedi P (2000) TASK (TWIK-related acid-sensitive K^+ channel) is expressed in glomerulosa cells of rat adrenal cortex and inhibited by angiotensin II. Mol Endocrinol 14:863–874
106. Czirjak G, Enyedi P (2002) TASK-3 dominates the background potassium conductance in rat adrenal glomerulosa cells. Mol Endocrinol 16:621–629
107. Heitzmann D, Derand R, Jungbauer S et al (2008) Invalidation of TASK1 potassium channels disrupts adrenal gland zonation and mineralocorticoid homeostasis. EMBO J 27: 179–187
108. Davies LA, Hu C, Guagliardo NA et al (2008) TASK channel deletion in mice causes primary hyperaldosteronism. Proc Natl Acad Sci U S A 105:2203–2208
109. Enyeart JJ, Xu L, Danthi S, Enyeart JA (2002) An ACTH- and ATP-regulated background K^+ channel in adrenocortical cells is TREK-1. J Biol Chem 277:49186–49199
110. Enyeart JA, Danthi SJ, Enyeart JJ (2004) TREK-1 K+ channels couple angiotensin II receptors to membrane depolarization and aldosterone secretion in bovine adrenal glomerulosa cells. Am J Physiol Endocrinol Metab 287:E1154–E1165
111. Danthi S, Enyeart JA, Enyeart JJ (2003) Modulation of native TREK-1 and Kv1.4 K^+ channels by polyunsaturated fatty acids and lysophospholipids. J Membr Biol 195:147–164
112. Burdakov D, Jensen LT, Alexopoulos H et al (2006) Tandem-pore K^+ channels mediate inhibition of orexin neurons by glucose. Neuron 50:711–722
113. Gonzalez JA, Jensen LT, Fugger L, Burdakov D (2008) Metabolism-independent sugar sensing in central orexin neurons. Diabetes 57:2569–2576
114. Williams RH, Jensen LT, Verkhratsky A, Fugger L, Burdakov D (2007) Control of hypothalamic orexin neurons by acid and CO_2. Proc Natl Acad Sci U S A 104:10685–10690
115. Kuwaki T (2008) Orexinergic modulation of breathing across vigilance states. Respir Physiol Neurobiol 164:204–212
116. Olschewski A, Li Y, Tang B et al (2006) Impact of TASK-1 in human pulmonary artery smooth muscle cells. Circ Res 98:1072–1080
117. Warth R, Barriere H, Meneton P et al (2004) Proximal renal tubular acidosis in TASK2 K^+ channel-deficient mice reveals a mechanism for stabilizing bicarbonate transport. Proc Natl Acad Sci U S A 101:8215–8220

Intricate Interaction Between Store-Operated Calcium Entry and Calcium-Activated Chloride Channels in Pulmonary Artery Smooth Muscle Cells

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Abstract Ca^{2+}-activated Cl^- channels (Cl_{Ca}) represent an important excitatory mechanism in vascular smooth muscle cells. Active accumulation of Cl^- by several classes of anion transporters results in an equilibrium potential for this ion about 30 mV more positive than the resting potential. Stimulation of Cl_{Ca} channels leads to membrane depolarization, which enhances Ca^{2+} entry through voltage-gated Ca^{2+} channels and leads to vasoconstriction. Cl_{Ca} channels can be activated by distinct sources of Ca^{2+} that include (1) mobilization from intracellular Ca^{2+} stores (ryanodine or inositol 1,4,5-trisphosphate [$InsP_3$]) and (2) Ca^{2+} entry through voltage-gated Ca^{2+} channels or reverse-mode Na^+/Ca^{2+} exchange. The present study was undertaken to determine whether Ca^{2+} influx triggered by store depletion (store-operated calcium entry, SOCE) activates Cl_{Ca} channels in rabbit pulmonary artery (PA) smooth muscle. Classical store depletion protocols involving block of sarcoplasmic reticular Ca^{2+} reuptake with thapsigargin (TG; 1 μM) or cyclopiazonic acid (CPA; 30 μM) led to a consistent nifedipine-insensitive contraction of intact PA rings and rise in intracellular Ca^{2+} concentration in single PA myocytes that required the presence of extracellular Ca^{2+}. In patch clamp experiments, TG or CPA activated a time-independent nonselective cation current (I_{SOC}) that (1) reversed between –10 and 0 mV; (2) displayed the typical "N"-shaped current–voltage relationship; and (3) was sensitive to the (I_{SOC}) blocker by SKF-96365 (50 μM). In double-pulse protocol experiments, the amplitude of I_{SOC} was varied by altering membrane potential during an initial step that was followed by a second constant step to +90 mV to register Ca^{2+}-activated Cl^- current, $I_{Cl(Ca)}$. The niflumic acid-sensitive time-dependent

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$I_{Cl(Ca)}$ at +90 mV increased in proportion to the magnitude of the preceding hyperpolarizing step, an effect attributed to graded membrane potential-dependent Ca^{2+} entry through I_{SOC} and confirmed in dual patch clamp and Fluo-5 experiments to record membrane current and free intracellular Ca^{2+} concentration simultaneously. Reverse-transcription polymerase chain reaction (RT-PCR) experiments confirmed the expression of several molecular determinants of SOCE, including transient receptor potential canonical (TRPC) 1, TRPC4, and TRPC6; stromal interacting molecule (STIM) 1 and 2; and Orai1 and 2, as well as the novel and probable molecular candidates thought to encode for Cl_{Ca} channels transmembrane protein 16A (TMEM16A) Anoctamin 1 (ANO1) and B (ANO2). Our preliminary investigation provides new evidence for a Ca^{2+} entry pathway consistent with store-operated Ca^{2+} entry signaling that can activate Ca^{2+}-activated Cl^- channels in rabbit PA myocytes. We hypothesize that this mechanism may be important in the regulation of membrane potential, Ca^{2+} influx, and tone in these cells under physiological and pathophysiological conditions.

Keywords Calcium-activated chloride channels • store-operated calcium entry (SOCE) • capacitative calcium entry (CCE) • vascular smooth muscle cells pulmonary arterial tone • contraction • intracellular calcium concentration • whole-cell patch clamp technique • TMEM16a • STIM1 • Orai • TRPC

1 Introduction

In many nonexcitable and excitable cell types, depletion of endoplasmic reticulum (ER) Ca^{2+} stores triggers the opening of channels in the plasma membrane that enable store replenishment. Physiologically, this process is activated by an agonist binding to a surface receptor, generally a G protein-coupled receptor (e.g., G_q) that triggers the synthesis of the second messenger diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) originating from the breakdown of the membrane phospholipid phosphatidylinositol. Whereas DAG serves as an activator of protein kinase C, an important signaling kinase, IP_3 binds to a Ca^{2+} channel receptor in the ER ($InsP_3$ or $InsP_3R$) that promotes Ca^{2+} mobilization, yielding a cellular response (e.g., contraction, secretion of a hormone or neurotransmitter, etc.).[1] The plasma membrane Ca^{2+} influx pathway stimulated by IP_3-mediated emptying of the Ca^{2+} stores or capacitative calcium entry (CCE), was originally proposed by Putney[2] and is now routinely referred to as store-operated calcium entry (SOCE). SOCE has been reported in a number of vascular smooth muscle cells, including pulmonary artery (PA) myocytes.[3–6]

The nature of the ionic conductance at the plasma membrane and mechanism by which SOCE mediates its activation is far from being understood but has received a lot of attention in light of the discovery of new molecular candidates participating in this pathway. Patch clamp studies revealed two different types of ionic current behaviors evoked by SOCE in different cell types: (1) a current mediated by a Ca^{2+} release-activated channel called I_{CRAC} (or CRAC) displaying high selectivity for Ca^{2+}

was first described in T lymphocytes and the commonly used Jurkat cell model[7]; (2) a nonselective cation channel (NSCC) current.[4,8,9] I_{CRAC} was the first SOCE-induced ionic current described. The underlying Ca^{2+}-selective channel displays strong inward rectification and a single-channel conductance that is below the resolution of the patch clamp technique and could only be indirectly inferred from fluctuation analysis of stationary and nonstationary analysis of I_{CRAC}. The NSCC current (I_{NSCC}) elicited by store depletion exhibits variable rectifying properties and a reversal potential lying between ~ –10 and +10 mV. The channels are mostly permeable to monovalent cations such as Na^+ and K^+ but exhibit significant permeability to Ca^{2+}. The single-channel conductance is also higher than that of CRAC channels. Relatively recent reports have provided evidence that homo- or heterotetrameric channels formed by one or several members of the canonical transient receptor potential (TRPC) family of genes may be an integral part of the pore-forming subunit of the NSCC activated by store depletion (TRPC1, TRPC4, TRPC5, and in some cases TRPC6).[9] Wide genomic screening techniques identified two new gene families, Orai and the stromal interacting molecule (STIM), which were suggested to form the molecular basis of I_{CRAC}.[10] Studies have shown that Orai proteins (Orai1–3) are transmembrane proteins found in a wide variety of cell types[11], and it has been suggested that Orai1 may be the pore-forming subunit of the I_{CRAC} channel.[12] In addition, overexpression studies also indicated that Orai2 is likely involved in the formation of I_{CRAC} channels as it also produces augmented currents but with lower efficacy than Orai1.[13] It seems doubtful that Orai3 is at all responsible for this current.[13] STIM1 and STIM2 are transmembrane proteins located in the ER and plasma membrane and speculated to act as the sensor for ER Ca^{2+} depletion, although the exact role of STIM2 in SOCE will require further investigation. Following store depletion, STIM1 would somehow aggregate near the plasma membrane and trigger the opening of Orai1 via a protein–protein interaction.[14] Evidence has also linked the activation of TRPC1 (and thus I_{NSCC}) to STIM1 through a dynamic trafficking process involving lipid rafts.[15] It has also been suggested that Orai1, TRPC1, and STIM1 may form a structural and dynamic trio of proteins allowing for a flexible array of SOCE patterns in the same cell.[16–18]

Since 2001, our group has focused on the elucidation of the biophysical properties, pharmacology, regulation, and functional significance of Ca^{2+}– activated Cl^- channels (Cl_{Ca}) in arterial and venous smooth muscle cells. These channels are believed to be important in signal transduction because their activation causes membrane depolarization.[19,20] This is due to an equilibrium potential for Cl^- that is about 20–30 mV more positive than the resting membrane potential of vascular smooth muscle cells and is the product of active accumulation of Cl^- by ion transporters. These channels are activated by a rise in intracellular Ca^{2+} concentration above about 180 n*M* and are modulated by voltage. Cl_{Ca} channels can be activated by distinct sources of Ca^{2+}, which include Ca^{2+} entry through voltage-gated Ca^{2+} channels or reverse-mode Na^+/Ca^{2+} exchange, or from mobilization of intracellular Ca^{2+} stores that may occur spontaneously due to short-lived spontaneous Ca^{2+} release events from ryanodine receptors in the sarcoplasmic reticulum (SR) or Ca^{2+} sparks (so-called STICs, standing for spontaneous transient inward currents) or evoked by stimulation by an agonist leading to the production of IP_3 and subsequent Ca^{2+} release from the SR.

The very low conductance Cl_{Ca} channel (~1–3 pS) displays strong outward rectification, especially at low to moderate levels of $[Ca^{2+}]_i$, and typical slow activation and deactivation kinetics at positive and negative potentials, respectively. The CLCA, Tweety, Bestrophin, and very recently the TMEM16A/B gene families have been proposed as molecular candidates encoding for Cl_{Ca} channels.[21–23] The CLCA family is most likely not a bona fide ion channel and is now speculated to be a regulator of Cl^- conductance. Some members of the Tweety family encode for Ca^{2+}-activated Cl^- channels, but their conductance is very high (>250 pS) and therefore does not fit the profile of the very-low-conductance Cl_{Ca} channel identified in native cells. Several members of the Bestrophin family of genes form Cl^--permeable small conductance channels that are activated by relatively high-affinity binding of Ca^{2+} ($K_d \approx 250$ n*M*); however the channels are voltage insensitive and lack time dependence. Three independent groups presented evidence suggesting that expression of TMEM16A (and TMEM16B in one study) in various heterologous expression cell systems generates a membrane current that is consistent with native Ca^{2+}-activated Cl^- currents recorded in many secretory and retinal epithelia, smooth muscle, and sensory neurons.[24–26] Common properties include (1) an identical anion permeability sequence ($SCN^- >> NO_3^- > I^- > Br^- > Cl^- >> F^- >>$ gluconate); (2) a voltage-sensitive K_d for activation by intracellular Ca^{2+}, (3) a low single-channel conductance (8.3 pS); and (4) time- and voltage-dependent macroscopic currents exhibiting outward rectification, slow activation on depolarization, and slow deactivation following repolarization.

Several groups of investigators have previously demonstrated the existence of SOCE in smooth muscle from PAs[3,27] and may thus represent an additional Ca^{2+} entry pathway for activation of Cl_{Ca} channels. The purpose of the present study undertaken in rabbit PA smooth muscle cells was twofold: (1) to test the hypothesis that Ca^{2+} entry elicited by store depletion can stimulate Cl_{Ca} channels and (2) to carry out a preliminary investigation of the expression profile of molecular candidates suspected to be involved in generating SOCE and Cl_{Ca} channels.

2 Materials and Methods

2.1 Isolation of Pulmonary Artery Myocytes

A similar method to that previously used by our group[28,29] was used to isolate smooth muscle cells. In brief, cells were prepared from the main and secondary PA branches dissected from New Zealand white rabbits (2–3 kg) killed by anesthetic overdose in accordance with British and American guidelines for animal care. After dissection and removal of connective tissue, the PAs were cut into small strips and incubated overnight (~16 h) at 4°C in a low-Ca^{2+} physiological salt solution (PSS; see composition in Section 3.2.5) containing 10 μ*M* $CaCl_2$ and about 1 mg/mL papain, 0.15 mg/mL dithiothreitol, and 1 mg/mL bovine serum albumin.

The next morning, the tissue strips were rinsed three times in low-Ca^{2+} PSS and incubated in the same solution for 5 min at 37°C. Cells were released by gentle agitation with a wide-bore Pasteur pipet and then stored at 4°C until used (within 10 h following dispersion).

2.2 Contractile Studies

The main branch of rabbit PA was dissected, placed in cold PSS, and cleaned of connective and adipose tissue. The artery was then denuded of endothelium by bubbling a gentle stream of O_2 through the intact vessels and was then cut into rings of about 3–5 mm, which were immersed in well-oxygenated PSS (95% O_2, 5% CO_2) at 37°C and mounted for tension measurements in a 30-mL bathing chamber. One of the rings was fixed via tungsten triangular mounting wire to the bottom of the chamber; the other end was hung via a similar tungsten triangular wire connected to a Grass Technologies FT03 force transducer to measure isometric force. The output of the force transducer was connected to a WPI TBM 4 Transbridge amplifier, and the amplified signal was recorded via a MP100 BIOPAC Systems analog-to-digital converter controlled by Acknowledge Software (v. 3.5.3) running on a Windows Millennium-based Pentium II Dell personal computer (PC). Rings were allowed to equilibrate for 60 min in normal PSS at a resting tension of 0.5 g before being challenged with two exposures (20 and 5 min, respectively) to 85.4 m*M* KCl to verify their responsiveness.

2.3 Patch Clamp Electrophysiology and Experimental Protocols

The nystatin-perforated or standard whole-cell configuration of the patch clamp technique was used to record macroscopic currents from freshly isolated vascular smooth muscle cells. Perforated patch access was achieved through dialysis of 400 mg nystatin per milliliter internal solution, which developed over the course of 10 min following pipet-to-cell sealing as monitored from the acceleration of the capacitative current transients elicited by repetitive 5- to 10-mV steps (100 Hz) from a holding potential (HP) of –50 mV applied by the internal pulse generator of the patch clamp amplifier. Patch pipets were manufactured from borosilicate glass and then fire polished to produce pipets with resistances of about 2 MΩ when filled with the perforated patch internal solution described in Section 3.2.5. Bathing solutions were gravity perfused from a 50-mL syringe barrel at an approximate flow rate of 1 mL/min, resulting in a complete bath exchange time of ~1 min.

All currents were monitored with an Axopatch 1D or Axopatch 200A patch clamp amplifier (Axon CNS, Molecular Devices) at room temperature (20–23°C). Output signals were filtered at 1 kHz by the patch clamp amplifier and sampled at 10 kHz via a Digidata 1322A acquisition system and pCLAMP 9.0 software

(Axon CNS, Molecular Devices) run on a Pentium IV Dell PC under Windows XP. L-type Ca^{2+} currents were elicited by stepping membrane potential to +20 mV every 20 s from an HP of –70 mV. Store-operated cation and Ca^{2+}-activated Cl^- currents induced by the store-depleting agent thapsigargin (TG) or cyclopiazonic acid (CPA) were recorded from an HP of 0 mV that consisted of a double-pulse protocol by which membrane potential was initially stepped for 1–2 s from one or a series of steps ranging from –100 to +60 mV. This initial step was used to measure the nonselective cation current in isolation and to alter the driving force for Ca^{2+} entry. A second step lasting 1–5 s to +90 or +130 mV served to elicit time-dependent Ca^{2+}-activated Cl^- current $I_{Cl(Ca)}$ and examine the impact of the preceding conditioning voltage step on the magnitude of $I_{Cl(Ca)}$. In some experiments, the second step was followed by a repolarizing step (500 ms) to –80 mV to record $I_{Cl(Ca)}$ tail.

2.4 Intracellular Ca^{2+} Concentration Measurements

Free intracellular Ca^{2+} concentration was measured with the Ca^{2+} indicator Fluo-4 or Fluo-5F. For the experiments described in Fig. 3.1c, cells were preincubated with 5 μ*M* of the ester form of the indicator (Fluo-4 AM) for 45 min at room temperature. Experiments were initiated after at least 30 min of washout of the indicator to allow for complete intracellular deesterification of the dye. These experiments were carried out using an ×40 Fluor Nikon objective (numerical aperture [NA] = 1.3) housed on an inverted Nikon Diaphot 300 microscope. Fluo-4 or Fluo-5F was excited at 490 nm with a 75-W xenon arc lamp, and epifluorescent light from the entire cell (restrained by a mechanical diaphragm) was measured at 520 nm. Epifluorescent light collected through the lateral port of the microscope was transmitted to a highly sensitive Hamamatsu photomultiplier tube housed in the spectral separator of a Solamere Technology Group SFX-2 Ratiometric Fluorometer system. The emitted Fluo-4 or Fluo-5F fluorescence signal at 520 nm was expressed relative to basal fluorescence recorded in the presence of nifedipine (F/F_0) and digitized via a Digidata 1322A acquisition system and pCLAMP 9.0 software (Axoscope or Clampex, Axon CNS, Molecular Devices) run on a Pentium IV Dell PC under Windows XP.

2.5 Solutions and Reagents

The composition of the normal PSS used in contractility experiments (Fig. 4.1a, b) was as follows (in m*M*): NaCl (120), KCl (4.2), $NaHCO_3$ (25; pH 7.4 after equilibration with 95% O_2/5% CO_2 gas), KH_2PO_4 (1.2), $MgCl_2$ (1.2), glucose (11), and $CaCl_2$ (1.8). All rings were challenged with a high-KCl solution made by substituting 80 m*M* NaCl of the above solution with 80 m*M* KCl. Single PA smooth muscle cells were isolated by incubating PA tissue strips in the following low-Ca^{2+} (10 μ*M*)

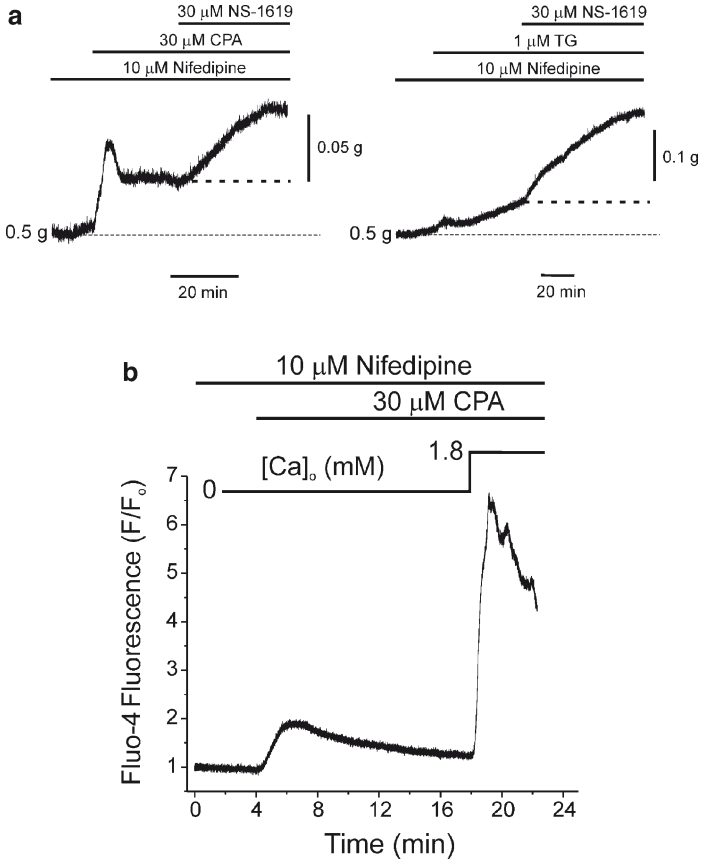

Fig. 3.1 Store depletion elicits contraction and Ca^{2+} transient in rabbit pulmonary artery smooth muscle. (**a**) Isometric tension recording showing the effects of 30 μ*M* cyclopiazonic acid (CPA) on a pulmonary arterial ring preincubated with 10 μ*M* nifedipine to inhibit L-type Ca^{2+} channels and the effect of the BK_{Ca} channel agonist NS-1619 in the continued presence of CPA and nifedipine. Thick bars above trace indicate drug applications. All solutions contained 1.8 m*M* Ca^{2+}. As indicated to the left of the trace, a 0.5-g resting tension was applied to the ring. Notice the transient and sustained contractions elicited by CPA and the enhancement of the CPA-induced sustained contraction by the hyperpolarization mediated by NS-1619. (**b**) Similar experiment to that shown in **a** except that store depletion was induced by 1 μ*M* thapsigargin (TG). (**c**) Plot of the time course of changes of normalized Fluo-4 fluorescence (F/F_0) in a freshly isolated pulmonary artery myocyte. The myocyte was preincubated for at least 10 min in Ca^{2+}-free solution prior to the beginning of the trace. As indicated, 30 μ*M* CPA evoked an initial Ca^{2+} transient consistent with Ca^{2+} leaking out of the sarcoplasmic reticulum due to blocking of Ca^{2+} reuptake. Readmitting extracellular Ca^{2+} ($[Ca]_o$ = 1.8 m*M*) in the continued presence of CPA and nifedipine led to the development of a robust Ca^{2+} transient consistent with SOCE

PSS (in m*M*): NaCl (120), KCl (4.2), $NaHCO_3$ (25; pH 7.4 after equilibration with 95% O_2/5% CO_2 gas), KH_2PO_4 (1.2), $MgCl_2$ (1.2), glucose (11), taurine (25), adenosine (0.01), and $CaCl_2$ (0.01 or 0.05). In the Fluo-4 experiments described in

Fig. 3.1c, single myocytes were superfused at room temperature with modified PSS composed of the following (in m*M*): NaCl (130), $NaHCO_3$ (10), KCl (4.2), KH_2PO_4 (1.2), $MgCl_2$ (0.5), $CaCl_2$ (0 or 1.8), glucose (5.5), and HEPES–NaOH (10; pH 7.35). The K^+-free bathing solution used in all patch clamp experiments had the following composition (in m*M*): NaCl (126), HEPES–NaOH (10; pH 7.35), tetraethyl-ammonium chloride (TEA) (8.4), glucose (20), $MgCl_2$ (1.2), and $CaCl_2$ (1.8). The pipet solution used in all perforated patch experiments had the following composition (in m*M*): Cs_2SO_4 (75), CsCl (55), HEPES–CsOH (10; pH 7.2), and $MgCl_2$ (5). Patch perforation was facilitated using the pore-forming antibiotic nystatin (400 mg/mL working concentration from a dimethyl sulfoxide (DMSO) stock solution of 60 mg/mL). For the dual patch clamp and fluorescence experiments (Fig. 3.4b), the pipet solution had the following composition (in m*M*): TEA (20), CsCl (106), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)–CsOH (10; pH 7.2), ethylene glycol tetraacetic acid (EGTA) (1), adenosine triphosphate (ATP).Mg (3), guanosine triphosphate (GTP).diNa (0.2), and K_5-Fluo-5F (0.2). All enzymes, analytical-grade reagents, nifedipine, NS-1619, and niflumic acid (NFA) were purchased from Sigma-Aldrich. CPA and TG were purchased from Calbiochem; Fluo-4 AM and the pentapotassium salt of Fluo-5F were purchased from Molecular Probes. NFA, nifedipine, Fluo-4, CPA, TG, and NS-1619 were initially prepared as a stock solution in DMSO, and an appropriate aliquot was added to the external solution to reach the final desired concentration. The maximal concentration of DMSO never exceeded 0.1%, a concentration that had no effect on all parameters measured.

2.6 Reverse-Transcriptase Polymerase Chain Reaction Experiments

Total RNA was isolated from homogenates of whole rabbit PA, rabbit brain, and mouse brain using PureZol (Bio-Rad). Prior to preparing complementary DNA (cDNA), the RNA was treated with DNase I (Invitrogen) to prevent genomic DNA contamination. The cDNA was prepared using oligodeoxy thymidylic acid (oligo-dT) and dNTP (deoxynucleotide triphosphate) mixtures with Superscript II, with a negative reverse-transcriptase (RT) control prepared for each tissue sample (Invitrogen).

Rabbit sequences were available for TRPC1, 2, and 5, and primers were designed accordingly. For all the other genes (TRPC3, 4, 6, and 7; Orai1–3; STIM1 and 2; and TMEM16 A and B), no rabbit sequences were available, so degenerate polymerase chain reaction (PCR) primers designed against an alignment of a combination of the sequences available for mouse, rat, dog, cow, chimp, monkey, and human were generated. All primers were synthesized by Operon Biotechnologies. Amplification of the cDNA was performed using Gotaq (Promega), which had an amplification profile of an initial step to 95°C for 2 min to activate the Amplitaq polymerase, followed by 36 cycles of denaturation at 95°C for 30 s, annealing at T_a (°C) for 30 s, extension at 72°C for 30 s, followed by a final extension step of 72°C for 5 min, where T_a is the optimal annealing temperature for each primer pair

(range 60–63°C). The amplified products (10 μL) were separated by electrophoresis on a 2% agarose/Tris (tris (hydroxy-methyl) amino-methane), acetic acid, ethylenediaminetetraacetic acid (EDTA) gel, and the DNA bands were visualized by ethidium bromide staining. Both a negative RT control (described in the first paragraph of this section) and a nontemplate control for the master mix of reagents made for each primer pair were also run on the gels to ensure that no contamination was present. Once DNA bands were observed at the expected size, the remainder of the PCR samples were sent for sequencing, and the sequences that were obtained were blasted against the NCBI (National Center for Biotechnology Information) database to confirm the identity of the products. From this, nested primers were designed for those sequences determined through the use of degenerate primers, and the PCR reaction was rerun as described.

2.7 Statistical Analysis

All data were pooled from *n* cells taken from at least two different animals. All data were first amalgamated in Excel and means exported to Origin 7.5 software for plotting and curve fitting. All graphs, contractility, fluorescence, and current traces were exported to CorelDraw 12 for final processing of the figures. Origin 7.5 software was also used to determine the statistical significance between two groups using a paired Student *t* test. We considered $P < 0.05$ as statistically significant.

3 Results

3.1 Demonstration of Store-Operated Ca^{2+} Entry

We first determined whether SOCE could be detected in intact rabbit PAs exposed to blockers of the SERCA pump to deplete the SR Ca^{2+} stores. Figure 3.1a shows two typical experiments demonstrating that store depletion can elicit a contraction, and that altering membrane potential pharmacologically can modulate the contractile response. In these experiments, endothelium-denuded arteries were exposed to a physiological Ca^{2+} concentration in the bathing solution (1.8 m*M*) and to 10 μ*M* nifedipine to block Ca^{2+} entry through voltage-gated L-type Ca^{2+} channels. In panel A, exposure of the preparation to 30 μ*M* CPA, a specific and reversible blocker of SERCA, induced a rapid transient increase in force, which stabilized to an elevated and sustained level of contraction after about 8 min. Exposure of the preparation to an activator of large conductance Ca^{2+}-activated K^+ channels (BK_{Ca}), NS-1619[30] in the continued presence of CPA and nifedipine more than doubled the magnitude of the contraction. In 15 rings from five animals, the initial transient and sustained contractions evoked by CPA were 53 ± 17 mg and 68 ± 36 mg, respectively;

NS-1619 enhanced the CPA-induced contraction in 12 of 15 rings by 20 ± 4 mg. In separate experiments, preincubating arterial rings to nominally Ca^{2+}-free solution in the presence of nifedipine led to the disappearance of the initial transient contraction evoked by 30 μ*M* CPA. However, readmitting Ca^{2+} in the bathing medium consistently produced a sustained contraction of similar magnitude to the ones measured in the experiments described in panel a (data not shown). One possible explanation for such a difference is that Ca^{2+} omission combined with nifedipine may have already induced depletion of the stores prior to exposure to CPA. As shown in panel B, although the response was typically slower in onset, similar results were obtained with the other SERCA inhibitor TG. In six rings from two animals, 1 μ*M* TG induced transient and sustained contractions of 130 ± 37 mg and 175 ± 35 mg, respectively. In three of the six rings onto which NS-1619 was tested, the BK_{Ca} channel activator elicited a potent additional contraction of 180 ± 20 mg.

We next tested whether SOCE can be observed in freshly isolated myocytes, which will be used in subsequent patch clamp experiments. Figure 3.1b illustrates the result of one of five typical experiments in which free intracellular Ca^{2+} concentration was measured with the fluorescent Ca^{2+} indicator Fluo-4. The cell was preincubated for more than 5 min in a Ca^{2+}-free solution containing 10 μ*M* nifedipine before the application of CPA. Application of CPA caused a transient increase in fluorescence (~ twofold), which slowly returned to baseline. Reintroducing Ca^{2+} in the presence of CPA led to a more than sixfold increase in fluorescence, which is consistent with SOCE. Taken together, the contraction and fluorescence data demonstrate convincingly the existence of SOCE in rabbit PAs. The initial transient response to CPA or TG was consistent Ca^{2+} leakage in the cytoplasm following inhibition of Ca^{2+} reuptake in the SR, whereas the sustained response was the hallmark of SOCE. Further support to the latter comes from the demonstration of a contraction associated with hyperpolarization mediated by BK_{Ca} channels by NS-1619, which increases the driving force for Ca^{2+} entry.

3.2 SOCE Activates Ca^{2+}-Activated Cl^- Conductance

The possible activation of Cl_{Ca} by SOCE was investigated by using the perforated variant of the patch clamp technique. In these experiments, K^+ was substituted with Cs^+ in the pipet solution, and a K^+-free extracellular solution containing TEA was used to inhibit K^+ channels. Figure 3.2a shows ionic currents (top traces) evoked by the voltage clamp protocol shown at bottom. From an HP of –70 mV, a 250-ms step

Fig. 3.2 (continued) for the nonselective cation current triggered by store depletion. (**d**) Graph showing *I–V*s for peak current measured during step 2 (see **b**) as a function of voltage during step 1. Both sets of data were fitted to a single exponential function. Although both followed a similar trend, the current recorded in the presence of TG was greatly enhanced by preconditioning hyperpolarizing steps

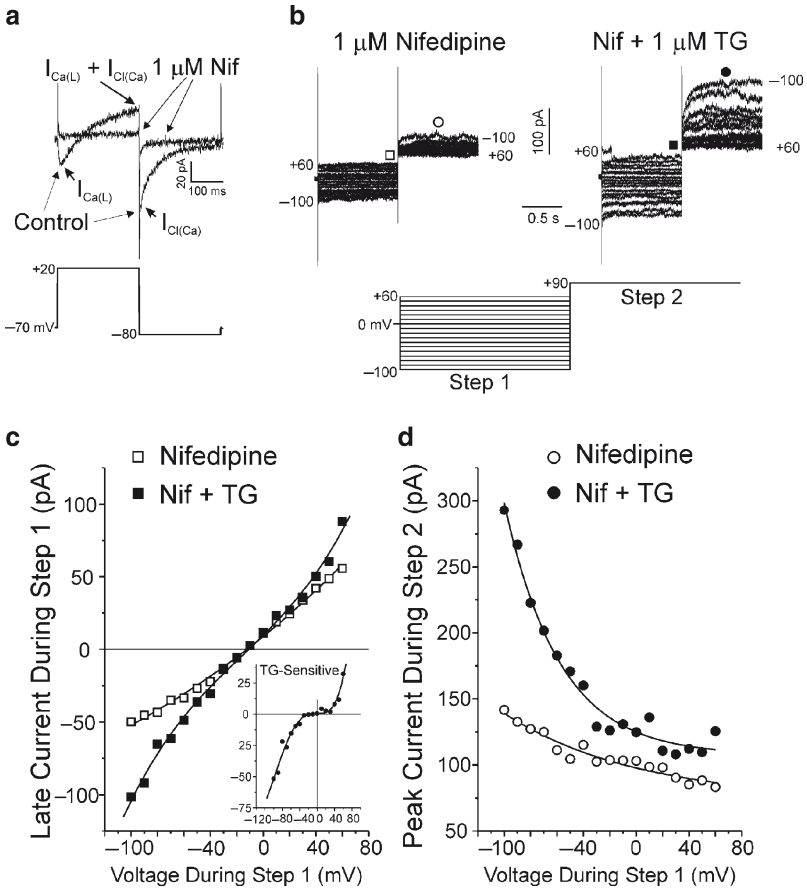

Fig. 3.2 Typical experiment demonstrating an interaction between a membrane current induced by store depletion and Ca^{2+}-activated Cl^- current in a rabbit pulmonary artery myocyte. All traces and graphs shown in **a**–**d** were derived from the same cell. (**a**) Superimposed membrane currents (*top*) elicited by the voltage clamp protocol shown at the *bottom,* which consisted of a 250-ms step to +20 mV from a holding potential of –70 mV, followed by a 250-ms return step to -80 mV. As indicated, the "Control" trace consists of an initial fast transient inward L-type Ca^{2+} current [$I_{Ca(L)}$] that reverses during inactivation to become an outward current composed of remaining $I_{Ca(L)}$ and Ca^{2+}-activated Cl^- current [$I_{Cl(Ca)}$]. The slow tail current is predominantly composed of $I_{Cl(Ca)}$ since deactivation of $I_{Ca(L)}$ is on the order of a few milliseconds. A 5-min application to 1 μ*M* nifedipine (Nif) abolished both components. (**b**) Two families of membrane currents recorded in the presence of 1 μ*M* nifedipine, before (*left*) and after (*right*) exposure to 1 μ*M* thapsigargin (TG) to deplete the stores. Each set of traces was generated by the double-pulse protocol shown below. The two steps were 1 s in duration. Notice that TG enhanced the magnitude of the time-independent current during step 1 and led to the appearance of a time-dependent Ca^{2+}-activated Cl^- current during step 2 (+90 mV) that grew with membrane hyperpolarization during the previous step. Symbols above traces correspond to where measurements were made and correspondingly plotted in **c** and **d**. (**c**) *I–V* relationships for the current measured at the end of step 1 (see **b**) in the absence (*open squares*) or presence (*filled squares*) of 1 μ*M* TG. Both *I–V*s reversed at –11 mV. *Inset:* Plot of the TG-sensitive *I–V* relationship obtained by subtraction of the data in the main graph. The *line* passing through the data points is a fourth-order polynomial fit. Notice the N-shaped relationship typical

to +20 mV elicited a rapid transient inward Ca^{2+} current that became outward after about 100 ms. Repolarization to −80 mV resulted in the appearance of a slow deactivating tail current, which reflects time-dependent closure of the outward current that developed during the preceding step. Figure 3.2a also shows that all time-dependent currents were abolished following the application of 1 μ*M* nifedipine. This experiment demonstrated the classical behavior in this and many other types of vascular smooth muscle cells (VSMCs) where Ca^{2+} influx associated with inward L-type current activates a slowly developing outward Ca^{2+}-activated Cl^- current, $I_{Cl(Ca)}$, during the step and inward $I_{Cl(Ca)}$ tail current following repolarization caused by Ca^{2+} removal and voltage-dependent deactivation. Traces shown on the left in Figure 3.2b were from the same cell as Figure 3.2a and were generated with the double-pulse protocol displayed below the traces. The same protocol was applied in the same cell after a 5-min exposure to 1 μ*M* TG in the presence of nifedipine (right traces) to induce SOCE. The closed and open symbols indicate where measurements were made to construct the corresponding current–voltage relationships (*I–V*) shown in Figure 3.2c and d. TG activated an instantaneous time-independent current during the first step, but more interestingly, it also triggered the appearance of a time-dependent outward current that is consistent with $I_{Cl(Ca)}$ in this preparation. Figure 3.2c shows the *I–V*s for the current obtained before (open squares) and after TG (filled squares). Both *I–V*s reversed at –11 mV, supporting the idea that the basal conductance under these conditions is nonselective. The inset displays the TG-sensitive *I–V* calculated by subtraction. The TG-activated conductance displayed a typical "N"-shaped *I–V*, showing signs of inward and outward rectification as reported in many other studies examining the properties of store depletion-activated ionic currents in VSMCs. Figure 3.2d shows two *I–V*s generated by plotting peak outward current during step 2 (+90 mV) as a function of voltage during step 1. The outward current declined exponentially in the presence of nifedipine alone from –100 to +60 mV, a process that was greatly accentuated by depleting the stores with TG.

Figure 3.3 shows a plot of the time course of changes of normalized peak outward current during step 2 for a typical experi ment carried out with CPA. Measurements from selected traces at the top labeled *a*, *b,* and *c* are correspondingly indicated on the plot below. A hint of time-dependent outward and inward tail currents were apparent in the presence of nifedipine alone (trace *a*). Application of CPA caused an initial transient increase in outward current followed by a delayed robust enhancement of the current. Such behaviors remarkably resemble those of SOCE described in Fig. 3.1. The enhancement of current was so great in this particular cell that the fully stimulated outward current (trace *b*) was time independent, and only a slowly deactivating tail current could be detected following repolarization. In this and all other similar experiments with CPA, the voltage dependence of the outward current exhibited a similar profile to that seen with TG (Fig. 3.2d), that is, a steep decline associated with membrane depolarization. NFA (100 μ*M*) potently inhibited the CPA-induced current to a level that was higher than that recorded in the absence of CPA and abolished the tail current (trace *c*). Even though the inward current during the step to –70 mV was also inhibited by NFA, we believe

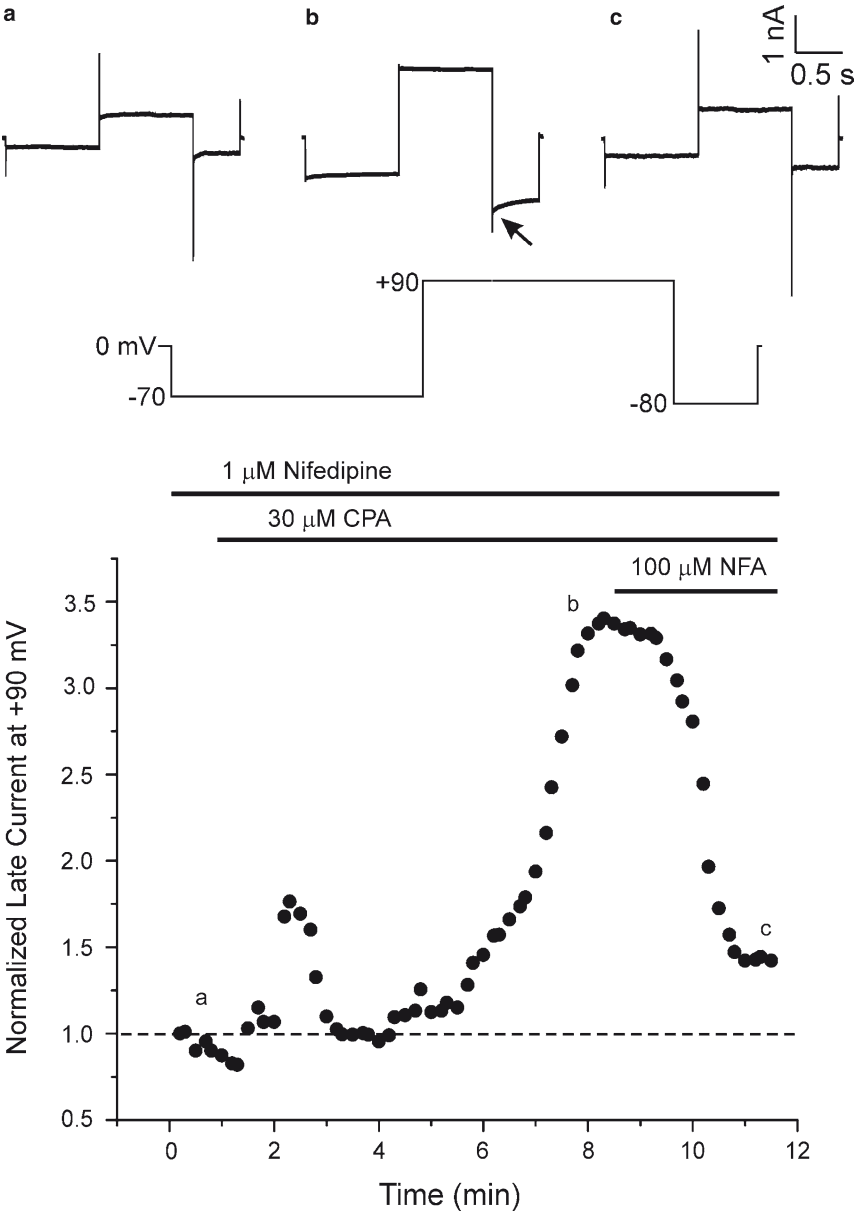

Fig. 3.3 The Ca^{2+}-activated Cl^- channel blocker niflumic acid (NFA) blocks a large component of membrane current activated by store depletion. Sample experiment from a rabbit pulmonary artery smooth muscle cell showing the effects of NFA on membrane currents evoked by the voltage clamp protocol shown below the traces. The three traces at the *top* were recorded in the presence of 1 μ*M* nifedipine alone (trace *a*) after full stimulation following exposure to 30 μ*M* cyclopiazonic acid (CPA; trace *b*) and following steady-state block by 100 μ*M* NFA (trace *c*). The *arrow* pointing at trace *b* indicates the appearance of a slow deactivating Ca^{2+}-activated Cl^- tail current. Measurements of current amplitude at the end of the step to +90 mV from these three traces were normalized to that recorded in the presence of nifedipine alone and are labeled in the graph below which shows the full time course of this experiment

that this reflected inhibition of $I_{Cl(Ca)}$ due to the very large basal activation of this current at the HP in this cell (0 mV). Indeed, significant deactivation of the current was evident at –70 mV in this cell (trace *b*). Analysis of the effects of NFA on the CPA-induced current at negative potentials revealed no significant differences between CPA and CPA plus NFA (e.g., at –50 mV, CPA 9.2 ± 3.0 pA/pF; CPA + NFA 8.8 ± 2.2 pA/pF; $P > 0.05$). These results suggest that NFA blocked $I_{Cl(Ca)}$ but not the SOCE-induced nonselective cation current. Finally, SKF-96365 (50 μ*M*), a widely used inhibitor of nonselective cation channels, suppressed the CPA-mediated time-independent current during the initial step (–100 to +60 mV), as well as time-dependent $I_{Cl(Ca)}$ during the second step to +90 mV (data not shown). In summary, our data provide evidence for a SKF-96365-sensitive SOCE pathway that allows for enhanced Ca^{2+} entry at negative potentials, which then serves as a trigger for activation of $I_{Cl(Ca)}$.

3.3 Voltage Dependence of SOCE and Impact on $I_{Cl(Ca)}$

We next sought to gain a better understanding of the properties of the source of Ca^{2+} entry activated by store depletion. In our initial voltage clamp experiments, we often observed that outward $I_{Cl(Ca)}$ reached a peak after a few hundred milliseconds and then slowly declined toward the end of the step. We hypothesized that activation and deactivation during the second step to +90 mV might be attributed to a complex pattern of voltage-dependent activation following the onset of the step and slow Ca^{2+} removal due to a decreased inwardly directed driving force for Ca^{2+} at positive potentials. This is better illustrated in the experiment shown in Fig. 3.4a. The voltage clamp protocol consisted of an initial 2-s step to –100 mV to increase Ca^{2+} entry following the application of CPA, followed by a 5-s step to +130 mV at which Ca^{2+} entry would be negligible. This panel clearly shows that after activation and stabilization for a few seconds, the current began to decrease progressively during the remainder of the step, in contrast with the maintenance of this current when intracellular Ca^{2+} is clamped with strong Ca^{2+} buffering with EGTA or 1,2-bis(o-aminophenoxy)ethane-N,N,N′N,N′-tetraacetic acid (BAPTA). To test this hypothesis more directly, we performed dual whole-cell voltage clamp experiments in myocytes dialyzed with the Ca^{2+} indicator Fluo-5 to monitor free $[Ca^{2+}]_i$ simultaneously. Figure 3.4b shows the results of one of three similar experiments carried out in the presence of nifedipine and CPA. While a step to +60 mV had no effect on the Fluo-5F signal and current, hyperpolarizing steps ranging from –60 to –100 mV led to voltage-dependent increases in $[Ca^{2+}]_i$ that were accompanied by time-dependent increases in inward current, most likely $I_{Cl(Ca)}$. Stepping to +90 mV caused an immediate exponential decline in $[Ca^{2+}]_i$. Again, progressively more negative voltage steps evoked progressively larger outward $I_{Cl(Ca)}$, which eventually declined over the course of the step, albeit at a slower rate than the Fluo-5F signal. These experiments lend support to the notion that store depletion evokes a Ca^{2+} entry pathway that is steeply dependent on the transmembrane Ca^{2+} driving force and that can stimulate Cl_{Ca} channels.

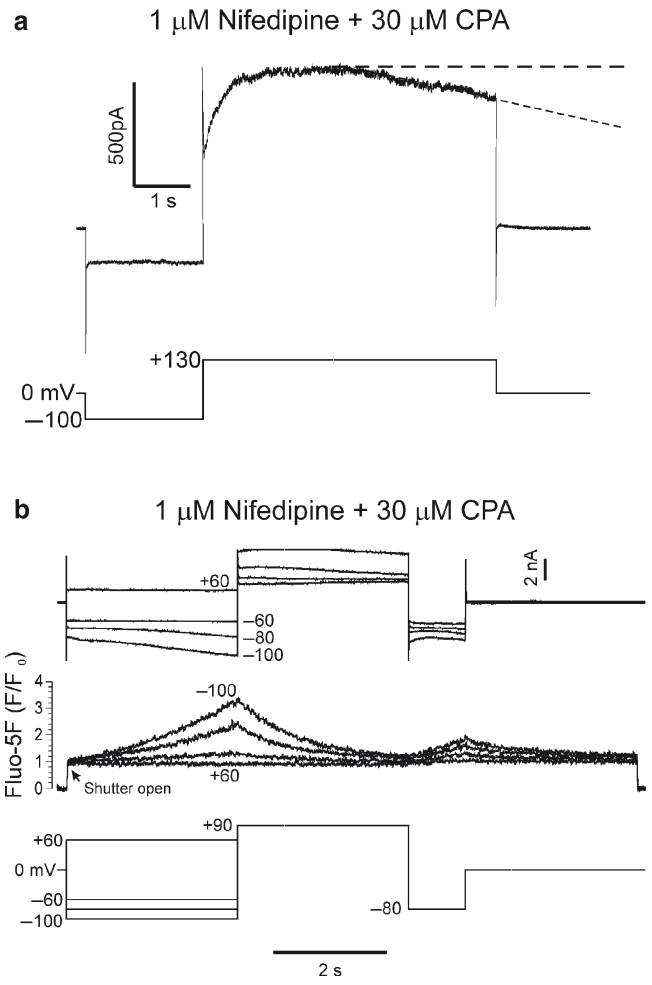

Fig. 3.4 Activation of Ca^{2+}-activated Cl^- conductance by store depletion depends on the transmembrane driving force for Ca^{2+}. (**a**) Typical membrane current (*top*) evoked by the protocol depicted below from pulmonary artery smooth muscle cells exposed to 1 μ*M* nifedipine and 30 μ*M* cyclopiazonic acid (CPA). The 2-s step to –100 mV was used to allow for Ca^{2+} entry through store-operated channels and was followed by a 5-s step to +130 mV (near the equilibrium potential for Ca^{2+}) to record Ca^{2+}-activated Cl^- current when Ca^{2+} entry is minimized. Notice the decline of time-dependent $I_{Cl(Ca)}$ (indicated by *dashed lines*) after activation and a temporary stabilization phase. (**b**) Dual-membrane current (*top traces*) and free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$; *middle traces*) recordings in a rabbit pulmonary artery myocyte dialyzed with 1 m*M* EGTA and 200 μ*M* Fluo-5 (K^+ salt form) and exposed to 1 μ*M* nifedipine and 30 μ*M* cyclopiazonic acid (CPA). The currents and Ca^{2+} transients were elicited by the voltage clamp protocol displayed below. Similar to Fig. 3.2, membrane current recorded during the second step to +90 mV increased with the magnitude of the preceding hyperpolarization and was consistent with the progressively larger Ca^{2+} transients detected. In contrast, stepping to +90 mV led to the immediate onset of an exponential decline in $[Ca^{2+}]_i$, which is consistent with store depletion triggering a Ca^{2+} entry pathway that is directly reliant on the Ca^{2+} driving force

3.4 Molecular Candidates for the Store-Operated Calcium Entry Pathway

Research has confirmed the importance of Orai, TRPC, and STIM as molecular entities involved in SOCE. We performed RT-PCR on cDNA made from isolated rabbit brain, rabbit PA, and mouse brain RNA, and nontemplate controls were run to control for primer contamination. Figure 3.5 shows the ethidium bromide-stained gel image for the PCR products. Gene expression for Orai1 was present in all three tissues, while Orai2 was confirmed only in the two brain samples (mouse and rabbit) and Orai3 only in the mouse brain (Fig. 3.5a). It is possible that the lack of evidence of Orai3 in the amplified rabbit cDNA samples is a facet of primer design rather than conclusive evidence that Orai3 is absent in the rabbit tissues. STIM1 and STIM2 gene expression was observed in mouse and rabbit brain and rabbit PA amplified cDNA samples (Fig. 3.5b). Various TRPC isoforms were detected in the amplified cDNA samples. In mouse brain, the TRPC1, 3, 4, 6, and 7 isoforms were detected. The primers for TRPC2 were not degenerate but designed against the rabbit sequence, suggesting that TRPC2 is not necessarily absent in this tissue, but that the primers were not specific enough to the species. With the exception of TRPC5 (not shown), all of the TRPC isoforms were detected in the rabbit brain (Fig. 3.5c). A similar pattern of expression was observed for the rabbit PA, although TRPC7 was undetectable (Fig. 3.5c). These data confirm the presence of many of the components necessary for a functional SOCE pathway to exist in the rabbit PA tissue.

3.5 Molecular Candidates for the Calcium-Activated Chloride Channel

We performed RT-PCR on cDNA made from isolated rabbit brain, rabbit PA, and mouse brain RNA, and nontemplate controls were run to control for primer contamination. Figure 3.5d shows evidence for detectable transcripts of both TMEM16A and TMEM16B in all three tissues, supporting a possible role for the TMEM16 family in the construction of the pore-forming subunit of Cl_{Ca} channels in the PA.

Fig. 3.5 (continued) rabbit sequences. (**b**) RT-PCR experiment carried out using degenerate primers for STIM1 and 2 (269 & 217 bp, respectively). Both STIM1 and 2 are present in the rabbit pulmonary artery, with additional unidentified bands observed. (**c**) RT-PCR experiment carried out using degenerate primers for TRPC3 (239 bp), 4 (242 bp), 6 (302 bp), and 7 (269 bp), and primers designed against available rabbit sequences for TRPC1 and 2 (479 and 235 bp, respectively). Most of the TRPC isoforms were present in the rabbit pulmonary artery (1–4 and 6); TRPC7 was absent. (**d**) RT-PCR experiment carried out using nested primers designed using rabbit sequences (obtained from previous experiments using degenerate primers) for TMEM16A (220 bp) and using degenerate primers for TMEM16B (377 bp). Both TMEM16A and TMEM16B are present in the rabbit pulmonary artery. Mouse brain and rabbit brain cDNA samples were consistently included in the experimental design as positive controls for the success of the degenerate primer design and their ability to detect sequences in rabbit tissues. All products obtained using degenerate primers were sequenced and blasted against the NCBI database to confirm identity

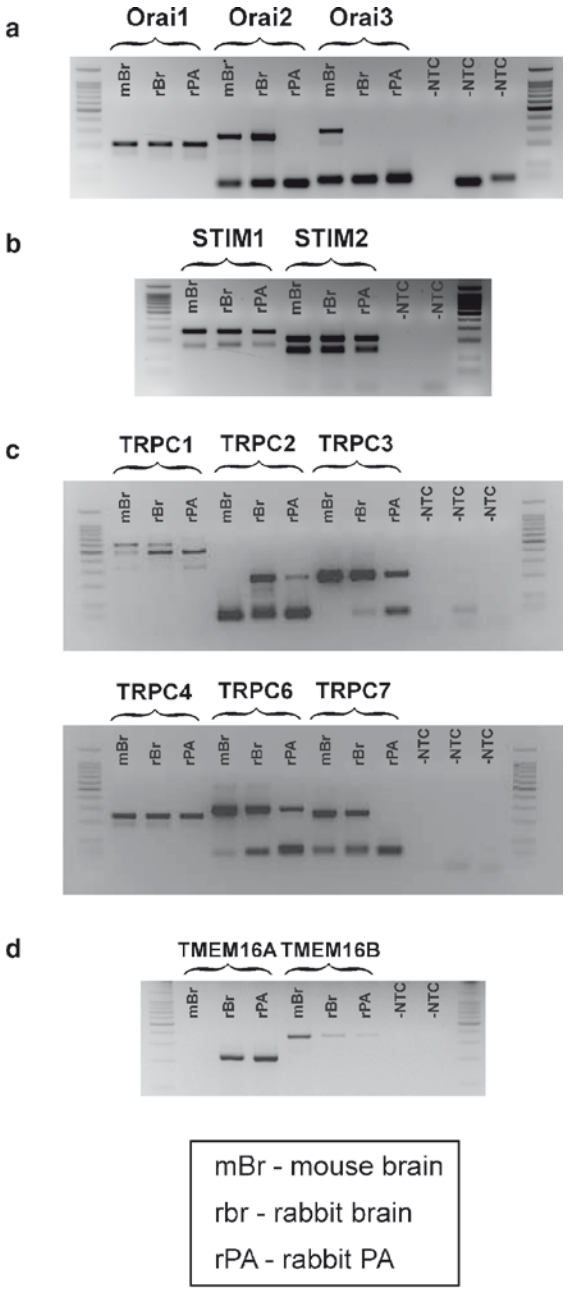

Fig. 3.5 Transcripts for some of the molecular candidates for store-operated calcium entry (SOCE) and the latest putative molecular candidate for the calcium-activated chloride channel (Cl_{Ca}) are expressed in rabbit pulmonary artery. (**a**) RT-PCR experiment carried out using degenerate primers for Orai1–3 (212, 238, and 313 bp, respectively). Only Orai1 appears to be present in the rabbit pulmonary artery, although the data for Orai3 remains inconclusive since these primer failed to detect a sequence in the rabbit brain, suggesting that they may be unsuccessful in detecting

4 Discussion

The primary objective of this investigation was to determine if store-operated Ca^{2+} entry is able to activate Ca^{2+}-activated Cl^- channels in PA myocytes, a major excitatory pathway in these vascular smooth muscle cells. We first showed that store depletion with the SR Ca^{2+}-ATPase (adenosine triphosphatase) inhibitor CPA or TG induced a sustained contraction of rabbit PAs that followed an initial transient contraction that was consistent with rapid leakage of Ca^{2+} out of the SR due to disruption of Ca^{2+} reuptake by TG or CPA. This contraction was independent of L-type Ca^{2+} channels since (1) nifedipine was present in all solutions and (2) cell hyperpolarization by activation of BK_{Ca} channels with NS-1619 led to enhancement of contraction instead of relaxation. Experiments carried out with freshly dispersed rabbit PA myocytes loaded with the Ca^{2+} indicator Fluo-4 demonstrated the persistence of an SOCE pathway following cell isolation, with an initial Ca^{2+} transient evoked by CPA in cells preincubated with nifedipine and the appearance of a large influx of Ca^{2+} following readmission of Ca^{2+} in the bathing medium. Store depletion with TG or CPA activated at least two distinct conductances in whole-cell path clamp experiments: (1) a time-independent SKF-96365-sensitive nonselective cation current reversing between –10 and 0 mV and (2) a time-dependent NFA-sensitive Cl_{Ca} current whose magnitude increased proportionately with preconditioning hyperpolarizing steps. Preliminary dual whole-cell patch clamp and Fluo-5F experiments to measure ionic currents and Ca^{2+} transients simultaneously showed that CPA-induced SOCE and activation of $I_{Cl(Ca)}$ were directly reliant on changes in the driving force for Ca^{2+}. Finally, our study provides strong evidence for the molecular expression of several key genetic components involved in SOCE and generation of Cl_{Ca} channels in rabbit PA smooth muscle.

4.1 Detection of SOCE-Induced Contraction in the Rabbit Pulmonary Arterial Vasculature

Store depletion induced by blocking Ca^{2+} reuptake into the SR, by releasing SR Ca^{2+} with caffeine, or following stimulation with G protein-coupled receptor agonists has been shown to induce Ca^{2+} influx or contraction in PAs[3,6,31] and smooth muscle cells from other vascular beds.[6,32] Our data in rabbit PA smooth muscle cells showed that exposure to a specific inhibitor of SERCA in Ca^{2+}-free medium elicits a Ca^{2+} transient that is manifest of Ca^{2+} leaking out of the SR when Ca^{2+} reuptake is impaired. Readmission of Ca^{2+} consistently led to a large insensitive elevation of intracellular free Ca^{2+} that is consistent with SOCE. As observed by others,[3,4] the Ca^{2+} signal was not sustained but partially declined to a stable elevated level that has been attributed to fast and slow Ca^{2+}-dependent inactivation modes of the store-operated channels.[33,34] In the presence of extracellular Ca^{2+}, the initial store depletion-induced Ca^{2+} transient and SOCE elicited corresponding contractions, indicating that SOCE is capable of generating PA tone in this species. Besides

insensitivity to block by the dihydropyridine nifedipine, which reportedly does not interfere with SOCE,[3] the store depletion-induced contraction was enhanced by NS-1619, a BK_{Ca} channel activator known to produce membrane hyperpolarization.[35] Such a behavior is anticipated for a Ca^{2+} entry pathway relying primarily on the electrochemical gradient for Ca^{2+}. This contrasts with the observation that NS-1619 causes relaxation of blood vessels contracted by agonists involving membrane depolarization and activation of voltage-gated Ca^{2+} channels, although there is evidence for direct block of Ca^{2+} channels.[35]

4.2 SOCE Activates Ca^{2+}-Activated Cl^- Current

The most interesting observation of the present study was the demonstration of a unique interaction between SOCE and Cl_{Ca} channels in our single-cell preparation. This was complicated by the scarcity and relatively poor specificity of pharmacological tools that rendered current separation difficult. We first took advantage of the voltage clamp technique to distinguish the store-operated cation current (I_{SOC}) and $I_{Cl(Ca)}$ on the basis that the former is known to generate sustained time-independent currents[3,9], whereas the latter displays pronounced outward rectification due to voltage- and time-dependent gating properties[20,29,36] and store depletion induced by TG or CPA elicited time-independent current that reversed between –10 and 0 mV. This is in accord with a nonselective cation current that would display significant permeability to Cs^+, Na^+, and Ca^{2+}, the major cation charge carriers used in our patch clamp experiments. The steady-state current–voltage relationship for this current measured at the end of 1-s steps from an HP of 0 mV exhibited the typical N shape, a behavior similar to that observed by others.[9] All our protocols consisted of an initial step to a range of potentials to evoke I_{SOC} followed by a second step at +90 mV or higher. The rationale for this second step was to minimize SOCE due to a dramatically reduced driving force for Ca^{2+} and to take advantage of the outwardly rectifying properties of $I_{Cl(Ca)}$. Keeping this second step constant enabled us to gauge Ca^{2+} entry through I_{SOC} and to monitor its effect on the magnitude of time-dependent $I_{Cl(Ca)}$ and indirectly free intracellular Ca^{2+} concentration.

Our data showed that in the presence of CPA or TG, activation of $I_{Cl(Ca)}$ at +90 mV followed a similar trend to that observed with the Ca^{2+} indicator Fluo-4 in single cells or contraction in PA rings with the detection of an initial and delayed and more robust component of outward current. A substantial fraction of this time-dependent outward current was blocked by NFA, a commonly used inhibitor of Cl_{Ca} channels.[19,20] Time-dependent $I_{Cl(Ca)}$ at +90 mV was also progressively enhanced by preconditioning hyperpolarizing steps. Whereas the relatively uncontaminated I_{SOC} elicited during the first step was insensitive to NFA, time-dependent $I_{Cl(Ca)}$ at +90mV and its slow deactivating tail current after repolarization to -80 mV were potently inhibited by the fenamate. Both I_{SOC} and $I_{Cl(Ca)}$ were strongly inhibited by SKF-96365, a potent blocker of SOCE and I_{SOC}.[9] Although this observation supports a possible role for Ca^{2+} entry through I_{SOC} in eliciting $I_{Cl(Ca)}$, we cannot rule out the

possibility of a direct inhibitory effect of this blocker on Cl_{Ca} channels that should be tested on $I_{Cl(Ca)}$ recorded in isolation by clamping $[Ca^{2+}]_i$ to known fixed elevated levels.

To obtain more direct proof of SOCE induced by hyperpolarization and subsequent activation of $I_{Cl(Ca)}$, we measured Ca^{2+} transients simultaneously with membrane current in whole-cell voltage-clamped PA myocytes dialyzed with Fluo-5 and exposed to CPA and nifedipine. Consistent with this hypothesis, graded Ca^{2+} entry was elicited in response to hyperpolarizing steps and was mirrored by the progressively larger amplitude of $I_{Cl(Ca)}$ during a following step to +90 mV. In agreement with this idea, $[Ca^{2+}]_i$ declined exponentially at positive potentials, an observation in line with the marked reduction in driving force for Ca^{2+} at positive potentials. The magnitude of the Ca^{2+} transient and $I_{Cl(Ca)}$ thus appears to follow the changes in Ca^{2+} driving force imposed by membrane potential during the initial step. A report in airway smooth muscle[37] has suggested that Ca^{2+} entry via reverse-mode Na^+/Ca^{2+} exchange was the main source of Ca^{2+} responsible for the contraction induced by SOCE. In this paradigm, Na^+ entry through I_{SOC} would raise intracellular Na^+ levels, causing a negative shift in the reversal potential of the exchanger ($E_{NCX} = 3E_{Na} - 2E_{Ca}$ assuming a stochiometry of $3Na^+$:1 Ca^{2+}) favoring net Ca^{2+} entry. We purposely dialyzed the myocyte with a Na^+-free pipet solution to minimize this effect. However, it is still possible that enough Na^+ entry through I_{SOC} during a hyperpolarizing step accumulates in a restricted compartment, allowing for Ca^{2+} to be transported into the cell by the exchanger and stimulate $I_{Cl(Ca)}$. This important aspect of the pathway will require further investigation by examining whether external Na^+ removal has any effect on $[Ca^{2+}]_i$ and $I_{Cl(Ca)}$.

4.3 Nature of the Ca^{2+} Entry Pathway Stimulating Cl_{Ca} Channels

The membrane current activated by CPA or TG in our experiments is clearly a nonselective cation current, and preliminary experiments suggest, as other studies have implied in vascular smooth muscle cells,[3,9] that external Na^+ and internal Cs^+ are the main charge carriers in our recording conditions. Several members of the TRPC family (TRPC1, TRPC4, TRPC5, and in some studies TRPC6) of ion channels have originally been suggested to form the basis of I_{SOC} in many cell types.

Using an antibody targeting an extracellular epitope and used as a blocker, Xu and Beech[4] initially hypothesized TRPC1 to be a major component of this current in vascular smooth muscle. There are also suggestions, mainly from studies of overexpression in heterologous cell systems, that I_{SOC} may be formed from TRPC1 partnering with TRPC4 or TRPC5 in heteromultimeric complexes.[9,38] More recent reports have convincingly demonstrated that more than one molecular mechanism may be responsible for SOCE in smooth muscle. One study challenged the previously considered dogma that TRPC1 is "the" protein responsible for SOCE in smooth muscle. Dietrich et al.[39] showed that SOCE in aortic and cerebral vascular smooth muscle cells from $TRPC1^{-/-}$ was indistinguishable from wild type. At least one report provided evidence for TRPC6 playing a role in determining SOCE in

cultured rat PA smooth muscle cells[40] as well as in receptor-operated channel activity activated by α_1-adrenoceptor stimulation leading to production of the second messenger DAG,[41,42] and stimulation of protein kinase C[5] or stretch-activated nonselective cation channels.[43]

In addition to the Orais, two STIM isoforms (STIM1 and 2) have been reported in relation to SOCE. Current evidence suggests that STIM1 is located in the membrane of the ER and acts as a Ca^{2+} sensor for the ER Ca^{2+} stores,[44,45] interacting directly with Orai1 to initiate SOCE. Current thinking suggests that STIM2 interacts with STIM1 to inhibit STIM1-mediated activation of SOCE.[46]

These exciting discoveries have unraveled a new level of complexity in cellular signaling. Our molecular data provided evidence for the expression of transcripts encoding for TRPC1, 4, and 6; Orai1 and Orai2 but not Orai3; as well as STIM1 and STIM2, all of which have been suggested to play a role in SOCE. Although TRPC5 has been detected and suggested to participate in SOCE in pial arterioles,[47] we were unable to identify messenger RNA (mRNA) for this subunit in our preparation, and this may or may not be due to the rather incomplete rabbit genome. There is now convincing evidence that STIM1 is an integral part of the molecular architecture sensing ER Ca^{2+} levels and leading to activation of TRPC1,[15,17,48] and that Orai1 can form a ternary dynamic complex with STIM1 and TRPC1 following store depletion dictating SOCE.[16,49,50] STIM2 was initially suggested to antagonize the effects of STIM1 on SOCE.[46] More recent reports indicated that STIM2 may also serve as an ER Ca^{2+} sensor and stimulate SOCE, but its dynamic range and role in controlling ER and cytoplasmic Ca^{2+} levels appears to be different.[51,52] Clearly, a lot more work lies ahead to determine the distribution and function of these proteins in PA smooth muscle cells.

As shown in this and previous studies from our group, rabbit PA smooth muscle cells exhibit a large $I_{Cl(Ca)}$ that is activated by an elevation in internal Ca^{2+}, displays strong outward rectification due to voltage-dependent gating, and is downregulated by phosphorylation.[28,29,36] A search for the molecular candidate underlying this channel has highlighted a number of possibilities to date. Previous studies have suggested that Tweety, *CLCA,* or Bestrophins may be contenders; however, these candidates generally produce membrane currents that are for the most part dissimilar to that recorded from native Cl_{Ca} channels.[20,22] More recent evidence suggested that TMEM16A (Anoctamin; ANO1) and possibly TMEM16B (ANO2) are better molecular candidates for this channel.

Yang et al.[26] showed that currents measured in HEK293T cells in which ANO1 had been overexpressed displayed a small unitary conductance, were outwardly rectifying, and exhibited Ca^{2+} and voltage dependence. Furthermore, 4, 4′-diisothiocyanatostilbene-2, 2′-disulfonic acid (DIDS) and niflumic acid both inhibited these currents. This work was supported by that of Schroeder et al.,[25] who also demonstrated that overexpression of either TMEM16A or TMEM16B results in the generation of Cl_{Ca} currents.

We previously demonstrated the expression of several Bestrophins in the rabbit PA.[20] The present study further extended this analysis by showing that TMEM16A and B are also expressed at the mRNA level. It will be necessary to determine the exact role of these proteins in generating $I_{Cl(Ca)}$ and assess whether Bestrophins interact physically and functionally with TMEM16 proteins.

Many questions remain unanswered with respect to the Ca^{2+} entry pathways. Is Ca^{2+} entering the myocyte through the nonselective TRPC1 (and possibly others), the Ca^{2+}-selective Orai1 (CRACM1), or both? Are Ca^{2+}-activated Cl^- channels (TMEM16A or B? Bestrophins?) located in the vicinity of the SOCE pathways and triggered by a preferential subsarcolemmal compartment accessible to both? There is also evidence for STIM1-induced TRPC1 translocation into lipid rafts and caveolae during store depletion[15] and for an important role played by caveolin-1 in this process in vascular smooth muscle cells.[53]

5 Conclusion

This study shed some light on a new mechanism of interaction between two excitatory ionic mechanisms in smooth muscle. Our data confirm that a Ca^{2+} entry pathway consistent with store-operated Ca^{2+} entry signaling can activate Ca^{2+}-activated Cl^- channels in PA myocytes (Fig. 3.6). We hypothesize that this mecha-

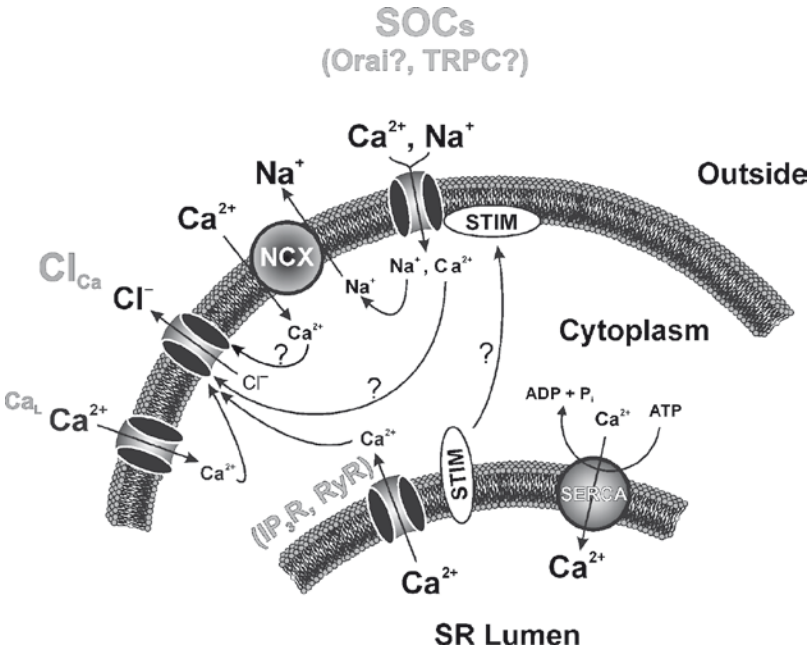

Fig. 3.6 Schematic diagram illustrating the different sources of Ca^{2+}, including those elicited by store depletion, that regulate Ca^{2+}-activated Cl^- channels in pulmonary artery smooth muscle cells. See text for explanations. Cl_{Ca} Ca^{2+}-activated Cl^- channels; Ca_L L-type Ca^{2+} channels; *SOCs* store-operated channels; Orai, STIM, and TRPC are the three candidate gene families proposed to support store-operated calcium entry in various cell types; *NCX* Na^+/Ca^{2+} exchanger; *SERCA* Ca^{2+}-ATPase in the sarcoplasmic reticulum (SR); IP_3R inositol trisphosphate receptor; *RyR* ryanodine receptor

nism may be important in the regulation of membrane potential, Ca^{2+} influx, and tone in these cells under physiological and pathophysiological conditions. Stimulation by Cl_{Ca} channels by SOCE may depolarize the cell toward E_{Cl} (~ –20 mV in smooth muscle) and thus reduce the transmembrane gradient for Ca^{2+}. Future studies should be undertaken to test the hypothesis that blocking Cl_{Ca} channels due to the ensuing hyperpolarization enhances SOCE.

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References

1. Bygrave FL, Roberts HR (1995) Regulation of cellular calcium through signaling cross-talk involves an intricate interplay between the actions of receptors, G-proteins, and second messengers. FASEB J 9:1297–1303
2. Putney JW, Broad LM, Braun FJ, Lievremont JP, Bird GSJ (2001) Mechanisms of capacitative calcium entry. J Cell Sci 114:2223–2229
3. Ng LC, Gurney AM (2001) Store-operated channels mediate Ca^{2+} influx and contraction in rat pulmonary artery. Circ Res 89:923–929
4. Xu SZ, Beech DJ (2001) TrpC1 is a membrane-spanning subunit of store-operated Ca^{2+} channels in native vascular smooth muscle cells. Circ Res 88:84–87
5. Albert AP, Large WA (2002) A Ca^{2+}-permeable non-selective cation channel activated by depletion of internal Ca^{2+} stores in single rabbit portal vein myocytes. J Physiol 538:717–728
6. Wilson SM, Mason HS, Smith GD et al (2002) Comparative capacitative calcium entry mechanisms in canine pulmonary and renal arterial smooth muscle cells. J Physiol 543:917–931
7. Prakriya M, Lewis RS (2003) CRAC channels: activation, permeation, and the search for a molecular identity. Cell Calcium 33:311–321
8. Albert AP, Large WA (2003) Store-operated Ca^{2+}-permeable non-selective cation channels in smooth muscle cells. Cell Calcium 33:345–356
9. Beech DJ, Muraki K, Flemming R (2004) Non-selective cationic channels of smooth muscle and the mammalian homologues of Drosophila TRP. J Physiol 559:685–706
10. Lewis RS (2007) The molecular choreography of a store-operated calcium channel. Nature 446:284–287
11. Feske S, Gwack Y, Prakriya M et al (2006) A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function. Nature 441:179–185
12. Prakriya M, Feske S, Gwack Y, Srikanth S, Rao A, Hogan PG (2006) Orai1 is an essential pore subunit of the CRAC channel. Nature 443:230–233
13. Mercer JC, DeHaven WI, Smyth JT et al (2006) Large store-operated calcium selective currents due to co-expression of Orai1 or Orai2 with the intracellular calcium sensor, Stim1. J Biol Chem 281:24979–24990
14. Luik RM, Wu MM, Buchanan J, Lewis RS (2006) The elementary unit of store-operated Ca^{2+} entry: local activation of CRAC channels by STIM1 at ER-plasma membrane junctions. J Cell Biol 174:815–825

15. Alicia S, Angelica Z, Carlos S, Alfonso S, Vaca L (2008) STIM1 converts TRPC1 from a receptor-operated to a store-operated channel: moving TRPC1 in and out of lipid rafts. Cell Calcium 44:479–491
16. Ong HL, Cheng KT, Liu X et al (2007) Dynamic assembly of TRPC1-STIM1-Orai1 ternary complex is involved in store-operated calcium influx. Evidence for similarities in store-operated and calcium release-activated calcium channel components. J Biol Chem 282:9105–9116
17. Li J, Sukumar P, Milligan CJ et al (2008) Interactions, functions, and independence of plasma membrane STIM1 and TRPC1 in vascular smooth muscle cells. Circ Res 103:e97–e104
18. Liao Y, Erxleben C, Abramowitz J et al (2008) Functional interactions among Orai1, TRPCs, and STIM1 suggest a STIM-regulated heteromeric Orai/TRPC model for SOCE/Icrac channels. Proc Natl Acad Sci U S A 105:2895–2900
19. Large WA, Wang Q (1996) Characteristics and physiological role of the Ca^{2+}-activated Cl^- conductance in smooth muscle. Am J Physiol 271:C435–C454
20. Leblanc N, Ledoux J, Saleh S et al (2005) Regulation of calcium-activated chloride channels in smooth muscle cells: a complex picture is emerging. Can J Physiol Pharmacol 83:541–556
21. Hartzell C, Putzier I, Arreola J (2005) Calcium-activated chloride channels. Annu Rev Physiol 67:719–758
22. Hartzell HC, Qu Z, Yu K, Xiao Q, Chien LT (2008) Molecular physiology of bestrophins: multifunctional membrane proteins linked to best disease and other retinopathies. Physiol Rev 88:639–672
23. Hartzell HC, Yu K, Xiao Q, Chien LT, Qu Z (2009) Anoctamin/TMEM16 family members are Ca^{2+}-activated Cl– channels. J Physiol 587(Pt 10):2127–2139
24. Caputo A, Caci E, Ferrera L et al (2008) TMEM16A, a membrane protein associated with calcium-dependent chloride channel activity. Science 322:590–594
25. Schroeder BC, Cheng T, Jan YN, Jan LY (2008) Expression cloning of TMEM16A as a calcium-activated chloride channel subunit. Cell 134:1019–1029
26. Yang YD, Cho H, Koo JY et al (2008) TMEM16A confers receptor-activated calcium-dependent chloride conductance. Nature 455:1210–1215
27. Lin MJ, Leung GPH, Zhang WM et al (2004) Chronic hypoxia-induced upregulation of store-operated and receptor-operated Ca^{2+} channels in pulmonary arterial smooth muscle cells — A novel mechanism of hypoxic pulmonary hypertension. Circ Res 95:496–505
28. Greenwood IA, Ledoux J, Sanguinetti A, Perrino BA, Leblanc N (2004) Calcineurin Aα but not Aβ augments $I_{Cl(Ca)}$ in rabbit pulmonary artery smooth muscle cells. J Biol Chem 279:38830–38837
29. Angermann JE, Sanguinetti AR, Kenyon JL, Leblanc N, Greenwood IA (2006) Mechanism of the inhibition of Ca^{2+}-activated Cl^- currents by phosphorylation in pulmonary arterial smooth muscle cells. J Gen Physiol 128:73–87
30. Edwards G, Niederstehollenberg A, Schneider J, Noack T, Weston AH (1994) Ion channel modulation by NS 1619, the putative BK_{Ca} channel opener, in vascular smooth muscle. Br J Pharmacol 113:1538–1547
31. McDaniel SS, Platoshyn O, Wang J et al (2001) Capacitative Ca^{2+} entry in agonist-induced pulmonary vasoconstriction. Am J Physiol Lung Cell Mol Physiol 280:L870–L880
32. Leung FP, Yung LM, Yao X, Laher I, Huang Y (2008) Store-operated calcium entry in vascular smooth muscle. Br J Pharmacol 153:846–857
33. Zweifach A, Lewis RS (1995) Slow calcium-dependent inactivation of depletion-activated calcium current. Store-dependent and -independent mechanisms. J Biol Chem 270:14445–14451
34. Zweifach A, Lewis RS (1995) Rapid inactivation of depletion-activated calcium current (i_{CRAC}) due to local calcium feedback. J Gen Physiol 105:209–226
35. Holland M, Langton PD, Standen NB, Boyle JP (1996) Effects of the BK_{Ca} channel activator, NS1619, on rat cerebral artery smooth muscle. Br J Pharmacol 117:119–129
36. Greenwood IA, Ledoux J, Leblanc N (2001) Differential regulation of Ca^{2+}-activated Cl^- currents in rabbit arterial and portal vein smooth muscle cells by Ca^{2+}-calmodulin-dependent kinase. J Physiol 534:395–408

37. Hirota S, Pertens E, Janssen LJ (2007) The reverse mode of the Na^+/Ca^{2+} exchanger provides a source of Ca^{2+} for store refilling following agonist-induced Ca^{2+} mobilization. Am J Physiol Lung Cell Mol Physiol 292:L438–L447
38. Vazquez G, Wedel BJ, Aziz O, Trebak M, Putney JW Jr (2004) The mammalian TRPC cation channels. Biochim Biophys Acta 1742:21–36
39. Dietrich A, Kalwa H, Storch U et al (2007) Pressure-induced and store-operated cation influx in vascular smooth muscle cells is independent of TRPC1. Pflugers Arch 455:465–477
40. Yu Y, Sweeney M, Zhang S et al (2003) PDGF stimulates pulmonary vascular smooth muscle cell proliferation by upregulating TRPC6 expression. Am J Physiol Cell Physiol 284:C316–C330
41. Inoue R, Okada T, Onoue H et al (2001) The transient receptor potential protein homologue TRP6 is the essential component of vascular α_1-adrenoceptor-activated Ca^{2+}-permeable cation channel. Circ Res 88:325–332
42. Jung S, Strotmann R, Schultz N, Plant TD (2002) TRPC6 is a candidate channel involved in receptor-stimulated cation currents in A7r5 smooth muscle cells. Am J Physiol Cell Physiol 282:C347–C359
43. Welsh DG, Morielli AD, Nelson MT, Brayden JE (2002) Transient receptor potential channels regulate myogenic tone of resistance arteries. Circ Res 90:248–250
44. Liou J, Kim ML, Heo WD et al (2005) STIM is a Ca^{2+} sensor essential for Ca^{2+}-store-depletion-triggered Ca^{2+} influx. Curr Biol 15:1235–1241
45. Zhang SL, Yu Y, Roos J et al (2005) STIM1 is a Ca^{2+} sensor that activates CRAC channels and migrates from the Ca^{2+} store to the plasma membrane. Nature 437:902–905
46. Soboloff J, Spassova MA, Hewavitharana T et al (2006) STIM2 is an inhibitor of STIM1-mediated store-operated Ca^{2+} entry. Curr Biol 16:1465–1470
47. Xu SZ, Boulay G, Flemming R, Beech DJ (2006) E3-targeted anti-TRPC5 antibody inhibits store-operated calcium entry in freshly isolated pial arterioles. Am J Physiol Heart Circ Physiol 291:H2653–H2659
48. Huang GN, Zeng W, Kim JY et al (2006) STIM1 carboxyl-terminus activates native SOC, I_{crac} and TRPC1 channels. Nat Cell Biol 8:1003–1010
49. Cheng KT, Liu X, Ong HL, Ambudkar IS (2008) Functional requirement for Orai1 in store-operated TRPC1-STIM1 channels. J Biol Chem 283:12935–12940
50. Jardin I, Lopez JJ, Salido GM, Rosado JA (2008) Orai1 mediates the interaction between STIM1 and hTRPC1 and regulates the mode of activation of hTRPC1-forming Ca^{2+} channels. J Biol Chem 283:25296–25304
51. Brandman O, Liou J, Park WS, Meyer T (2007) STIM2 is a feedback regulator that stabilizes basal cytosolic and endoplasmic reticulum Ca^{2+} levels. Cell 131:1327–1339
52. Stathopulos PB, Zheng L, Ikura M (2009) Stromal interaction molecule (STIM) 1 and STIM2 calcium sensing regions exhibit distinct unfolding and oligomerization kinetics. J Biol Chem 284:728–732
53. Ingueneau C, Huynh-Do U, Marcheix B et al (2008) TRPC1 is regulated by caveolin-1 and is involved in oxidized LDL-induced apoptosis of vascular smooth muscle cells. J Cell Mol Med Nov 14 [Epub ahead of print]

The Role of Intracellular Ion Channels in Regulating Cytoplasmic Calciumin Pulmonary Arterial Mmooth Muscle: Which Store and Where?

A. Mark Evans

Abstract The mobilisation of intracellular Ca^{2+} stores plays a pivotal role in the regulation of arterial smooth muscle function, paradoxically during both contraction and relaxation. Moreover, different spatiotemporal Ca^{2+} signalling patterns may trigger differential gene expression while mediating the same functional response. These facts alone serve to highlight the importance of the growing body of evidence in support of the view that different Ca^{2+} storing organelles may be selected by the discrete or co-ordinated actions of multiple Ca^{2+} mobilising messengers. In this respect, it is generally accepted that sarcoplasmic reticulum stores may be mobilised by the ubiquitous messenger inositol 1,4,5 trisphosphate. However, relatively little attention has been paid to the role of Ca^{2+} mobilising pyridine nucleotides in arterial smooth muscle, namely cyclic adenosine diphosphate-ribose and nicotinic acid adenine dinucleotide phosphate. This review will, therefore, focus on the role of these novel Ca^{2+} mobilising messengers in pulmonary arterial smooth muscle, with particular reference to hypoxic pulmonary vasoconstriction.

Keywords hypoxia • AMPK • NAADP • cADPR • ryanodine receptor • sarcoplasmic reticulum • lysosomes • artery • smooth muscle

1 Introduction

We know that agonist-specificity is determined, in part, by the release of Ca^{2+} from intracellular stores in a manner dependent on second messengers and their associated Ca^{2+} release channels. During pharmaco-mechanical coupling in smooth muscle, it has long been accepted that many G protein-coupled receptors induce the

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production of inositol 1,4,5-trisphosphate (IP_3), which leads to the activation of one or more of the known IP_3 receptor (IP_3R) sub-types on the sarcoplasmic reticulum (SR) and release of Ca^{2+} from this store.[1] However, there is a growing body of evidence to support a role for the Ca^{2+} mobilising pyridine nucleotides nicotinic acid adenine dinucleotide phosphate (NAADP) and cyclic adenosine diphosphate-ribose (cADPR) in the regulation of intracellular Ca^{2+} signalling in a number of cell types, including smooth muscle.[2–4] Furthermore, studies have raised the possibility that the spatiotemporal pattern of Ca^{2+} signals may also be determined via the selection of different intracellular Ca^{2+} stores in a manner dependent on the nature of the Ca^{2+}-mobilising messengers recruited by a given stimulus.[5–7]

Consideration of the role of Ca^{2+} signalling by pyridine nucleotides in arterial smooth muscle and of the Ca^{2+}-storing organelles that may be accessed by these messengers will therefore be central to advances in this field for some time to come. Our studies have revealed that in this respect the processes involved in pulmonary arterial smooth muscle are more complex than one might expect. In the context of pulmonary artery constriction and dilation, therefore, this chapter focuses on the role of pyridine nucleotide Ca^{2+}-mobilizing messengers, their receptors and the functional segregation of the Ca^{2+}-storing organelles they target.

2 Hypoxic Pulmonary Vasoconstriction

2.1 Regulation by Hypoxia of Calcium Mobilisation from Sarcoplasmic Reticulum Calcium Stores in Pulmonary Artery Smooth Muscle

In isolated pulmonary arteries, hypoxic pulmonary vasoconstriction (HPV) is biphasic when induced by switching from a normoxic to a hypoxic gas mixture [Fig. 4.1a(i)]. Thus, hypoxia induces an initial transient constriction (phase 1) and a slow tonic constriction (phase 2).[8,9] Both phases of constriction are superimposed on each other; that is, they are discrete events and are both initiated immediately on exposure to hypoxia. The initial transient constriction peaks within 5–10 min of the hypoxic challenge, whilst the underlying, tonic constriction peaks after 30–40 min. When the endothelium is removed, the gradual amplification of phase 2, which is driven by the release of an endothelium-derived vasoconstrictor, is not observed, and the phase 1 constriction now declines to a maintained plateau[8] [Fig. 4.1a(ii)]. Several investigations have suggested that phase 1 (first 5–10 min) of HPV is mediated, at least in part, by the release of Ca^{2+} from SR stores via ryanodine receptors (RyRs), but in general these studies did not demonstrate that this was an endothelium-independent process (see Chap. 12). The most significant study in this respect was that of Salvaterra and Goldman.[10] They determined that hypoxia triggered SR Ca^{2+} release in cultured pulmonary arterial smooth muscle cells, and that this led to consequent activation of a verapamil- and nifedipine-

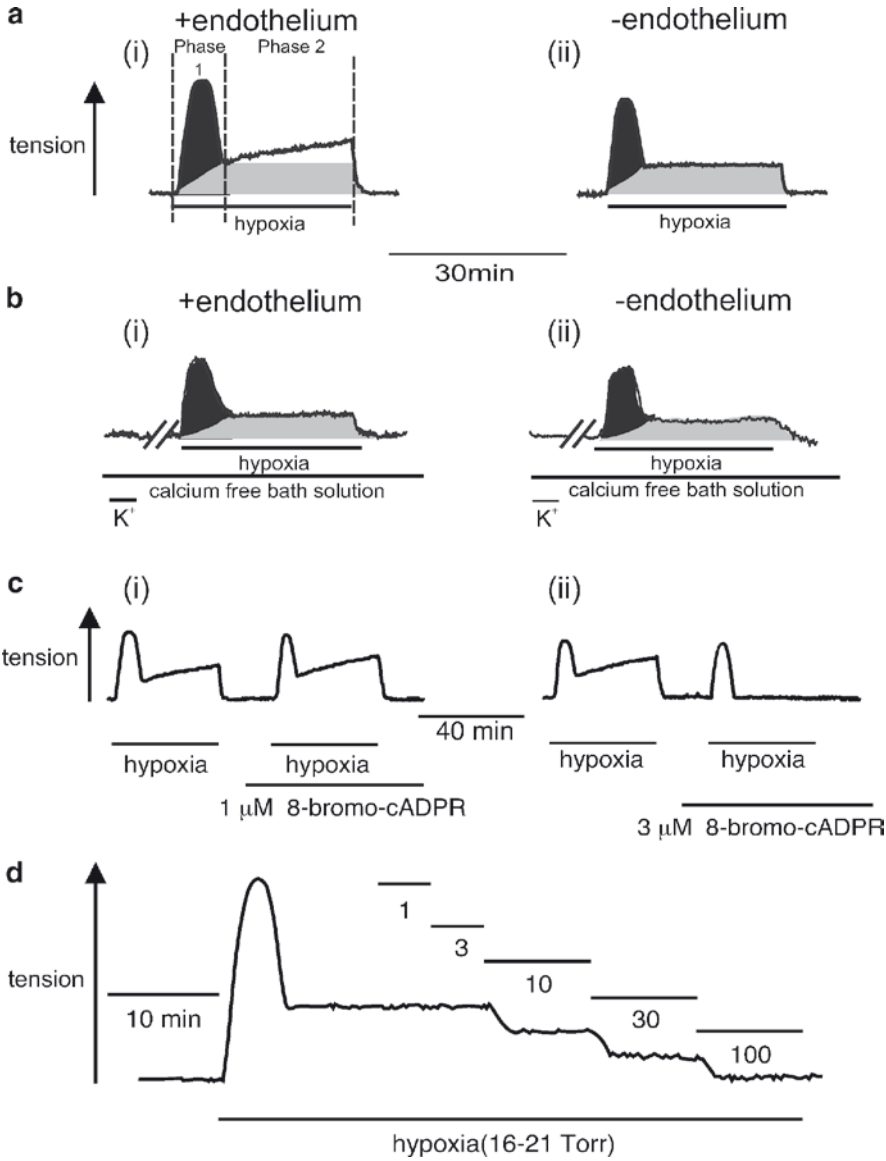

Fig. 4.1 Hypoxia triggers cADPR-dependent Ca^{2+} release from smooth muscle sarcoplasmic reticulum stores. **a**(i) Record indicating phase 1 and phase 2 of the response of an intact pulmonary artery ring to hypoxia, and the three identified components of HPV cADPR-independent SR Ca^{2+} release (*black*) cADPR-dependent SR Ca^{2+} release (*grey*) and endothelium-dependent (*white*). **a**(ii) Constriction by hypoxia of a pulmonary artery ring without endothelium. **b**(i) Constriction by hypoxia of an intact pulmonary artery ring in the absence of extra-cellular Ca^{2+}. **b**(ii) Constriction by hypoxia of a pulmonary artery ring without endothelium in the absence of extra-cellular Ca^{2+}. (**c**) Pre-incubating intact pulmonary arteries with 8-bromo-cylic ADP-ribose, a cADPR antagonist, at (i) 1 μ*M* has no effect but produces all-or-none block of phase 2 at (ii) 3 μ*M*. (**d**) Concentration-dependent reversal of maintained HPV by 8-bromo-cylic ADP-ribose in an artery without endothelium

insensitive transmembrane Ca^{2+} influx pathway that was not "modulated" by hypoxia. In short, hypoxia triggered SR Ca^{2+} release and store-operated Ca^{2+} entry in a manner that was mimicked by the SR Ca^{2+} ATPase (SERCA) antagonist thapsigargin and blocked by pre-incubation of cells with thapsigargin.

Some years later, we provided compelling evidence in support of a pivotal role for continued smooth muscle SR Ca^{2+} release via RyRs in the induction (phase 1 and 2) and maintenance (phase 2) of HPV in isolated pulmonary arteries both with and without endothelium.[8] Briefly, both phase 1 and phase 2 of HPV were shown to be abolished following block of SR Ca^{2+} release via RyRs with ryanodine and caffeine, whilst constriction in response to membrane depolarisation (80 m*M* K^+) and consequent voltage-gated Ca^{2+} influx remained unaffected (not shown). Furthermore, when the endothelium was absent, hypoxia triggered both the transient and maintained phases of constriction after removal of extra-cellular Ca^{2+}, despite the fact that constriction induced by depolarisation (by K^+) was abolished [Fig. 4.1b(ii)]. Thus, it would appear that smooth muscle Ca^{2+} release from ryanodine-sensitive SR stores underpins pulmonary artery smooth muscle constriction by hypoxia. It is notable, however, that maintained constriction of pulmonary artery rings was attenuated by up to 50% in Ca^{2+}-free medium,[11] consistent with the view that HPV is supported by consequent activation of store-depletion-activated Ca^{2+} entry.[12]

These findings suggested that in the intact artery the mobilisation by hypoxia of SR Ca^{2+} stores via RyRs was mediated by mechanisms intrinsic to pulmonary arterial smooth muscle cells. We therefore considered the possibility that cADPR, an endogenous regulator of RyRs,[13] may play a role in this process.

2.2 ADP-Ribosyl Cyclase and cADPR Hydrolase Activities Are Differentially Distributed in Pulmonary Versus Systemic Artery Smooth Muscle

Our initial findings were striking in that the enzyme activities for the synthesis and metabolism of cADPR were at least an order of magnitude higher in homogenates of pulmonary artery smooth muscle than in those of aortic or mesenteric artery smooth muscle.[14] Of further significance was the finding that the level of these enzyme activities was inversely related to pulmonary artery diameter. Thus, the differential distribution of these enzyme activities may offer, via amplification of the initial stimulus, the pulmonary selectivity required of a mediator of HPV and underpin, at least in part, the inverse relationship between the magnitude of constriction by hypoxia and pulmonary artery diameter.[15] This proposal was supported by direct measurement of cADPR content (estimated basal level ≥5 μ*M*) using a [^{32}P]cADPR binding assay. Hypoxia (16–21 Torr) increased cADPR levels twofold in second-order branches of the pulmonary arterial tree and tenfold in third-order branches.[14] Thus, like constriction by hypoxia and the distribution of the enzyme activities for cADPR synthesis, the

increase in cADPR content induced by hypoxia was inversely related to pulmonary artery diameter.

The mechanism by which hypoxia promotes cADPR accumulation in pulmonary artery smooth muscle remains to be confirmed. However, we have provided evidence to suggest that increased β-NADH beta-nicotinamide adenine dinucleotide formation under hypoxic conditions may facilitate cADPR formation from β-NAD^+ by augmenting adenosine diphosphate (ADP)-ribosyl cyclase or inhibiting cADPR hydrolase activities.[14] An alternative proposal is that hypoxia may initiate a paradoxical increase in reactive oxygen species (ROS) generation by mitochondria and thereby elicit SR Ca^{2+} release.[16] Most recently, however, we have obtained data consistent with the view that the metabolic sensor adenosine monophosphate (AMP)-activated protein kinase may couple the inhibition of mitochondrial oxidative phosphorylation by hypoxia to cADPR-dependent SR Ca^{2+} release in pulmonary arterial smooth muscle.[11] It is my view that the mobilisation by hypoxia of SR Ca^{2+} stores is mediated by the combinatorial effects of AMPK activation and β-NADH accumulation,[17] not only via the regulation of cADPR accumulation but also due to direct regulation by AMPK of, for example, RyRs, SERCA function and ion channels in the plasma membrane. Further investigations are, however, required to define the precise mechanisms involved.

2.3 *The cADPR Antagonist 8-Bromo-cADPR Identifies cADPR-Independent and cADPR-Dependent Phases of Smooth Muscle SR Ca^{2+}Release by Hypoxia*

The effects of a cADPR antagonist, 8-bromo-cADPR, on HPV in isolated pulmonary artery rings were quite different from the effects of ryanodine and caffeine. In arteries with and without endothelium, 8-bromo-cADPR had no effect on phase 1 of HPV. However, it abolished phase 2 in the presence of the endothelium and blocked the maintained constriction observed in arteries without endothelium[18] [Fig. 4.1c(ii)]. Thus, while cADPR-dependent SR Ca^{2+} release is required for the initiation and maintenance of phase 2 of acute HPV in isolated pulmonary artery rings, cADPR is not required to support the majority of SR Ca^{2+} release during the phase 1 constriction.

2.4 *8-Bromo-cADPR Blocks Phase 2 of HPV in an All-or-None Manner*

An unexpected and surprising observation of ours was that when arteries were pre-incubated with the cADPR antagonist 8-bromo-cADPR, phase 2 of HPV was blocked in an all-or-none manner.[18] Briefly, following pre-incubation of isolated pulmonary arteries with 1 μ*M* 8-bromo-cADPR, HPV remained unaltered [Fig. 4.1c(i)], but pre-incubation with 3 μ*M* 8-bromo-cADPR abolished the maintained

constriction observed during phase 2 [Fig. 4.1c(ii)]. This is entirely incompatible with the block by a competitive antagonist, such as 8-bromo-cADPR, of a simple process of "agonist"-receptor coupling.

The aforementioned finding was all the more curious given that, once initiated, the maintained phase of constriction, in pulmonary arteries without endothelium, was reversed by 8-bromo-cADPR in a concentration-dependent manner and with complete block only being attained at a concentration of 100 μ*M*,[18] nearly an order of magnitude higher than required for all-or-none block following pre-incubation with 8-bromo-cADPR (Fig. 4.1d). Unlike the all-or-none block observed following pre-incubation, this concentration-dependent reversal of maintained HPV is entirely consistent with the inhibition by a competitive antagonist of agonist-receptor coupling at a single population of receptors.

We concluded that these findings are reminiscent of the block by α-bungarotoxin of transmission at the neuromuscular junction, where greater than 45% of skeletal muscle nicotinic acetylcholine receptors must be blocked before neuromuscular transmission is compromised, and we proposed that a similar "margin of safety" may therefore be built into HPV. At the time, we suggested that the cADPR-dependent component of HPV may be initiated in an all-or-none manner, and that the all-or-none block of HPV by 8-bromo-cADPR could be due to it "blocking the activation by cADPR of a certain proportion of RyRs" or by the block of "cADPR-dependent Ca^{2+} mobilisation from a sub-population of RyRs" that are pivotal to the initiation of HPV.[2,18]

3 NAADP Induces Global Ca^{2+} Waves and Smooth Muscle Contraction in an All-or-None Manner

3.1 NAADP Triggers Ca^{2+}Bursts from Lysosome-Related Acidic Stores that Are Amplified by Ca^{2+}-induced Ca^{2+} release from the SR via RyRs

One possible explanation of the all-or-none block of HPV by 8-bromo-cADPR could be revealed by studies on NAADP, which is also synthesised by ADP-ribosyl cyclase, if NAADP or Ca^{2+} mobilisation from the store it accesses acts in concert with cADPR to mediate intracellular Ca^{2+} signalling by hypoxia in pulmonary arterial smooth muscle. This is clear from the fact that NAADP initiates global Ca^{2+} waves in an all-or-none manner and via a relatively complex two-pool system[19] comprised of the mobilisation of acidic, lysosome-related Ca^{2+} stores and subsequent Ca^{2+}-induced Ca^{2+} release (CICR) from the SR via RyRs.[5]

That NAADP may selectively elicit Ca^{2+} signals from lysosome-related Ca^{2+} stores in pulmonary arterial smooth muscle cells is supported by the fact that selective depletion of acidic Ca^{2+} stores by bafilomycin A1, which blocks the vacuolar H^+ ATPase (adenosine triphosphatase), abolishes NAADP-dependent Ca^{2+} signal-

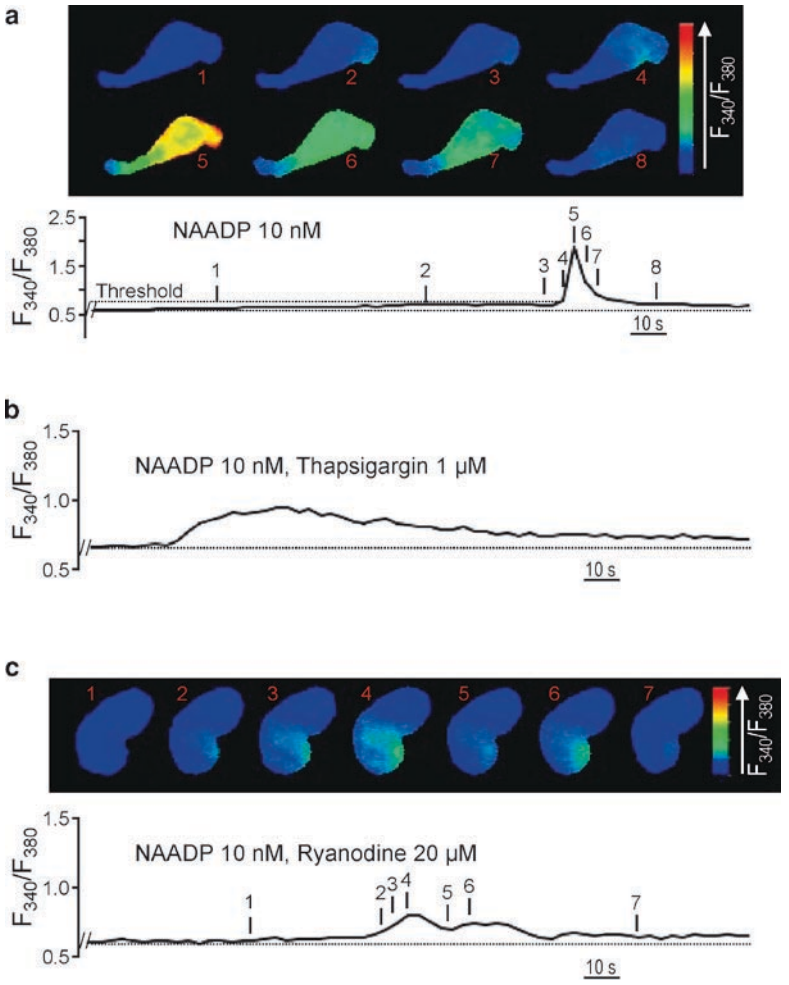

Fig. 4.2 NAADP triggers Ca^{2+} signals via lysosome-related stores in a manner that leads to subsequent Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum (SR) via ryanodine receptors (RyRs) in isolated pulmonary arterial smooth muscle cells. (**a**) *Upper panel* shows a series of pseudocolour images of the fura-2 fluorescence ratio (F_{340}/F_{380}) recorded in a pulmonary artery smooth muscle cell during intracellular dialysis of 10 n*M* NAADP. Note that a spatially localised 'Ca^{2+} burst' precedes the global Ca^{2+} wave (image 4). *Lower panel* shows the fura-2 fluorescence ratio against time. (**b**) Effect of intracellular dialysis of 10 n*M* NAADP after pre-incubation (20 min) of cells with 1 μ*M* thapsigargin. (**c**) *Upper panel* shows a series of pseudocolour images and the *lower panel* indicates F_{340}/F_{380} against time, obtained during the intracellular dialysis of 10 n*M* NAADP after pre-incubation (20 min) of cells with 20 μ*M* ryanodine. *Lower panel* shows the fura-2 fluorescence ratio against time. Note that in the absence of functional RyRs or SR stores replete in Ca^{2+}, spatially restricted Ca^{2+} bursts are triggered without initiation of a global Ca^{2+} wave

ling without effect on SR Ca^{2+} release via either RyRs or IP_3Rs.[19] Significantly, however, depletion of SR Ca^{2+} stores by inhibition of the sarcoendoplasmic reticulum Ca^{2+} pump (SERCA) with thapsigargin or block of RyRs with ryanodine (Fig. 4.2a-c) revealed spatially restricted bursts of Ca^{2+} release in response to NAADP that failed to propagate away from their point of initiation in the absence of either SR stores replete in Ca^{2+} or functional RyRs.[5,19] Thus, NAADP initiates highly localised "Ca^{2+} bursts" from lysosome-related stores that may either decline back to basal levels or precede and then trigger a global Ca^{2+} wave due to subsequent CICR from the SR via RyRs. NAADP-induced Ca^{2+} bursts must therefore breach a given threshold to elicit a global Ca^{2+} wave by CICR via RyRs on the SR, and this may be facilitated indirectly if Ca^{2+} bursts also serve to prime SR stores by increasing their Ca^{2+} load via SERCA-dependent uptake of a proportion of released Ca^{2+} into the SR (see section 5 see below). However, before this point can be discussed further we must consider the ways in which cADPR and Ca^{2+} may modulate RyR function.

3.2 Possible Role of cADPR in the Modulation of NAADP-Dependent Ca^{2+}Signalling

By sensitising RyRs to Ca^{2+}, cADPR may determine the threshold for CICR via RyRs in response to Ca^{2+} bursts elicited from lysosomes by NAADP, or any other stimulus, and the degree of amplification of the initial Ca^{2+} burst by CICR.[2] The set point for these processes will likely be determined by the basal cADPR concentration (≥ 5 μM) and the local Ca^{2+} concentration but will be open to modulation by stimulus-dependent changes in cADPR synthesis or degradation. Thus, it is quite possible that a competitive cADPR antagonist, such as 8-bromo-cADPR, could block in an all-or-none manner the amplification of NAADP-dependent Ca^{2+} bursts into global Ca^{2+} waves by raising the threshold for CICR via RyRs. Subsequent to the initiation of a global Ca^{2+} wave, however, increased cADPR accumulation alone in response to a given stimulus could also provide for the maintenance of regenerative Ca^{2+} waves and smooth muscle contraction by cADPR-dependent CICR via RyRs.[2] Such a paradigm would then allow for the concentration-dependent reversal of maintained constriction by 8-bromo-cADPR.

The process of CICR is therefore pivotal. This refers to the fact that RyRs may be activated by Ca^{2+} in its own right or to the fact that Ca^{2+} release via RyRs may be facilitated by released Ca^{2+} via positive feedback.[20–22] Thus, CICR offers cells the facility to amplify small, highly localised Ca^{2+} signals into global Ca^{2+} waves via the recruitment of neighbouring RyR complexes. This can be achieved in a highly regulated manner due to the limitations placed on Ca^{2+} diffusion (≤ 5 μm)[23] by the buffering capacity within the cytoplasm. CICR may therefore recruit, in concert, discrete clusters of RyRs to initiate highly localised, elementary Ca^{2+} release events such as Ca^{2+} sparks.[24,25] Alternatively, once a given threshold concentration is breached Ca^{2+} may induce a propagating global Ca^{2+} wave by the progressive

recruitment by CICR of RyR clusters distant from the site of initiation. As mentioned, cADPR, like Ca^{2+}, may act as an endogenous regulator of RyRs[13] and may also either activate RyRs directly or facilitate CICR via RyRs.[26,27] Thus, it is not surprising that Ca^{2+} also sensitises RyRs to activation by cADPR.[28] Therefore, when considering the regulation by cADPR of RyRs in a given cell type, the combinatorial effects of Ca^{2+} and cADPR are of fundamental importance,[4] not least with respect to the threshold for activation of RyRs by either agent. And, the threshold for CICR via RyRs may also be modulated by the luminal Ca^{2+} concentration of the SR,[29–33] which could in turn be primed by Ca^{2+} supplied by preceding lysosome-related Ca^{2+} release events.

The situation presented in vascular smooth muscle is more complex still given that RyR sub-types 1, 2 and 3 are highly co-expressed in these cell types,[34,35] not least because all three RyR sub-types can be expressed in a cADPR-sensitive form and each may exhibit different sensitivities to both Ca^{2+} and cADPR. Therefore, the RyR sub-type targeted by Ca^{2+} signals from lysosomes could affect markedly the characteristics of any subsequent amplification process, determine to a great extent the all-or-none initiation of global Ca^{2+} signals by NAADP in pulmonary arterial smooth muscle and, if a sub-population of RyRs on the SR were targeted, confer all-or-none block of this amplification step by pre-incubation with 8-bromo-cADPR (see below see section 4.1).

4 Lysosome-Sarcoplasmic Reticulum Junctions Form a Trigger Zone for Ca^{2+} Signalling by NAADP

4.1 Lysosomes Co-localise with a Sub-population of RyRs

Using LysoTracker Red as a fluorescent label for acidic organelles in acutely isolated pulmonary arterial smooth muscle cells, we demonstrated that a large proportion of lysosomes form tight clusters in a manner consistent with the spatially restricted nature of Ca^{2+} bursts triggered by NAADP. Importantly, lysosomal clusters were closely associated with a sub-population of RyRs labelled with Bodipy-Ryanodine [Fig. 4.3a(i)] and appear to be separated from these RyRs by a narrow junction or cleft that is beyond the resolution of deconvolution microsopy (<1 μm).[5] We proposed, therefore, that lysosomal clusters and RyRs may form a highly organised "trigger zone," or intracellular synapse, for Ca^{2+} signalling by NAADP in arterial smooth muscle. The presence of this trigger zone may explain, in part, why Ca^{2+} bursts by NAADP induce global Ca^{2+} signals in an all-or-none manner by further CICR from the SR via RyRs. This tight coupling of lysosomal Ca^{2+} stores to a sub-population of RyRs could also serve to provide the aforementioned "margin of safety" with respect to the initiation of HPV, should lysosome-related Ca^{2+} release play a role, and confer all-or-none block of HPV by 8-bromo-cADPR due to the consequent increase in the threshold for CICR.

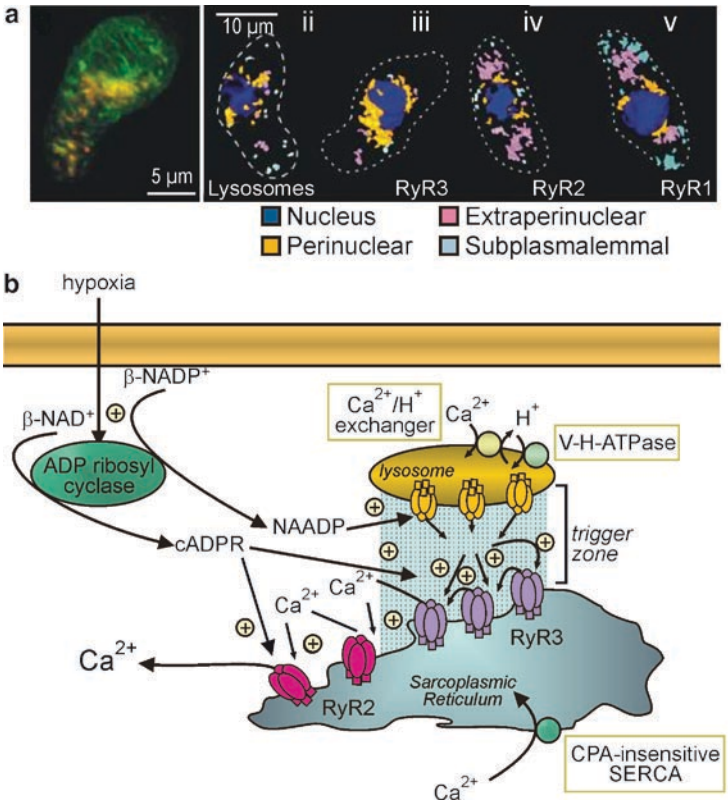

Fig. 4.3 Lysosomes preferentially co-localise with ryanodine receptor sub-type 3 to form a trigger zone for calcium signalling in response to NAADP. (**a**) 3D reconstruction of a deconvolved Z-stack of images from a pulmonary artery smooth muscle cell showing co-localisation (*yellow*) of lysosomes labelled with LysoTracker Red (*red*) and ryanodine receptors (RyRs) labelled with Bodipy-Ryanodine (*green*). (**b**) 3D reconstruction of deconvolved Z-stacks of images showing the distribution of individual volumes of lysosomal (αlgp120), RyR3, RyR2 and RyR1 labelling coloured to indicate their respective distribution in defined regions of the cell: the perinuclear volume, extra-perinuclear volume and sub-plasmalemmal volume. (**c**) Schematic diagram depicts the proposed trigger zone for Ca^{2+} signalling by NAADP and the possible amplification of NAADP-dependent Ca^{2+} signals by cADPR. *NAADP* nicotinic acid adenine dinucleotide phosphate; *cADPR* cyclic adenosine diphosphate-ribose; *SERCA* sarcoendoplasmic reticulum Ca^{2+} ATPase; *RyR* ryanodine receptor; *V-H-ATPase* vacuolar proton pump

4.2 Lysosomes Co-localise with RyR Sub-type 3 to Form a Trigger Zone for Ca^{2+}Signalling by NAADP in Pulmonary Arterial Smooth Muscle

Our most recent studies sought to determine whether lysosomes selectively couple to one of the three RyR sub-types expressed in arterial smooth muscle, namely, RyR1, RyR2, or RyR3.[36] The distribution of labelling for a given protein by density was determined for each of three defined regions of the cell relative to the nucleus (defined by DAPI 4'-6-Diamidino-2-phenylindole labelling), namely,

the perinuclear (within 1.5 μm of the nucleus), the sub-plasmalemmal (within 1.5 μm of the plasma membrane) and the extra-perinuclear region (the remaining volume of the cytoplasm). The density of labelling for the lysosome marker (αlgp120) was about twofold greater in the perinuclear than observed within the extra-perinuclear region and about fourfold greater than was observed in the sub-plasmalemmal region of cells, with dense clusters of labelling evident in the perinuclear region compared to a more diffuse distribution of labelling outside this region [Fig. 4.3a(ii)]. In common with the distribution of lysosomes, but to an even greater extent, the density of RyR3 labelling was concentrated within the perinuclear region of the cell, where it was about 4- and ~14-fold greater than that in the extra-perinuclear and sub-plasmalemmal regions, respectively. Furthermore, the density of RyR3 labelling within the perinuclear region was about twofold higher than that for either RyR1 or RyR2 [Fig. 4.3a(iii-v)].

Further insight was provided by analysis of the density of co-localisation between lysosomes and each RyR sub-type. Within the perinuclear region of the cell, RyR3 was found to co-localise with about 41% of the total volume of lysosome labelling, with the density of co-localisation about 4- and about 60-fold greater than that observed in the extra-perinuclear or sub-plasmalemmal regions, respectively. In marked contrast, labelling for RyR2 and RyR1 co-localised with only 13 and 14%, respectively, of the total volume of lysosome labelling within the perinuclear region, and their respective density of co-localisation was approximately twofold lower than that for RyR3. Furthermore, the mean volume of co-localisation between RyR3 and lysosomes was about twofold greater than that for either RyR1 or RyR2. We concluded, therefore, that lysosomal clusters preferentially co-localise with RyR3 in the perinuclear region of the cell to form a trigger zone for Ca^{2+} signalling by NAADP.

4.3 Why Might RyR3 Be Targeted to Lysosome–SR Junctions?

Determining factors in this respect could be the relative sensitivity of each RyR sub-type to CICR, the maximum gain in response to Ca^{2+} and the relative sensitivity of each receptor sub-type to inactivation by Ca^{2+}.[37,38] The threshold for activation of RyR1, RyR2 and RyR3 is similar, with channel activation at cytoplasmic Ca^{2+} concentrations above 100 n*M*. However, estimates of the EC_{50} are different, with half-maximal activation at about 250 n*M* for RyR2 and about 400 n*M* for RyR3. The higher EC_{50} exhibited by RyR3 could be significant because this would provide for a higher "margin of safety" with respect to the all-or-none amplification of Ca^{2+} bursts from lysosomal Ca^{2+} stores by CICR via RyRs at the lysosome–SR junction; that is, the probability of false events being initiated would be lower for RyR3 than for RyR2. Another factor that may be of significance is that whilst the mean open times versus cytoplasmic Ca^{2+} concentration for RyR2 and RyR3 are comparable and increase approximately tenfold over their activation range, the mean open time for RyR1 is much lower and increases only twofold over its activation range. Furthermore, comparison of the Po versus cytoplasmic Ca^{2+} concentration curves

shows that RyR3 (0–1) exhibits a higher gain in Po than does RyR2 (0–0.9), whilst RyR1 (0–0.2) exhibits relatively little gain in Po with increasing cytoplasmic Ca^{2+} concentration. Thus, once the threshold for activation is breached, RyR3 would offer greater amplification of Ca^{2+} bursts from lysosomal Ca^{2+} stores than would RyR2, whilst amplification via RyR1 would be marginal. There is also marked variation in the relative sensitivity of each RyR sub-type to inactivation by Ca^{2+}. RyR3 exhibits the lowest sensitivity to inactivation by Ca^{2+} with an IC_{50} of 3 m*M* whilst that for RyR2 is 2 m*M*; in each case, channel activity may still be observed at concentrations above 10 m*M*. In marked contrast, RyR1 inactivation occurs within the micromolar range, and full inactivation is achieved by 1 m*M* Ca^{2+}; this may, in part, explain the low gain in Po for RyR1 in response to activation by Ca^{2+}. Its sensitivity to inactivation by Ca^{2+} would therefore render RyR1 unsuitable for a role in the amplification of Ca^{2+} bursts at lysosome-SR junctions because the local Ca^{2+} concentration may exceed the threshold for RyR1 inactivation. Thus, the functional properties of RyR3 make it best suited to a role in the amplification of Ca^{2+} bursts at lysosome–SR junctions.

4.4 How May Ca^{2+}Signals Propagate Away from Lysosome–SR Junctions to the Wider Cell If RyR3 Is Targeted to the Perinuclear Region of Cells?

Significantly, the density of RyR3 labelling declines markedly (between 4- and 14-fold by region) outside the perinuclear region of the cell.[36] It seems unlikely, therefore, that RyR3 functions to carry a propagating Ca^{2+} wave far beyond the point of initiation of CICR within the proposed trigger zone for Ca^{2+} signalling via lysosomes. Given this finding, it may be of significance that the density of labelling for RyR2 increases markedly in the extra-perinuclear region when compared to the perinuclear region and exhibits about a threefold greater density of labelling within this region than observed for either RyR3 or RyR1. This suggests that RyR2, but not RyR1, may function to receive Ca^{2+} from RyR3 at the interface of the lysosome–SR junction and thereby allow for further propagation of the Ca^{2+} signal via CICR. Such a role would be supported by the lower EC_{50} for CICR via RyR2, which would ensure that once initiated a propagating Ca^{2+} wave would be less prone to failure. Furthermore, relative to RyR1, its greater intrinsic gain and lower sensitivity to inactivation by Ca^{2+} would render RyR2 most suitable to a role in the wider propagation of a global Ca^{2+} wave.

If clusters of RyR3 do indeed sit within the lysosome–SR junction and an array of RyR2 carries propagating Ca^{2+} signals away from this and Ca^{2+} signalling via this junction triggers HPV, pre-incubation of pulmonary arteries with 8-bromo-cADPR could block HPV in an all-or-none manner by increasing the threshold for CICR via RyR3 or RyR2. Furthermore, once initiated, if regenerative, propagating Ca^{2+} waves via RyR2 are maintained by an increase in cADPR accumulation in the

absence of further Ca^{2+} release from lysosome-related stores, 8-bromo-cADPR could reverse associated pulmonary artery constriction in a concentration-dependent manner (Fig. 4.3c).

4.5 RyR1 Is may be the predominant RyR subtype in the Sub-plasmalemmal Region of the Cell

The even distribution of RyR1 across the three specified regions of pulmonary artery smooth muscle cells suggests that it may contribute in some way to the regulation of Ca^{2+} signalling within each region. However, RyR1 is predominantly targeted (three- to fivefold by density of labelling) to the sub-plasmalemmal region and may therefore play a prominent role in Ca^{2+} signalling between the SR and Ca^{2+}-sensitive ion channels in the plasma membrane.[36]

5 Discrete SR Compartments Underpin Ca^{2+}-Dependent Vasodilation and Vasoconstriction

5.1 Cyclopiazonic Acid and 8-Bromo-cADPR Reveal Two Functionally Segregated SR Ca^{2+}Stores

An unexpected observation during our studies on the role of cADPR in HPV was that the SERCA pump antagonist cyclopiazonic acid blocked the phase 1 constriction but had no effect on phase 2 (Fig. 4.4a-b),[18] even though Ca^{2+} release from ryanodine-sensitive SR stores in the smooth muscle underpins both phases of HPV.[8] This was precisely the reverse of the effect of 8-bromo-cADPR, which abolished phase 2 of HPV without effect on phase 1 (Fig. 4.4c, d).[18] At the time, we concluded that phase 1 might be mediated by the mobilisation of an SR compartment served by a cyclopiazonic acid-sensitive SERCA that may be inhibited by hypoxia due to a fall in adenosine triphosphate (ATP) supply, and that to allow for this and a second phase of maintained cADPR-dependent SR Ca^{2+} release, one would require the presence of a second, spatially segregated SR Ca^{2+} store that is served by a discrete, cyclopiazonic acid-insensitive SERCA pump.[2,18]

We began to square this circle when studying the effect on cytoplasmic Ca^{2+} concentration of intracellular dialysis of cADPR (from a patch pipette). High concentrations of cADPR (100 μM) induced a small but sustained (unlike NAADP or IP_3)[19] and global increase in intracellular Ca^{2+} concentration (unpublished data). However, relatively low concentrations (20 μM) only increased cytoplasmic Ca^{2+} concentration at the perimeter of the cell and elicited a concomitant membrane hyperpolarisation (not shown).[39] The hyperpolarisation was reversed by the highly selective BK_{Ca} channel antagonist iberiotoxin, by chelating intracellular Ca^{2+} with BAPTA, by selective block of RyRs with ryanodine and by pre-incubation with

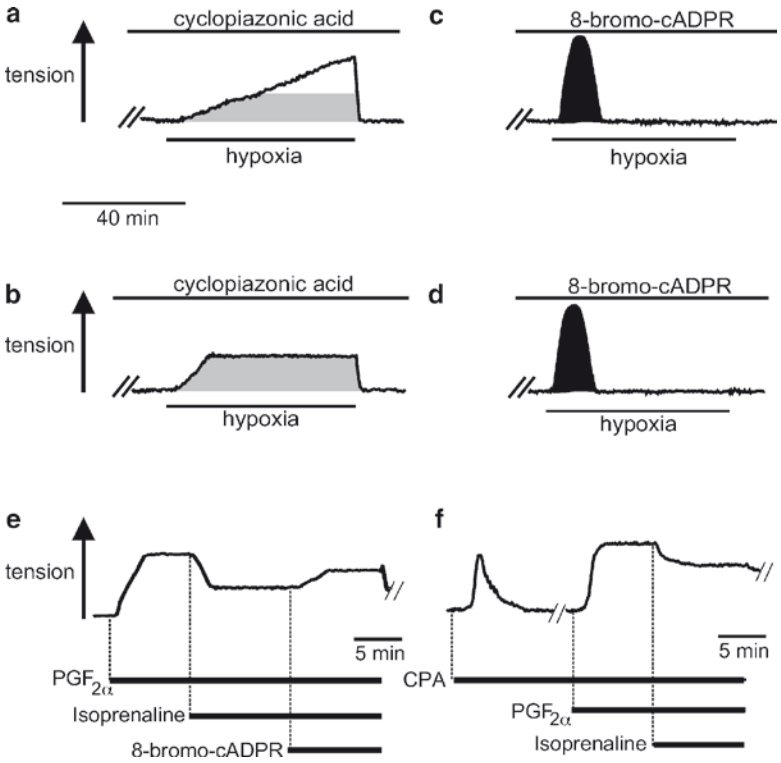

Fig. 4.4 Pharmacologically distinct smooth muscle sarcoplasmic reticulum Ca^{2+} stores underpin pulmonary artery dilation and constriction. Constriction by hypoxia (16–21 Torr) of a pulmonary artery ring (**a**) with and (**b**) without endothelium following pre-incubation (20 min) with cyclopiazonic acid (CPA) (10 μ*M*); (**c**) with and (**d**) without endothelium following pre-incubation (20 min) with 8-bromo-cADPR (300 μ*M*). Vasodilation by isoprenaline (100 n*M*) of a pulmonary artery ring, without endothelium, following pre-constriction with prostaglandin F2α (50 μ*M*) and the effect of (**e**) 8-bromo-cADPR (300 μ*M*) and (**f**) pre-incubation (20 min) with CPA (10 μ*M*)

cyclopiazonic acid. Most importantly, hyperpolarisation by cADPR was blocked by two different cADPR antagonists. Given that cADPR synthesis is up-regulated in a cAMP- (cyclic adenosine monophosphate-) and protein kinase A- (PKA)-dependent manner in cardiac muscle,[40] it seemed likely that cADPR could mediate hyperpolarisation by adenylyl cyclase-coupled receptors, such as β-adrenoceptors. Consistent with this proposal and previous studies on smooth muscle from a variety of tissues (for review, see Ref. [24]), we found that isoprenaline and cAMP induced hyperpolarisation in isolated pulmonary arterial smooth muscle cells and demonstrated that in each case hyperpolarisation exhibited a similar pharmacology to that induced by cADPR.[39] Strikingly, however, the selective PKA antagonist H89 blocked hyperpolarisation by both isoprenaline and cAMP, respectively, but was without effect on hyperpolarisation by cADPR. Thus, it would appear that cADPR is a downstream element in this signalling cascade. Further support for this proposal

was derived from studies of isolated pulmonary artery rings without endothelium. Vasodilation evoked in response to β-adrenoceptor activation by isoprenaline was inhibited (~50%) by blocking cADPR with the membrane-permeant antagonist 8-bromo-cADPR (Fig. 4.4e), RyRs with ryanodine and, consistent with the hyperpolarisation, by pre-incubation with cyclopiazonic acid (Fig. 4.4f). We concluded that cADPR-dependent Ca^{2+} signalling via RyRs on a cyclopiazonic acid-sensitive SR store was responsible, in part, for BK_{Ca}-dependent vasodilation by isoprenaline in isolated pulmonary arteries.[39]

Although these findings provided evidence of functionally segregated stores and allowed for further interpretation of our anomalous findings, they presented us with a paradox. That is, our data suggested that cADPR-dependent SR Ca^{2+} release via RyRs mediates both vasodilation and vasoconstriction of pulmonary arteries in a stimulus-dependent manner. We concluded that this could only be explained if (1) β-adrenoceptor signalling targets, via PKA-anchoring proteins, PKA-dependent cADPR synthesis to a particular RyR sub-type, possibly RyR1 (section 4.5 see above), in the "peripheral" SR that is in close apposition to BK_{Ca} channels in the plasma membrane (2) cADPR-dependent vasoconstriction results from the activation of discrete RyR sub-types localised in the "central" SR. Clearly, however, our data suggest that these discrete SR compartments would have to be served by different SERCA pumps. More precisely, an SR compartment in close apposition to the plasma membrane would be served by a SERCA pump that is sensitive to cyclopiazonic acid and, by contrast, a central SR compartment in close apposition to the contractile apparatus would be served by a SERCA pump that is relatively insensitive to cyclopiazonic acid.[2,39] This conclusion is supported by the fact that both SR Ca^{2+} release in response to hypoxia[10] and HPV (unpublished data) are abolished following SR store depletion by block of SERCA with thapsigargin.

In complete agreement with our proposal, previous studies on smooth muscle, the pulmonary vasculature included, have provided evidence of discrete SR compartments.[41–46] Most significantly, a number of these studies shared one common and convincing piece of evidence: The SERCA pump antagonist cyclopiazonic acid selectively depletes one of at least two functionally segregated SR compartments. We therefore sought to determine whether multiple SERCA were expressed in pulmonary arterial smooth muscle and, if so, their respective spatial distribution.

5.2 SERCA2a and SERCA2b Serve Discrete SR Compartments in Pulmonary Arterial Smooth Muscle

Western blots and immunocytochemistry carried out with sequence-specific antibodies raised against each SERCA isoform identified protein bands for SERCA2a and SERCA2b but not SERCA1 or SERCA3 (not shown). In agreement with previous studies on vascular smooth muscle,[47] therefore, it would appear that only SERCA2a and SERCA2b are functionally expressed in pulmonary arterial smooth muscle.

Astonishingly clear differences in the spatial organisation of SERCA2a and SERCA2b, respectively, were evident even on visual inspection of deconvolved Z sections and three-dimensional (3D) reconstructions of all cells labelled for SERCA2a and SERCA2b. This fact was confirmed by determining the distribution by density of labelling for each SERCA isoform within the sub-plasmalemmal (within 1 μm of the plasma membrane), the perinuclear (within 1.5 μm of the nucleus) and the extra-perinuclear (remainder) volumes.[48] The vast majority of SERCA2b labelling, about 70%, lay within the sub-plasmalemmal region, with only about 8% and about 20% of labelling present in the extra-perinuclear and perinuclear regions, respectively (Fig. 4.5a). In marked contrast, SERCA2a labelling was almost entirely (~90%) restricted to the perinuclear region of pulmonary arterial smooth muscle cells (Fig. 4.5a). These data suggest, therefore, that native SERCA2b may be sensitive to cyclopiazonic acid and supply an SR compartment that sits proximal to the plasma membrane and underpins Ca^{2+}-dependent vasodilation via adenylyl cyclase-coupled receptors, while SERCA2a may supply a central SR compartment and represent a cyclopiazonic acid-insensitive, thapsigargin-sensitive SERCA that underpins pulmonary artery constriction by hypoxia (Fig. 4.5b).

5.3 Possible Role of cADPR-Independent and cADPR-Dependent Phases of SR Ca^{2+}Release by Hypoxia

Given that vasodilation in response to activation of adenylyl cyclase-coupled receptors and phase 1 of HPV are inhibited by cyclopiazonic acid, they likely utilise a common SR store. It is possible, therefore, that SR Ca^{2+} release by hypoxia serves two purposes. Hypoxia may primarily trigger constriction by cADPR-dependent Ca^{2+} release from a central SR compartment that is in close apposition to the contractile apparatus and served by a cyclopiazonic acid-insensitive SERCA pump (SERCA2a). A secondary action of hypoxia may be to deplete a peripheral SR compartment by inhibition of a cyclopiazonic acid-sensitive SERCA pump (SERCA2b) in close apposition to the plasma membrane and that normally mediates vasodilation by releasing Ca^{2+} proximal to the plasma membrane to trigger membrane hyperpolarisation and thereby facilitate Ca^{2+} sequestration via plasma membrane Ca^{2+} ATPases and the Na^{+}/Ca^{2+} exchanger. This would explain why pulmonary vasodilation by β-adrenoceptor activation is abolished by hypoxia[49] and why maintained HPV is enhanced by cyclopiazonic acid[50] but abolished by thapsigargin (unpublished data).

6 Summary

In pulmonary arterial smooth muscle, considerations on the direct regulation of Ca^{2+} release from a single SR compartment are insufficient to explain current experimental observations of intracellular Ca^{2+} signalling. With respect to vasocon-

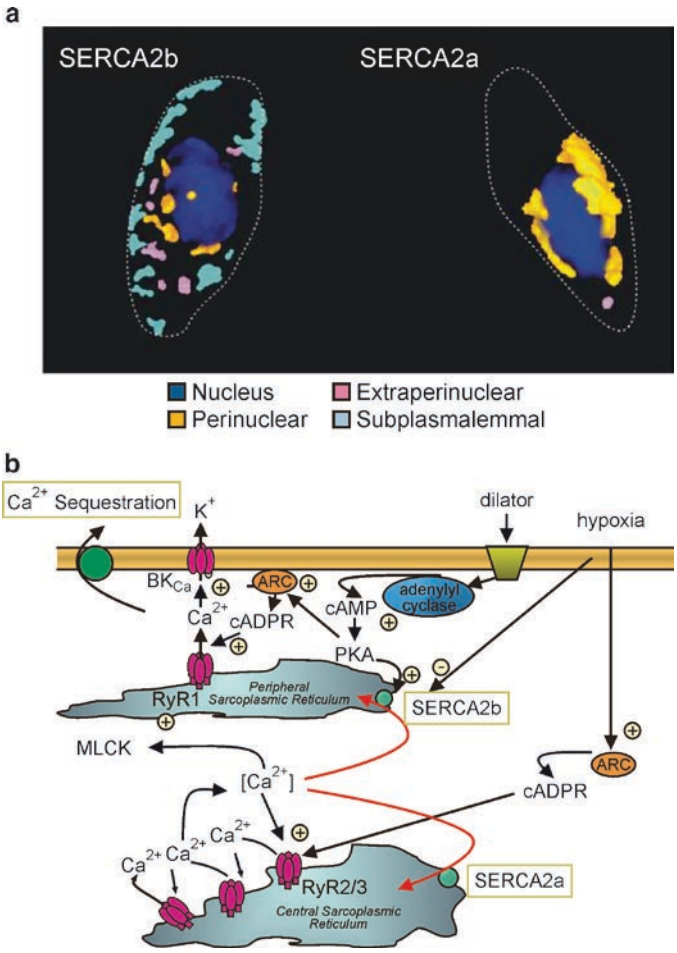

Fig. 4.5 SERCA2a and SERCA2b are differentially distributed within isolated pulmonary arterial smooth muscle cells and may serve functionally segregated SR Ca^{2+} stores. (**a**) 3D reconstruction of deconvolved Z-stacks of images showing the distribution of individual volumes of SERCA2b and SERCA2a labelling coloured to indicate distribution by defined regions of the cell: the perinuclear volume, extra-perinuclear volume and sub-plasmalemmal volume. (**b**) Schematic representation of the proposed spatial and functional compartmentalisation of the sarcoplasmic reticulum in a pulmonary arterial smooth muscle cell: *SR* Ca^{2+} *ATPase* SERCA; *ARC* ADP-ribosyl cyclase; *cADPR* cyclic adenosine diphosphate-ribose; *RyR* ryanodine receptor; BK_{Ca} Ca^{2+}-activated potassium channel; *cAMP* cyclic adenosine monophosphate; *PKA* protein kinase A

striction, it is now clear that Ca^{2+} signals may be initiated via a trigger zone, or intracellular synapse, between lysosome-related Ca^{2+} stores and the SR. Thus, mobilisation of lysosome-related stores by NAADP or other stimuli may be amplified by subsequent CICR via RyR3 and RyR2 on a central SR compartment that is served by SERCA2a and that feeds the contractile apparatus. Both cADPR and IP_3 may modulate Ca^{2+} release from this SR store independently or may coordinate

Ca^{2+} signals in concert with each other or NAADP. A second SR compartment may, however, lie in close apposition to the plasma membrane, is likely served by SERCA2b and supports cADPR-dependent Ca^{2+} release via a discrete RyR subtype (possibly RyR1) to recruit plasmalemmal BK_{Ca} channels to elicit smooth muscle cell hyperpolarisation and pulmonary artery dilation. In each case, however, the view proposed is most likely an oversimplification of the Ca^{2+} signalling apparatus available to the cell, and this point is emphasised by the fact that lysosome-related Ca^{2+} stores are mobile. In short, the complex nature and versatility of cellular Ca^{2+} signalling mechanisms should not be underestimated.

References

1. Berridge MJ (2008) Smooth muscle cell calcium activation mechanisms. J Physiol 586:5047–5061
2. Evans AM, Wyatt CN, Kinnear NP, Clark JH, Blanco EA (2005) Pyridine nucleotides and calcium signalling in arterial smooth muscle: from cell physiology to pharmacology. Pharmacol Ther 107:286–313
3. Lee HC (2004) Multiplicity of Ca^{2+} messengers and Ca^{2+} stores: a perspective from cyclic ADP-ribose and NAADP. Curr Mol Med 4:227–237
4. Morgan AJ, Galione A (2008) Investigating cADPR and NAADP in intact and broken cell preparations. Methods 46:194–203
5. Kinnear NP, Boittin FX, Thomas JM, Galione A, Evans AM (2004) Lysosome-sarcoplasmic reticulum junctions. A trigger zone for calcium signaling by nicotinic acid adenine dinucleotide phosphate and endothelin-1. J Biol Chem 279:54319–54326
6. Churchill GC, Okada Y, Thomas JM, Genazzani AA Patel S, Galione A (2002) NAADP mobilizes Ca^{2+} from reserve granules, lysosome-related organelles, in sea urchin eggs. Cell 111:703–708
7. Yamasaki M, Masgrau R, Morgan AJ et al (2004) Organelle selection determines agonist-specific Ca2+ signals in pancreatic acinar and beta cells. J Biol Chem 279:7234–7240
8. Dipp M, Nye PC, Evans AM (2001) Hypoxic release of calcium from the sarcoplasmic reticulum of pulmonary artery smooth muscle. Am J Physiol Lung Cell Mol Physiol 281:L318–L325
9. Robertson TP, Aaronson PI, Ward JP (1995) Hypoxic vasoconstriction and intracellular Ca^{2+} in pulmonary arteries: evidence for PKC-independent Ca^{2+} sensitization. Am J Physiol 268:H301–H307
10. Salvaterra CG, Goldman WF (1993) Acute hypoxia increases cytosolic calcium in cultured pulmonary arterial myocytes. Am J Physiol 264:L323–L328
11. Evans AM, Mustard KJ, Wyatt CN et al (2005) Does AMP-activated protein kinase couple inhibition of mitochondrial oxidative phosphorylation by hypoxia to calcium signaling in O_2-sensing cells? J Biol Chem 280:41504–41511
12. Weigand L, Foxson J, Wang J, Shimod LA, Sylvester JT (2005) Inhibition of hypoxic pulmonary vasoconstriction by antagonists of store-operated Ca^{2+} and nonselective cation channels. Am J Physiol Lung Cell Mol Physiol 289:L5–L13
13. Galione A, Lee HC, Busa WB (1991) Ca^{2+}-induced Ca^{2+} release in sea urchin egg homogenates: modulation by cyclic ADP-ribose. Science 253:1143–1146
14. Wilson HL, Dipp M, Thomas JM, Lad C, Galione A, Evans AM (2001) ADP-ribosyl cyclase and cyclic ADP-ribose hydrolase act as a redox sensor. A primary role for cyclic ADP-ribose in hypoxic pulmonary vasoconstriction. J Biol Chem 276:11180–11188
15. Kato M, Staub NC (1966) Response of small pulmonary arteries to unilobar hypoxia and hypercapnia. Circ Res 19:426–440

16. Waypa GB, Chandel NS, Schumacker PT (2001) Model for hypoxic pulmonary vasoconstriction involving mitochondrial oxygen sensing. Circ Res 88:1259–1266
17. Evans AM (2006) AMP-activated protein kinase and the regulation of Ca^{2+} signalling in O_2-sensing cells. J Physiol 574:113–123
18. Dipp M, Evans AM (2001) Cyclic ADP-ribose is the primary trigger for hypoxic pulmonary vasoconstriction in the rat lung in situ. Circ Res 89:77–83
19. Boittin FX, Galione A, Evans AM (2002) Nicotinic acid adenine dinucleotide phosphate mediates Ca^{2+} signals and contraction in arterial smooth muscle via a two-pool mechanism. Circ Res 91:1168–1175
20. Endo M, Tanaka M, Ogawa Y (1970) Calcium induced release of calcium from the sarcoplasmic reticulum of skinned skeletal muscle fibres. Nature 228:34–36
21. Fabiato A, Fabiato F (1975) Contractions induced by a calcium-triggered release of calcium from the sarcoplasmic reticulum of single skinned cardiac cells. J Physiol 249:469–495
22. Sutko JL, Airey JA (1996) Ryanodine receptor Ca^{2+} release channels: does diversity in form equal diversity in function? Physiol Rev 76:1027–1071
23. Allbritton NL, Meyer T, Stryer L (1992) Range of messenger action of calcium ion and inositol 1,4,5-trisphosphate. Science 258:1812–1815
24. Jaggar JH, Porter VA, Lederer WJ et al (2000) Calcium sparks in smooth muscle. Am J Physiol Cell Physiol 278:C235–C256
25. Niggli E (1999) Localized intracellular calcium signaling in muscle: calcium sparks and calcium quarks. Annu Rev Physiol 61:311–335
26. Meszaros LG, Bak J, Chu A (1993) Cyclic ADP-ribose as an endogenous regulator of the non-skeletal type ryanodine receptor Ca^{2+} channel. Nature 364:76–79
27. Tanaka Y, Tashjian AH Jr (1995) Calmodulin is a selective mediator of Ca^{2+}-induced Ca^{2+} release via the ryanodine receptor-like Ca^{2+} channel triggered by cyclic ADP-ribose. Proc Natl Acad Sci U S A 92:3244–3248
28. Panfoli I, Burlando B, Viarengo A (1999) Cyclic ADP-ribose-dependent Ca^{2+} release is modulated by free $[Ca^{2+}]$ in the scallop sarcoplasmic reticulum. Biochem Biophys Res Commun 257:57–62
29. Gilchrist JS, Belcastro AN, Katz S (1992) Intraluminal Ca^{2+} dependence of Ca^{2+} and ryanodine-mediated regulation of skeletal muscle sarcoplasmic reticulum Ca^{2+} release. J Biol Chem 267:20850–20856
30. Gyorke I, Gyorke S (1998) Regulation of the cardiac ryanodine receptor channel by luminal Ca^{2+} involves luminal Ca^{2+} sensing sites. Biophys J 75:2801–2810
31. Tripathy A, Meissner G (1996) Sarcoplasmic reticulum lumenal Ca^{2+} has access to cytosolic activation and inactivation sites of skeletal muscle Ca^{2+} release channel. Biophys J 70:2600–2615
32. Ching LL, Williams AJ, Sitsapesan R (2000) Evidence for Ca^{2+} activation and inactivation sites on the luminal side of the cardiac ryanodine receptor complex. Circ Res 87:201–206
33. Beard NA, Sakowska MM, Dulhunty AF, Laver DR (2002) Calsequestrin is an inhibitor of skeletal muscle ryanodine receptor calcium release channels. Biophys J 82:310–320
34. Herrmann-Frank A, Darling E, Meissner G (1991) Functional characterization of the Ca^{2+}-gated Ca^{2+} release channel of vascular smooth muscle sarcoplasmic reticulum. Pflügers Arch 418:353–359
35. Neylon CB, Richards SM, Larsen MA, Agrotis A, Bobik A (1995) Multiple types of ryanodine receptor/Ca^{2+} release channels are expressed in vascular smooth muscle. Biochem Biophys Res Commun 215:814–821
36. Kinnear NP, Wyatt CN, Clark JH et al (2008) Lysosomes co-localize with ryanodine receptor subtype 3 to form a trigger zone for calcium signalling by NAADP in rat pulmonary arterial smooth muscle. Cell Calcium 44:190–201
37. Li P, Chen SR (2001) Molecular basis of Ca^{2+} activation of the mouse cardiac Ca^{2+} release channel (ryanodine receptor). J Gen Physiol 118:33–44
38. Chen SR, Li X, Ebisawa K, Zhang L (1997) Functional characterization of the recombinant type 3 Ca^{2+} release channel (ryanodine receptor) expressed in HEK293 cells. J Biol Chem 272:24234–24246

39. Boittin FX, Dipp M, Kinnear NP, Galione A, Evans AM (2003) Vasodilation by the calcium-mobilizing messenger cyclic ADP-ribose. J Biol Chem 278:9602–9608
40. Higashida H, Egorova A, Higashida C et al (1999) Sympathetic potentiation of cyclic ADP-ribose formation in rat cardiac myocytes. J Biol Chem 274:33348–33354
41. Tribe RM, Borin ML, Blaustein MP (1994) Functionally and spatially distinct Ca^{2+} stores are revealed in cultured vascular smooth muscle cells. Proc Natl Acad Sci U S A 91:5908–5912
42. Golovina VA, Blaustein MP (1997) Spatially and functionally distinct Ca^{2+} stores in sarcoplasmic and endoplasmic reticulum. Science 275:1643–1648
43. Ethier MF, Yamaguchi H, Madison JM (2001) Effects of cyclopiazonic acid on cytosolic calcium in bovine airway smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 281:L126–L133
44. Janiak R, Wilson SM, Montague S, Hume JR (2001) Heterogeneity of calcium stores and elementary release events in canine pulmonary arterial smooth muscle cells. Am J Physiol Cell Physiol 280:C22–C33
45. Iino M, Kobayashi T, Endo M (1988) Use of ryanodine for functional removal of the calcium store in smooth muscle cells of the guinea-pig. Biochem Biophys Res Commun 152:417–422
46. Yamaguchi H, Kajita J, Madison JM (1995) Isoproterenol increases peripheral $[Ca^{2+}]_i$ and decreases inner $[Ca^{2+}]_i$ in single airway smooth muscle cells. Am J Physiol 268:C771–C779
47. Eggermont JA, Wuytack F, Verbist J, Casteels R (1990) Expression of endoplasmic-reticulum Ca^{2+}-pump isoforms and of phospholamban in pig smooth-muscle tissues. Biochem J 271:649–653
48. Clark JH, Wuytack F, Evans AM (2007) SERCA2A and SERCA2B exhibit discrete spatial distributions in rat pulmonary arterial smooth muscle cells. Proc Br Pharmacol Soc 4:173P http://wwwpA2onlineorg/abstracts
49. McIntyre RC Jr, Banerjee A, Hahn AR, Agrafojo J, Fullerton DA (1995) Selective inhibition of cyclic adenosine monophosphate-mediated pulmonary vasodilation by acute hypoxia. Surgery 117:314–318
50. Morio Y, McMurtry IF (2002) Ca^{2+} release from ryanodine-sensitive store contributes to mechanism of hypoxic vasoconstriction in rat lungs. J Appl Physiol 92:527–534

Ca^{2+} Oscillations Regulate Contraction Of Intrapulmonary Smooth Muscle Cells

Michael J. Sanderson, Yan Bai, and Jose Perez-Zoghbi

Abstract Pulmonary blood pressure is a function of the resistance of the intrapulmonary blood vessels. Consequently, the mechanisms controlling blood vessel smooth muscle cell (SMC) contraction serve as potential sites for hypertension therapy. To explore these mechanisms, access to the intrapulmonary vessels is required and this is provided by the observation of a unique lung slice preparation with microscopy. There are 2 major processes that determine SMC tone; the intracellular Ca^{2+} concentration and the sensitivity of the SMCs to Ca^{2+}. Agonist-induced increases in Ca^{2+} occur in the form of propagating Ca^{2+} oscillations that predominately utilize internal Ca^{2+} stores and inositol trisphosphate receptors. The frequency of these Ca^{2+} oscillations correlates with contraction. Agonists also increase Ca^{2+} sensitivity of SMCs to enhance contraction. Changes in membrane potential mediated by KCl also stimulate contraction via slow Ca^{2+} oscillations and increased sensitivity. However, these slow Ca^{2+} oscillations rely on Ca^{2+} influx to drive the cyclic release of over-filled Ca^{2+} stores via the ryanodine receptor. The relaxation of SMC tone can be induced by the reduction of the frequency of the Ca^{2+} oscillations and the Ca^{2+} sensitivity by b_2-adrenergic agonists or nitric oxide.

Keywords Pulmonary hypertension • Confocal microscopy • Lung slices • Mouse • Arterioles • Airways

1 Introduction

Increased pulmonary vasculature resistance is a key parameter associated with the various forms of pulmonary hypertension.[1] In this chapter, we address the normal Ca^{2+}-based mechanisms that lead to acute vasoconstriction and increased vasculature resistance.

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A major concern associated with the investigation of the mechanisms of pulmonary vasoconstriction is that the small intrapulmonary arterioles, which are thought to respond to hypoxia and other molecular stimuli (agonists) associated with pulmonary hypertension,[2] are the focus of attention. This issue is not easily addressed because these vessels are widely distributed and form an integral part of the lung tissue. A common technique used to investigate the responses of these blood vessels within the lung has been the measurement of pulmonary blood pressure of whole lungs (either in vivo or in vitro) in response to blood-borne agonists. This approach has a number of disadvantages when attempting to understand smooth muscle cell (SMC) physiology. For example, the responses to the blood-borne agonist are likely to be filtered by endothelial cell responses. More important, the measured parameter of pressure provides little information regarding the mechanisms that regulate contraction. The usual alternative to overcome this problem is the study of isolated or cultured individual SMCs. But, this approach also has its limitations. While changes in intracellular signals are accessible in single cells, the correlation of these responses with the contraction of the blood vessel is lost. In addition, the isolation of SMCs from blood vessels leads to the loss of their extracellular matrix and interactions with neighboring cells and the lung parenchyma. In addition, even short periods of cell culturing can alter SMC function, and the adherence of cells to the culture surface limits their contractility. Moreover, the isolation of pure vascular SMCs from small intrapulmonary blood vessels is extremely difficult because, within the lung, the arteries are very close to the airways; therefore, the isolation of SMCs does not render a purified population of vascular cells. The use of isolated large vessels, such as the extrapulmonary arteries, can facilitate the isolation of specific SMCs, but these SMCs would not seem to be the most relevant cells with which to study physiological responses related to pulmonary hypertension. Of course, without cell culture techniques, many important advances in SMC biology would not have been possible, but in this case, where the cumulative output of the cell response is a change in blood vessel diameter, the disparate scales of cell physiology and blood vessel dynamics need to be bridged.

2 Lung Slices

One experimental approach to study intracellular signals in vascular SMCs while retaining the morphology and the dynamic state of the blood vessel is the use of thin, live lung slices.[3,4] Lung slices are prepared by filling the alveolar spaces with warm (~37°C) fluid agarose via the trachea.[5] The gelling of the agarose by cooling transforms the spongy soft lung parenchyma into a more solid gel that can be sectioned with a vibratome. Lung slices ranging from 75 to 250 μm thick can be cut and are suitable to investigate SMC physiology. However, slices cut too thin can result in damage to long cells, such as the SMCs, that may spiral around the lumen of the vessels. Each lung slice predominantly consists of alveoli tissue; within this, the cross sections of arteries, veins, and airways can be found (Fig. 5.1). The airways are easily identified by their epithelium with active cilia. The arteries or

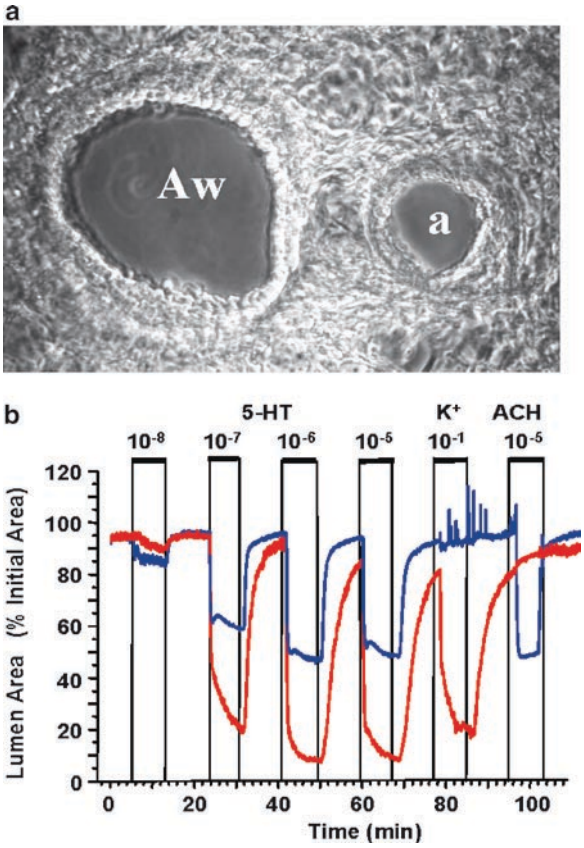

Fig. 5.1 (**a**) The appearance of an arteriole (*a*) and airway (*Aw*) in a mouse lung slice as observed with phase-contrast microscopy. (**b**) The contractile response of the arteriole and airway to a range of increasing concentrations of 5-hydroxytryptamine (5-HT), high K^+, and acetylcholine (ACh)(molar). The extent of contraction is expressed as a percentage of the initial lumen size. The arteriole (*red trace*) displays a slow contraction and relaxation rate in response to 5-HT in comparison to the airway (*blue trace*). Although K^+ induces twitching in both the airway and arteriole SMCs, a greater cumulative contraction is observed in the arteriole. The arteriole does not contract in response to ACh. Hanks' balanced salt solution (HBSS) is used to wash the lung slice between agonist applications

arterioles are also easily found because these vessels partner the airways through the lung (Fig. 5.1). Veins are generally found as independent structures. Using this technique, live lung slices have been prepared from a variety of animals, including mice,[6] rats,[7] guinea pigs,[8] and humans.[8,33]

The ability to retain blood vessel morphology in mouse lung slices has been more challenging than for the airways. For the most part, the airways are found, after slicing, to be fully relaxed and open (mouse and rat). However, the mouse blood vessels have a tendency to collapse and appear to have undergone an irreversible contraction. The underlying cause of this response is not fully understood, but it does underscore that there is a significant difference in the way airway and blood vessel SMCs respond to the same stimulation (cutting). To obtain noncollapsed blood vessels, we

modified the preparation of the lungs for sectioning by the additional instillation of warm liquid gelatin into the blood vessels via the pulmonary artery.[4] This has the effect of resisting the contraction that appears to occur in response to sectioning. Gelatin was used instead of agarose because gelatin dissolves at 37°C and, as a result, can be easily removed after slicing, leaving the blood vessel lumen empty and without lumen resistance to contraction for future experiments. However, we observed that some blood vessels also collapsed after the gelatin was dissolved at 37°C. From a practical point of view, the retention of the agarose in the alveoli serves as a replacement for pleural pressure and cannot be removed, otherwise the whole slice would collapse. The collapse of the blood vessels appears to result from a change in its interaction with the lung parenchyma, as indicated by an open or expanded appearance of the vessel adventitia. By contrast, the airways do not show this morphology and may have a stronger connection with the lung parenchyma and the associated tethering forces that maintains their relaxed or open state. In view of weak tethering of blood vessels, a positive pulmonary blood pressure, although normally low, may be more important for the dilation of the blood vessels. As a result, the use and retention of agarose in the blood vessels is being evaluated.

Lung slices provide many experimental advantages. These include the ability to examine the responses of different size vessels[9] and the applicability of the technique to a variety of animals and humans. Lung slices are viable for several days and appear to retain most of their physiological responses to agonists. The adjacent location of the blood vessel to the airway also provides a unique opportunity for comparative studies of vascular and airway SMC activity.[4,10,11] This is particularly useful because each SMC type serves as a control for the other in experiments in which activities of blood vessels and airways are recorded simultaneously. Perhaps the most important aspect of the lung slice is that individual cells are readily observed by microscopy techniques. In combination with fluorescence Ca^{2+} reporter dyes and confocal or two-photon microscopy, the changes in $[Ca^{2+}]_i$ (intracellular calcium concentration) have been imaged in individual SMCs, and this activity has been correlated with blood vessel and airway contraction.[4,12]

3 Response of Arterioles to Agonists

Perfusion of mouse lung slices with a variety of agonists, such as 5-hydroxytryptamine (5-HT) (100–1,000 n*M*), induces contraction of the blood vessels (Fig. 5.1). Although this is expected, the advantage of the lung slices is that they provide the ability to assess the dynamics and magnitude of this contraction, especially because the activity can be compared with the airway SMCs. In comparison to the airway, 5-HT induces a large but slower contraction of the blood vessel. Interestingly, on the removal of 5-HT, the relaxation rate of the blood vessel is also slower than the airway (Figs. 5.1 and 5.2). Blood vessels also strongly, but slowly, contract in response to low concentrations (10 n*M*) of endothelin (ET)[12] (Fig. 5.3). The response to ET is only slowly reversed by extensive washing. However, mouse intrapulmonary blood vessels do not respond to acetylcholine (Fig. 5.1) or phenylephrine.[4]

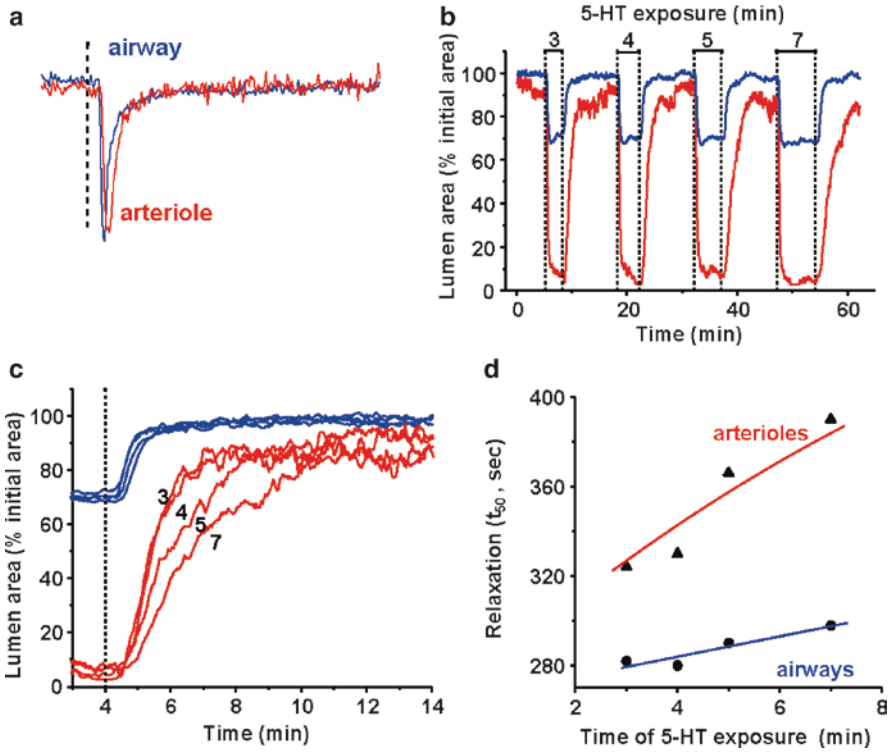

Fig. 5.2 The relaxation rate of arteriole and airway SMCs as a function of agonist exposure. (**a**) The dynamics of a similar size transient contraction induced by 20 m*M* caffeine in an arteriole (*red*) and airway (*blue*) appear similar. (**b**) The sequential contraction of an arteriole (*red*) and airway (*blue*) in response to increasing exposure times (minutes) of 1 μ*M* 5-HT. (**c**) The alignment (at 5-HT washout time) of the four maximal contractions induced by 5-HT shown in **b** to demonstrate the effect of contraction time on the relaxation time of both the airway and arteriole. (**d**) The time to attain 50% of relaxation (t_{50}) as a function of the exposure time to 5-HT from the data shown in **c**. In the airway, the duration of relaxation is slightly increased, whereas in the arteriole the relaxation time substantially increases with exposure time to 5-HT

A simple explanation for a slower relaxation rate of the arteries with respect to the airways is the idea that the blood vessels are weakly tethered to the lung parenchyma compared to the airways. This, in addition to the lack of vascular lumen pressure in the lung slices, would be consistent with the constriction of the blood vessels during sectioning. However, the rates of relaxation of an arteriole and an airway were comparable when transiently contracted to a similar extent using caffeine to induce a transient release of internal Ca^{2+} in both SMC types (Fig. 5.2a) This response suggests that during Ca^{2+}-induced contraction, similar tethering forces act on the airway and arteriole.

On the other hand, the rate of blood vessel relaxation appears to be influenced by the duration of agonist stimulation (Fig. 5.2). The relaxation time of the arterioles, after reaching a similar contractile state, was increased by extending the duration of sustained contraction induced by 5-HT (Fig. 5.2d). This behavior would not be

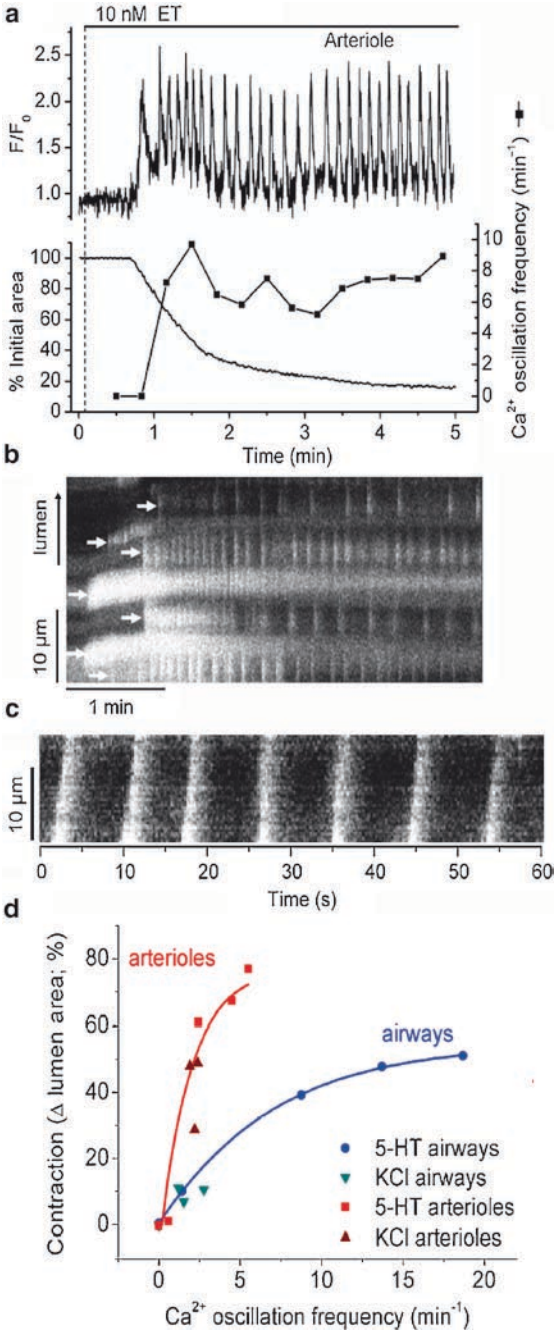

Fig. 5.3 (**a**) Stimulation of Ca^{2+} oscillations in a single SMC (*top*) and contraction (*bottom*, *continuous line*) in a mouse arteriole in a lung slice in response to endothelin (ET). The increase in the frequency of the Ca^{2+} oscillations in response to ET is shown (*bottom*, *squares* and *line*).

expected to result from loose or weak tethering or a lack of blood pressure. Because the relaxation rate after a brief contraction was initially fast (Fig. 5.2a), it would appear that the blood vessel SMCs have stiffened to a greater extent than airways in proportion to the duration of agonist exposure. This observation suggests a difference in the intrinsic mechanism for maintaining a contractile force between arteriole and airway SMCs. The development in the arterioles of a more extensive latch state than in the airways could explain this difference, and this property may be a useful mechanism to help arterioles develop a sustained contraction against blood pressure.

4 Ca^{2+} Signaling of Intrapulmonary SMCs

It has been well established that increases in intracellular calcium concentration ($[Ca^{2+}]_i$) stimulate the contraction of SMCs. However, due to the lack of imaging technologies with adequate spatial and temporal resolution and the use of cultured cells to study Ca^{2+} signaling, the dynamics of SMC Ca^{2+} signaling in situ appears to have been greatly underestimated. The classic "textbook" description of agonist-induced increases in $[Ca^{2+}]_i$, in both vascular and airway SMCs, consists of an initial spike followed by a decreased and sustained plateau and force production by the SMCs correlated with the sustained phase of Ca^{2+} increase. This activity is in stark contrast to the Ca^{2+} signals we have recorded from blood vessel and airway SMCs in lung slices with confocal or two-photon laser scanning microscopy and recording rates of 15–30 images per second. We found that the agonists 5-HT and ET induced Ca^{2+} signaling in the SMCs consisting of a series of Ca^{2+} oscillations[4,12] (Fig. 5.3). The frequency of these Ca^{2+} oscillations increases with agonist concentration; importantly, the magnitude of the blood vessel and airway contraction increases with the increasing frequency of the Ca^{2+} oscillations (Fig. 5.3). The frequency of the Ca^{2+} oscillations in arteriole SMCs reaches a maximum of about 10 per minute (10 n*M* ET after 5 min at room temperature).

Each Ca^{2+} oscillation also has a spatial organization; in general, the Ca^{2+} oscillation begins as an increase in Ca^{2+} at one end of the cell, and this propagates as a wave of Ca^{2+} to the other end of the cell (Fig. 5.3). It is not uncommon for the direction

Fig. 5.3 (continued) (**b**) The asynchronous nature of Ca^{2+} oscillations occurring in multiple adjacent SMCs represented by a line-scan analysis. The line of interest (distance, *vertical axis*) is oriented parallel to the vessel and across the circumferential SMCs. Each individual cell is represented by a horizontal trace (time) indicated by a *white arrow*. Each Ca^{2+} oscillation appears as a *white vertical line* and occurs asynchronously in each cell. The initiation of the Ca^{2+} oscillations by ET is also asynchronous. (**c**) A line-scan analysis of a single SMC demonstrating that with each Ca^{2+} oscillation a Ca^{2+} wave is propagated along the length of the cell. In this case, the line of interest is aligned along the cell (vertical distance). Because the Ca^{2+} wave propagates along the cell, the trace has a *slope* to the *right* (with respect to time) that indicates wave propagation velocity. (**d**) The relationship between the frequency of the Ca^{2+} oscillations and the contraction of arterioles (*red*) and airways (*blue*) in response to 5-HT and KCl. Slow Ca^{2+} oscillations induced in arterioles by 5-HT and KCl induce a larger contraction compared to the 5-HT-induced contraction of airways that is mediated by faster Ca^{2+} oscillations. Slow Ca^{2+} oscillations induced by KCl in airways induce little contraction

of the wave propagation to reverse. This usually occurred when a Ca^{2+} wave prematurely dissipated and failed to propagate along the full length of the cell. Under these circumstances, a second initiation site emerged near the opposite end of the cell and propagated a Ca^{2+} wave in the opposite direction. This spatial behavior of Ca^{2+} waves has interesting implications; the initiation of Ca^{2+} waves at the end of the cells suggests that these regions have the highest pacemaker activity, and this may be the result of a narrowing of the SMC toward its ends. It is possible that the concentrations of the internal messengers (in this case, inositol 1,4,5-trisphosphate, IP_3) are higher in the smaller cell volumes (assuming equal production per unit membrane) near the end of the cell. A higher concentration of IP_3 would be predicted to lead to the earlier activation of the IP_3 receptors (IP_3Rs) and the origination of the Ca^{2+} waves (Fig. 5.4). An alternative possibility is that the density of the IP_3Rs is higher toward the ends of the cell.

The spatial behavior also emphasizes that the mechanism propagating the Ca^{2+} wave is regenerative, and this is believed to be mediated by Ca^{2+}-induced Ca^{2+} release (CICR) from the sarcoplasmic reticulum (SR) via IP_3-sensitized IP_3Rs (Fig. 5.4). Although the Ca^{2+} waves spread throughout the cell, they do not appear to be propagated to adjacent cells. As a result, agonist-induced Ca^{2+} oscillations appear asynchronously in multiple SMCs (Fig. 5.3). It is important to point out that each agonist-induced Ca^{2+} oscillation does not normally initiate a wave of contraction or "twitch" response in each SMC. This implies that the regulation of the cellular contractile apparatus by the phosphorylation state of the myosin light chain (MLC) has a time constant significantly slower than the frequency of the Ca^{2+} oscillations and therefore integrates the Ca^{2+} oscillations into an average response (Fig. 5.4). This has the advantage that sustained contraction can be maintained without a sustained increase in $[Ca^{2+}]_i$, which can be deleterious to the cell. In a similar way, the asynchronous Ca^{2+} oscillations may help the muscle tissue, as a whole, to further integrate the individual Ca^{2+} pulses into a steady, sustained contraction.

Fig. 5.4 (continued) phospholipase C (PLC) to synthesize IP_3 from membrane lipids phosphatidylinositol 4,5-bisphosphate (PIP_2). IP_3 stimulates the release of Ca^{2+} from the sarcoplasmic reticulum (SR) via the IP_3 receptor (IP_3R). Ca^{2+} is returned to the SR via the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA). The cyclic release and reuptake of Ca^{2+} leads to Ca^{2+} oscillations. Ryanodine receptors (RyRs) do not seem to be activated by Ca^{2+} released from the IP_3R during agonist stimulation. Ca^{2+} oscillations are integrated via calmodulin to stimulate myosin light chain kinase (MLCK) which in turn phosphorylates the myosin light chain (MLC) and initiate contraction ("on rate"). Agonists may also inactivate MLC phosphatase (MLCP) via receptors, protein kinase C (PKC) or Rho kinase (ROK) to decrease the rate of MLC dephosphorylation ("off rate") and enhance contraction. SMC relaxation can be induced by reducing the Ca^{2+} oscillation frequency via the action of cGMP/cAMP on the IP_3R. In addition, cAMP can induce relaxation by stimulating MLCP activity. (**b**) Hypothetical relative speeds of MLCK-mediated contraction and MLCP-mediated relaxation; mouse arterioles have a slower relaxation rate than airways but similar contraction rates. (**c** and **d**) Contraction in arteries (*red line*) and airways (*blue line*) induced by slow Ca^{2+} oscillations (*gray lines*) is cumulative in arterioles but transient (twitching) in airways. The fast relaxation rate of airway SMCs allows them to fully relax in the time between each Ca^{2+} oscillation. (**e**) Sustained airway contraction (*blue*) is achieved in response to fast agonist-induced Ca^{2+} oscillations because the time between each oscillation is insufficient to allow relaxation

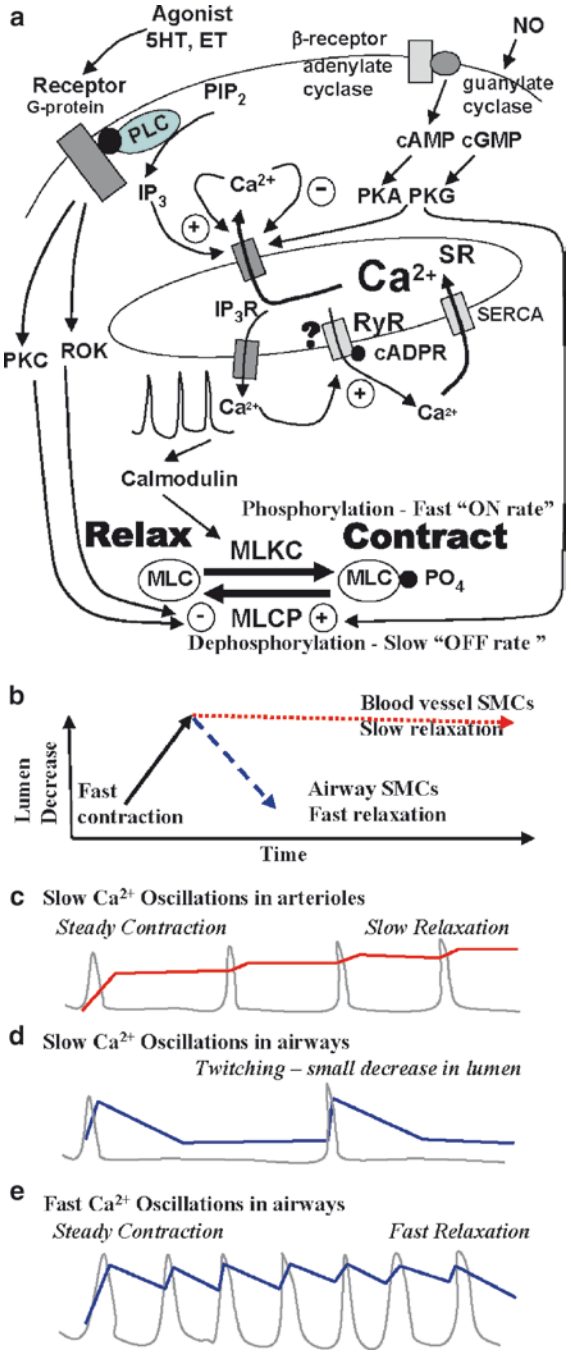

Fig. 5.4 (**a**) A general scheme of the molecular mechanisms contributing to contraction of arteriole SMCs. Agonists (5-HT, ET) stimulate their specific G protein-coupled receptors to stimulate

4.1 Roles of Ca^{2+} Influx and Internal Ca^{2+} Release

A second classical idea that features in many studies aimed at understanding the regulatory mechanisms of pulmonary SMC contraction is that the steady plateau phase of elevated $[Ca^{2+}]_i$, responsible for maintaining force, is primarily determined by Ca^{2+} influx via membrane channel activity. The loss of force after the removal of extracellular Ca^{2+} is consistent with this view.

In our studies with lung slices, it is clear that the steady-state contraction is related to the frequency of the Ca^{2+} oscillations rather than a steady level of $[Ca^{2+}]_i$. Consequently, the mechanism generating these Ca^{2+} oscillations is vital to understanding SMC physiology. The first issue to address is the source of the Ca^{2+} used in the Ca^{2+} oscillations. In the absence of extracellular Ca^{2+}, Ca^{2+} oscillations can both be initiated by agonist and can persist for some time. As a result, a substantial contraction is observed under Ca^{2+}-free conditions. However, in the prolonged absence of Ca^{2+}, the Ca^{2+} oscillations run down and terminate. The frequency of the Ca^{2+} oscillations was not altered by nifedipine, a voltage-dependent Ca^{2+} channel blocker, but the Ca^{2+} oscillations were abolished by agents such as thapsigargin or cyclopiazonic acid (CPA) that inhibit sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (adenosine triphosphatase) (SERCA) pumps and lead to an emptying of internal Ca^{2+} stores (Fig. 5.4). The conclusion that can be drawn from these experiments is that Ca^{2+} oscillations primarily utilize internal Ca^{2+} stores. Each Ca^{2+} oscillation requires a release of Ca^{2+} from the SR, and although most of this Ca^{2+} is returned to the SR after the Ca^{2+} release terminates (Fig. 5.4), invariably, some Ca^{2+} is lost to the extracellular environment. As a result, Ca^{2+} oscillations are dependent on external Ca^{2+} to refill the SR store to prevent its depletion. Thus, the classical view would benefit from a redefinition of the roles of Ca^{2+} release and Ca^{2+} influx related to the cellular mechanism of maintaining SMC contraction, and the role of Ca^{2+} influx needs to be investigated in terms of its effect on Ca^{2+} oscillation frequency rather than the steady state $[Ca^{2+}]_i$.

The oscillatory nature of the Ca^{2+} signals in blood vessel SMCs is consistent with the release of Ca^{2+} from the SR via the IP_3R (Fig. 5.4). This process is found in vascular SMCs from other types of blood vessels.[13] With lung slices, we have also found that airway SMCs display Ca^{2+} oscillations in response to agonist (5-HT and acetylcholine [ACh]), and that the frequency of these Ca^{2+} oscillations also correlates with contraction[10] (Fig. 5.3). The Ca^{2+} oscillations in airway SMCs are similar in many ways to those of the pulmonary blood vessels and require external Ca^{2+} for prolonged activity. Importantly, these oscillations can be initiated or increased in frequency by the photolytic release of IP_3 in the SMCs.[14,15] Also consistent with the idea that Ca^{2+} oscillations are mediated by increases in IP_3 is the finding that 5-HT appears to act via $5\text{-}HT_2$ receptors that are linked via G proteins to the activation of phospholipase-β (PLC-β) to produce IP_3. The $5\text{-}HT_2$-receptor-specific antagonist ketanserin blocked the action of 5-HT, whereas 2, 5-dimethoxy-4-iodoamphetamine (DOI), an agonist of this receptor, induced 5-HT-like changes.[4] By contrast, an agonist of the $5\text{-}HT_3$ receptor that mediates Ca^{2+} influx had little effect.

Likewise, the use of specific agonists and antagonists indicates that Ca^{2+} oscillations induced by ET are mediated by an equal contribution by ET_A and ET_B receptors[12] that act via activation of PLC-β to produce IP_3.

4.2 Effect of KCl on Arteriole SMC Physiology

From these data, it appears that pulmonary arteriole SMCs utilize SR Ca^{2+}. However, another idea, which is frequently incorporated into experimental regimes, is that SMC depolarization is a major control signal for the contraction of pulmonary SMCs. Because a common way to experimentally induce membrane depolarization in SMCs is their exposure to high extracellular concentrations of KCl (~100 m*M*), we examined the responses of SMCs in lung slices to this treatment.[4,10]

Although exposure to KCl induces pulmonary arterioles to contract, this contraction is usually slower and smaller in magnitude than that induced by agonists and often displays some regional twitching of the blood vessel. The contractile response to KCl was abolished in the absence of extracellular Ca^{2+} or presence of nifedipine and Ni^{2+}, indicating Ca^{2+} influx is a primary mediator of the response and consistent with the idea that membrane depolarization leads to the opening of voltage-dependent Ca^{2+} channels.

To confirm these ideas, we examined the Ca^{2+} responses of the SMCs to KCl and were surprised to observe that, in place of a steady-state elevation of $[Ca^{2+}]_i$, which might be expected with sustained depolarization, there occurred a series of Ca^{2+} oscillations. However, these Ca^{2+} oscillations were significantly different from those induced by agonists; their frequency was very slow (1–3 per minute), and the duration of the Ca^{2+} increase associated with each Ca^{2+} oscillation was greatly extended.[4,10] Extremely similar Ca^{2+} oscillations were observed in airway SMCs in response to KCl, and in contrast to agonist-induced Ca^{2+} oscillations, a clear correlation between each Ca^{2+} oscillation and an airway SMC twitch could be made. While a similar effect can be observed in vascular SMCs, this effect is reduced. Such twitching behavior associated with KCl-induced Ca^{2+} oscillations suggests that their slow frequency is at the limits of the integration time of the enzymes of the contractile process; in other words, at slow frequencies, especially in the absence of agonist, contraction begins to follow the changes in $[Ca^{2+}]_i$.

A closer examination of the KCl-induced Ca^{2+} oscillations with higher-speed recordings revealed that a series of elemental and localized Ca^{2+} signals with increasing frequency occurred prior to the development of a propagating Ca^{2+} wave or Ca^{2+} oscillation.[4,10] Immediately after a Ca^{2+} oscillation had subsided, these elemental Ca^{2+} signals could not be detected, but with time, they began to reappear with increasing frequency until the next Ca^{2+} oscillation was triggered. Similar activity was observed in airway SMCs. Elemental Ca^{2+} signaling, in the form of Ca^{2+} sparks, has been observed in other SMCs and cardiomyocytes, and these signals have been determined to be the result of localized Ca^{2+} release from the SR via a small grouping of ryanodine receptors (RyRs), specialized SR Ca^{2+} channels.[16,17] Although the

Ca^{2+} elemental events we observed are somewhat bigger than those associated with traditional Ca^{2+} sparks, we found that ryanodine (a RyR antagonist) abolished KCl-induced Ca^{2+} oscillations. Again, similar results were observed in airway SMCs.

From these data, we propose the following mechanism to explain KCl-induced Ca^{2+} oscillations: KCl depolarizes the cell membrane and initiates an initial influx of Ca^{2+} into the cell. To compensate and restore the $[Ca^{2+}]_i$, the cell transports the excess Ca^{2+} from the cytosol not only to the extracellular medium but also to the SR. This redistribution of Ca^{2+} will continue while there is a Ca^{2+} influx, with the result that the SR Ca^{2+} content increases. RyRs appear to be sensitive to this SR Ca^{2+} content and become more likely to open.[16] The opening of a RyR releases Ca^{2+}, which itself can stimulate adjacent sensitized RyRs, with the result that a localized Ca^{2+} event occurs. Because the Ca^{2+} discharge associated with these local events does not counter the accumulation of Ca^{2+} in the SR, the SR Ca^{2+} content continues to increase with time. This in turn further sensitizes the RyRs to elicit more Ca^{2+} elemental events. However, when the Ca^{2+} content of the SR reaches a threshold level and the RyRs are very sensitive to Ca^{2+}, an elemental Ca^{2+} release event quickly turns into a propagating Ca^{2+} wave by the process of CICR. This global increase in $[Ca^{2+}]_i$ has the effect of lowering the Ca^{2+} content of the SR, which desensitizes the RyRs, and the process can begin again to generate the slow Ca^{2+} oscillations.

4.3 Ca^{2+} Sparks: A Relaxation Mechanism in Pulmonary SMCs?

In almost all respects, the contractility and Ca^{2+} signaling of the SMCs in the lung slices is highly reproducible and consistent. The tissue morphology appears normal, and as mentioned, the SMCs retain their cell contacts and extracellular matrix. In our opinion, lung slices appear to represent a healthy preparation of lung tissue. Yet, we have failed to observe Ca^{2+} sparks in the multitude of SMCs we have examined in relaxed blood vessels, even though Ca^{2+} sparks have been suggested to serve as a fundamental relaxation mechanism for vascular SMCs. One explanation could be that the sensitivity of our confocal or two-photon microscope systems is inadequate. However, we clearly observed elemental signals associated with KCl-induced Ca^{2+} oscillations and Ca^{2+} sparks within cardiomyocytes in the myocardial sheath surrounding the pulmonary vein in mouse lung slices.[18]

The Ca^{2+} sparks are believed to induce SMC relaxation by hyperpolarizing the cell membrane via the localized activation of spontaneous transient outward currents (STOCs) carried by K^+ though Ca^{2+}-activated K^+ channels[19]; hyperpolarization would counter Ca^{2+} influx via voltage-dependent Ca^{2+} channels. However, contraction of intrapulmonary vascular SMCs appears to be regulated by internal Ca^{2+} release in the form of agonist-induced Ca^{2+} oscillations rather than by membrane depolarization and activation of voltage-dependent Ca^{2+} channels.[20] Thus, membrane hyperpolarization would appear to have a minimal contribution to relaxation in intrapulmonary blood vessel SMCs.

4.4 The Role of Ryanodine Receptors

The inhibition of elemental Ca^{2+} signaling in cells exposed to KCl by ryanodine supports the idea that RyRs are present in intrapulmonary SMCs. However, if Ca^{2+} sparks do not strongly feature in intrapulmonary SMC physiology, what role might the RyR have? Because RyRs can be gated open by increases in $[Ca^{2+}]_i$, RyRs may contribute to the propagation of Ca^{2+} waves associated with Ca^{2+} oscillations by amplifying or replacing the Ca^{2+} release initiated via IP_3Rs (Fig. 5.4). Surprisingly, we have found that ryanodine has no influence on the frequency or form of the agonist-induced Ca^{2+} oscillations of both blood vessel and airway SMCs.[34] Similar findings apply to agonist-induced Ca^{2+} oscillations in other SMCs.[21,22] This suggests that the RyR does not respond to the local changes in $[Ca^{2+}]_i$ associated with agonist-induced Ca^{2+} oscillations. Part of the reason for this might be that agonist-induced Ca^{2+} oscillations lower the SR Ca^{2+} content, and as a result, the RyRs become desensitized.[16] In airway SMCs, it has been suggested that increased cyclic adenosine diphosphate (cADP)-ribose production associated with inflammation can sensitize the RyR for it to take part in cell signaling.[23] It is therefore possible that the RyR contributes more to the pathologic changes associated with the SMCs in pulmonary hypertension, such as those of cell growth and proliferation, when the activity of CD38 may be increased.

5 Frequency-Modulation as Method for Contraction Regulation

In blood vessels, as well as in airways, an increase in the frequency of the Ca^{2+} oscillations correlated with an increase in contraction (Fig. 5.3). This has led to the suggestion that extent of contraction is regulated by a frequency-modulated (FM) mechanism. This approach has the potential advantage of generating sustained contraction without sustained increases in $[Ca^{2+}]_i$. In addition, a reliance on distinct Ca^{2+} oscillations rather than steady-state $[Ca^{2+}]_i$ may be a more accurate way to detect regulatory signals because Ca^{2+} oscillations will be less susceptible to small variations in cytoplasmic $[Ca^{2+}]_i$.

Multiple steps of phosphorylation of Ca^{2+}/calmodulin-dependent protein kinase II (CaM-kinase II) in response to sequential Ca^{2+} oscillations have been proposed as a specialized mechanism mediating FM regulation.[24] However, the concept of FM regulation only requires that the forward activation of the system ("on rate") has faster kinetics than the reverse inactivation ("off rate"). Rather than requiring a specialized single molecule such as CaM-Kinase II, this arrangement can be achieved by a pair of antagonistic enzymes that have different kinetics. In this respect, SMCs appear to have the ideal molecular control to take advantage of FM regulation. The forward step, stimulated by Ca^{2+}, is mediated by calmodulin and

MLCK (myosin light chain kinase) and results in phosphorylation of MLC. The reverse step, dephosphorylation of MLC, is mediated by MLCP (MLC phosphatase); slower kinetics of this enzyme will result in sustained contraction during the presence of Ca^{2+} oscillations (Fig. 5.4).

Although it appears, at first sight, that the frequency of Ca^{2+} oscillations dominates the control of contraction (perhaps because of their visual impact and reliable observation), comparative studies with different agonists, KCl, or airway SMCs revealed that their influence on the extent of contraction is variable. In mouse blood vessels, the Ca^{2+} oscillations occurred with frequencies up to a maximum of 10 per minute and induced a large contraction (about 80% with ET). Yet, in airway SMCs, the Ca^{2+} oscillations occurred at higher frequencies (25 per minute) but were associated with smaller contractions (Fig. 5.3d). The Ca^{2+} oscillations induced by KCl were very slow and induced a small contraction with pronounced twitching in airway SMCs but greater contraction in blood vessels with less twitching (Fig. 5.3d).

To explain these variable responses, we have proposed a hypothesis based on the duration of the off rate of the control of contraction (Fig. 5.4). If we assume that the on rate of contraction is similar between SMCs, an idea that mathematical modeling of the Ca^{2+}-dependent kinetics of calmodulin and MLCK activation upholds,[25] then the off rate (MLC dephosphorylation by MLCP) will determine the duration and extent of contraction. With a slow off rate, slow Ca^{2+} oscillations can stimulate a sustained-steady contraction; this is the case of agonist-induced contraction in blood vessels. With a fast off-rate, slow Ca^{2+} oscillations can only stimulate a weak, twitch contraction; this is the case with KCl-induced contraction in airways. Fast Ca^{2+} oscillations are required for a sustained contraction with a fast off rate; this is the case with agonist-induced contraction of airways (Fig. 5.4).

Because the activity of MLCP is the major determinate of the off rate, MLCP activity should be considered to be equally important as the Ca^{2+}-based mechanisms that regulate SMC contraction. It should also be noted that although most agonists that regulate contraction commonly stimulate Ca^{2+} oscillations and increases in $[Ca^{2+}]_i$, they also simultaneously regulate MLCP activity.

6 The Influence of Ca^{2+} Sensitivity on Contraction

The variability in the extent of contraction that occurs in the presence of a constant level of $[Ca^{2+}]_i$ (i.e., a constant on rate) is referred to as the Ca^{2+} sensitivity of the SMCs (i.e., the off rate). A high Ca^{2+} sensitivity implies a strong contraction and hence low MLCP activity, whereas low Ca^{2+} sensitivity indicates a tendency to relax or give weak contraction and is mediated by high MLCP activity. In keeping with its definition, the method of measuring Ca^{2+} sensitivity requires the stimulation of SMCs with a constant level of $[Ca^{2+}]_i$ and the measurement of the extent of contraction. This can only be achieved if the $[Ca^{2+}]_i$ of the SMCs can be experimentally controlled.

6.1 A Unique Nondestructive Technique for Ca^{2+} Permeabilization

Control of $[Ca^{2+}]_i$ requires the circumvention of the ability of the cells to regulate a Ca^{2+} gradient across its membranes. This can be achieved by "permeabilizing" the cell membrane with bacterial toxins or mild detergents. However, such treatments have the disadvantage that they also increase the permeability of the membrane to a number of other ions and cytoplasmic constituents, including important messenger molecules, by forming large pores in the plasma membrane. In addition, it is unclear if these treatments alter the membrane permeability of intracellular organelles, including the SR.

We have adapted an alternative approach in our laboratory to specifically elevate membrane Ca^{2+} permeability that exploits the ion channels of the cells and induces little membrane damage. This technique involves the treatment of lung slices simultaneously with caffeine and ryanodine.[11] Caffeine serves as an agonist of the RyR and stimulates its activation. By contrast, ryanodine serves as an antagonist of the RyR, and when RyR is activated by caffeine, ryanodine irreversibly locks the RyR in an open state. As a result, the SR is depleted of Ca^{2+}. Normally, the emptying of Ca^{2+} from the SR leads to the opening of store-operated channels (SOCs) in the plasma membrane, allowing a Ca^{2+} influx that can be used to replace the Ca^{2+} lost from the SR.[26] However, after caffeine and ryanodine treatment, any Ca^{2+} returned to the SR will not accumulate because of the open RyRs. Thus, the cell has a persistent influx of Ca^{2+} via SOCs. The $[Ca^{2+}]_i$ can now be manipulated by varying external $[Ca^{2+}]$. The advantage of this technique is that the cell and tissue structure are unaffected, and agonist receptor function remains viable. In addition, Ca^{2+} mobilization by agonists is prevented because the SR remains empty. There appears to be no loss of cell constituents with this technique; as a result, the Ca^{2+}-permeabilized lung slices can be experimentally used for many hours to investigate the influence of Ca^{2+} sensitivity.[11]

6.2 Agonist-Induced Increases in Ca^{2+} Sensitivity

After Ca^{2+} permeablization with caffeine and ryanodine and the exposure of the lung slices to Ca^{2+}-free saline, the $[Ca^{2+}]_i$ falls to zero, and the blood vessels, as well as the airways in both mouse and rat lung slices, are relaxed (Fig. 5.5). On exposure to high extracellular $[Ca^{2+}]$, the $[Ca^{2+}]_i$ is increased in the SMCs, and the mouse blood vessel partially contracts. This indicates that resting arteriole SMCs have a basal level of sensitivity to Ca^{2+}, implying that the activity of the MLCP is not high. Interestingly, under the same conditions, the mouse airway initially contracts but subsequently relaxes even though the $[Ca^{2+}]_i$ remained high (Fig. 5.5). This result implies that in mouse airway SMCs the increase in $[Ca^{2+}]_i$, in addition to initially activating MLCK, also slowly activated MLCP activity, to a level that eventually fully countered the activity of MLCK.[11,25] Because rat airways, rat blood vessels,

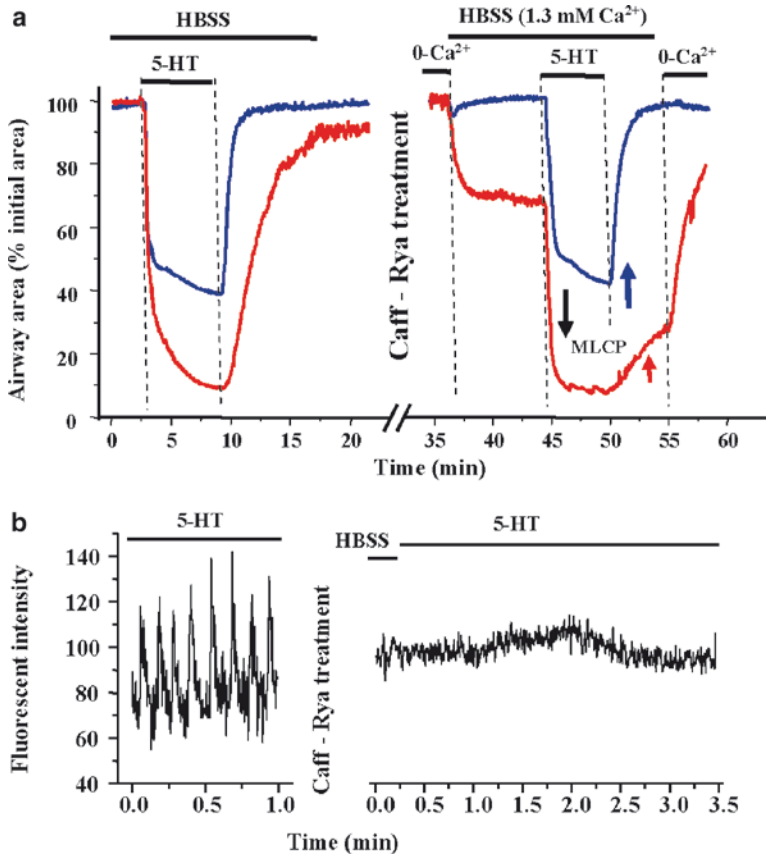

Fig. 5.5 Changes in Ca^{2+} sensitivity induced by increases in $[Ca^{2+}]_i$ and agonists in mouse arterioles (*red*) and airways (*blue*). (**a**) The contraction induced by 5-HT in normal arterioles and airways (*left*) and in Ca^{2+}-permeabilized lung slices (*right*) following treatment with caffeine and ryanodine. In zero extracellular Ca^{2+}, the arteriole and airway are relaxed. An increase in $[Ca^{2+}]_i$ induced by the addition of HBSS containing 1.3 m*M* $CaCl_2$ induces a partial contraction of the arteriole. By contrast, the airway transiently contracts and relaxes. On the addition of 5-HT, both the arteriole and airway display contraction to a level equivalent to that observed in normal lung slices prior to caffeine/ryanodine treatment. Both the airway and arteriole relax on the removal of 5-HT. Because there are no changes in $[Ca^{2+}]_i$ during the addition and removal of 5-HT, the rates of change of the airway and arteriole lumen size indicate the rates of inactivation (*black arrow*, contraction) and reactivation (relaxation; *blue arrow*, airway; *red arrow*, arteriole) of MLCP activity. The relaxation rate of the arteriole is slower than that of the airway. (**b**) Representative changes in $[Ca^{2+}]_i$ in the SMCs (both airway and artery) associated with the treatments shown in **a**. In normal lung slices (*left*), 5-HT induced Ca^{2+} oscillations to mediate contraction. In Ca^{2+}-permeablized slices (*right*), the $[Ca^{2+}]_i$ was sustained at an elevated level after HBSS addition but did not change on the addition or removal of agonist (5-HT)

and human airways all respond to a sustained increase in $[Ca^{2+}]_i$ in a manner similar to mouse blood vessels, it appears that a Ca^{2+}-induced increase in MLCP activity is a species-specific (mouse) response.

If these same Ca^{2+}-permeabilized lung slices are subsequently exposed to a contractile agonist, the blood vessels show a further substantial increase in contraction, albeit without any change in $[Ca^{2+}]_i$. Perhaps more impressively, the mouse airway also displays an increase in contraction that is similar in magnitude to that displayed in a normal slice (Fig. 5.5). The implication is that agonist, acting via the same G protein-coupled receptors that mediate increases in $[Ca^{2+}]_i$ via Ca^{2+} oscillations (now disabled by caffeine/ryanodine treatment), decreases the MLCP activity (increases Ca^{2+} sensitivity) of both arteriole and airway SMCs. Similar findings apply to rat and human airway SMCs. The exact degree of this enhancement of the Ca^{2+} sensitivity by agonist, like the exact stimulation of Ca^{2+} oscillations by agonist, is both agonist and species specific. Thus, the final contraction is a function of the precise changes in Ca^{2+} signaling and Ca^{2+} sensitivity (Fig. 5.3d).

As might be expected, the removal of agonist results in the relaxation of arterioles and airways and implies a reactivation of the MLCP. We had proposed that the relaxation rates differ between arterioles and airways, and that this accounted for differences in the Ca^{2+} oscillation frequency-contraction relationships. With the Ca^{2+}-permeablized lung slices, this difference in relaxation rate can be observed (Fig. 5.5). With a constant MLCK activity, induced by the sustained elevated $[Ca^{2+}]_i$, the removal of 5-HT reveals that the airways quickly and fully relax, indicating a fast and strong reactivation of the MLCP activity (Fig. 5.5, blue arrow). By contrast, the arteriole displays a slow and partial relaxation in the same time (red arrow). Thus, with a similar Ca^{2+} stimulation, the arterioles display greater contraction than airways as a result of their MLCP activity.

7 Relaxation Mechanisms of SMCs

A major goal of therapy for pulmonary hypertension is a reduction in arteriole resistance. In conditions for which increased vessel resistance is primarily due to increased muscle tone rather than changes in arteriole structure, a suppression of Ca^{2+} signaling and Ca^{2+} sensitivity may be an effective therapy. A similar goal is addressed in asthma; acute airway SMC relaxation and relief of dyspnea is achieved by the inhalation of β_2-agonists (steroids are required to counter inflammation for the long-term control of asthma). In lung slices, we have shown that β_2-agonists such as isoproterenol,[14] albuterol,[27] and formoterol[33,35] all induced airway SMC relaxation by reducing the frequency of the Ca^{2+} oscillations. Although β_2-agonists have little effect on blood vessels, we have found similar results with nitric oxide (NO) that reduces Ca^{2+} oscillations in both airways[28] and arterioles of mouse lung slices. The implication of these findings is that the mechanisms of the Ca^{2+} oscillations serve as key contractile signals in arterioles and therefore represent a therapeutic target. The investigation of Ca^{2+} influx, without reference to its role in Ca^{2+} oscillations, is less likely to identify a key regulator.

The mechanism of Ca^{2+} oscillations in pulmonary arteriole SMCs is not well understood, but it is likely that it has many features in common with Ca^{2+} oscillations

in airway SMCs or systemic arterioles.[16] Our experiments with β_2-agonists and NO indicate that a reduction in the frequency of the Ca^{2+} oscillations is achieved by a reduction in the sensitivity of the IP_3R to IP_3.[14,28] This mechanism appears to involve phosphorylation by either cyclic adenosine monophosphate (cAMP)/protein kinase A or cyclic guanosine monophosphate (cGMP)/protein kinase G (PKG) (Fig. 5.4 a) A similar conclusion has been reached with bovine tracheal SMCs, in which IP_3R-associated cGMP kinase substrate (IRAG) protein phosphorylated by PKG associates with and downregulates the IP_3R.[29] However, the frequency of the Ca^{2+} oscillation can be influenced by Ca^{2+} influx as this is required to fully replenish the SR Ca^{2+} content; the period of the Ca^{2+} oscillation is determined, in part, by the time taken to refill the SR.[30] The channels directly contributing to the process of Ca^{2+} influx appear to be associated with the recently identified Orai proteins which are, in turn, regulated by a Ca^{2+} sensor, STIM1 (stromal interacting molecule 1), within the SR.[31] In addition, transient receptor potential (TRP) channels may contribute to this process.[32] While it is reasonable that Ca^{2+} influx via a variety of channels may contribute to the frequency of the Ca^{2+} oscillations, a direct demonstration of this connection would be a major step toward identifying specific channels playing a significant role in contraction and relaxation of pulmonary blood vessels.

In addition to the Ca^{2+} oscillations, as we have emphasized, Ca^{2+} sensitivity plays a major role in contraction. Therefore, it might be no surprise to point out that β_2-agonists exert a substantial part of their relaxing effect on airway SMCs by simultaneously decreasing Ca^{2+} sensitivity by presumably increasing MLCP activity.[27] However, NO appears to much have less effect on Ca^{2+} sensitivity in airway SMCs, and this correlates with its weak action on airway relaxation.[28] The role of MLCP in SMC contractility makes it an attractive therapeutic target that has perhaps been overlooked by our focus on Ca^{2+}.

8 Conclusions

Contraction of intrapulmonary blood vessel SMCs is regulated by dynamic Ca^{2+} signaling in the form of agonist-induced Ca^{2+} oscillations and waves rather than by steady-state $[Ca^{2+}]_i$. The extent of this contraction is also strongly regulated by agonist-induced changes in Ca^{2+} sensitivity. While Ca^{2+} influx via a variety of ion channels can occur, it is unclear how this contributes to contraction. An understanding of the relationship of this Ca^{2+} influx with Ca^{2+} oscillations and contraction would be valuable as this would help identify pivotal therapeutic sites to induce SMC relaxation. It must also be emphasized that SMC contraction and, importantly, relaxation belong to an active process driven by MLCP activity. Because this mechanism can have an equal contribution to SMC contraction as Ca^{2+}-based mechanisms, it also has important therapeutic potential.

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References

1. Aaronson PI, Robertson TP, Knock GA, Becker S, Lewis TH, Snetkov V, Ward JPT (2006) Hypoxic pulmonary vasoconstriction: mechanisms and controversies. J Physiol 570:53–58
2. Moudgil R, Michelakis ED, Archer SL (2005) Hypoxic pulmonary vasoconstriction. J Appl Physiol 98:390–403
3. Held HD, Martin C, Uhlig S (1999) Characterization of airway and vascular responses in murine lungs. Br J Pharmacol 126:1191–1199
4. Perez JF, Sanderson MJ (2005) The contraction of smooth muscle cells of intrapulmonary arterioles is determined by the frequency of Ca^{2+} oscillations induced by 5-HT and KCl. J Gen Physiol 125:555–567
5. Martin C, Uhlig S, Ullrich V (1996) Videomicroscopy of methacholine-induced contraction of individual airways in precision-cut lung slices. Eur Respir J 9:2479–2487
6. Martin C, Uhlig SU, V (1996) Videomicroscopy of methacholine-induced contraction of individual airways in precision-cut lung slices. Eur Respir J 9:2479–2487
7. Bai Y, Sanderson MJ (2009) The contribution of Ca^{2+} signaling and Ca^{2+} sensitivity to the regulation of airway smooth muscle contraction is different in rats and mice. Am J Physiol Lung Cell Mol Physiol 296:L947–L958
8. Ressmeyer AR, Larsson AK, Vollmer E, Dahlen SE, Uhlig S, Martin C (2006) Characterisation of guinea pig precision-cut lung slices: comparison with human tissues. Eur Respir J 28:603–611
9. Bai Y, Zhang M, Sanderson MJ (2007) Contractility and Ca^{2+} signaling of smooth muscle cells in different generations of mouse airways. Am J Respir Cell Mol Biol 36:122–130
10. Perez JF, Sanderson MJ (2005) The frequency of calcium oscillations induced by 5-HT, ACH, and KCl determine the contraction of smooth muscle cells of intrapulmonary bronchioles. J Gen Physiol 125:535–553
11. Bai Y, Sanderson MJ (2006) Modulation of the Ca^{2+} sensitivity of airway smooth muscle cells in murine lung slices. Am J Physiol Lung Cell Mol Physiol 291:L208–L221
12. Perez-Zoghbi JF, Sanderson MJ (2007) Endothelin-induced contraction of bronchiole and pulmonary arteriole smooth muscle cells is regulated by intracellular Ca^{2+} oscillations and Ca^{2+} sensitization. Am J Physiol Lung Cell Mol Physiol 293:L1000–L1011
13. Wier WG, Morgan KG (2003) $_1\alpha$-Adrenergic signaling mechanisms in contraction of resistance arteries. Rev Physiol Biochem Pharmacol 150:91–139
14. Bai Y, Sanderson MJ (2006) Airway smooth muscle relaxation results from a reduction in the frequency of Ca^{2+} oscillations induced by a cAMP-mediated inhibition of the IP_3 receptor. Respir Res 7:34
15. Sneyd J, Tsaneva-Atanasova K, Reznikov V, Bai Y, Sanderson MJ, Yule DI (2006) A method for determining the dependence of calcium oscillations on inositol trisphosphate oscillations. Proc Natl Acad Sci U S A 103:1675–1679
16. Berridge MJ (2008) Smooth muscle cell calcium activation mechanisms. J Physiol 586:5047–5061
17. Zhuge R, Fogarty KE, Baker SP, McCarron JG, Tuft RA, Lifshitz LM, Walsh JV Jr (2004) Ca^{2+} spark sites in smooth muscle cells are numerous and differ in number of ryanodine receptors, large-conductance K^+ channels, and coupling ratio between them. Am J Physiol Cell Physiol 287:C1577–C1588
18. Mueller-Hoecker J, Beitinger F, Fernandez B, Bahlmann O, Assmann G, Troidl C, Dimomeletis I, Kaab S, Deindl E (2008) Of rodents and humans: a light microscopic and ultrastructural study on cardiomyocytes in pulmonary veins. Int J Med Sci 5:152–158
19. Jaggar JH, Porter VA, Lederer WJ, Nelson MT (2000) Calcium sparks in smooth muscle. Am J Physiol Cell Physiol 278:C235–C256
20. Janssen LJ (2002) Ionic mechanisms and Ca^{2+} regulation in airway smooth muscle contraction: do the data contradict dogma? Am J Physiol Lung Cell Mol Physiol 282:L1161–L1178

21. Lamont C, Wier WG (2004) Different roles of ryanodine receptors and inositol (1,4,5)-trisphosphate receptors in adrenergically stimulated contractions of small arteries. Am J Physiol Heart Circ Physiol 287:H617–H625
22. MacMillan D, Chalmers S, Muir TC, McCarron JG (2005) IP_3-mediated Ca^{2+} increases do not involve the ryanodine receptor, but ryanodine receptor antagonists reduce IP_3-mediated Ca^{2+} increases in guinea-pig colonic smooth muscle cells. J Physiol 569:533–544
23. Jude JA, Wylam ME, Walseth TF, Kannan MS (2008) Calcium signaling in airway smooth muscle. Proc Am Thorac Soc 5:15–22
24. Dupont G, Houart G, De Koninck P (2003) Sensitivity of CaM kinase II to the frequency of Ca^{2+} oscillations: a simple model. Cell Calcium 34:485–497
25. Wang I, Politi AZ, Tania N, Bai Y, Sanderson MJ, Sneyd J (2008) A mathematical model of airway and pulmonary arteriole smooth muscle. Biophys J 94:2053–2064
26. Parekh AB, Putney JW Jr (2005) Store-operated calcium channels. Physiol Rev 85:757–810
27. Delmotte P, Sanderson MJ (2008) Effects of albuterol isomers on the contraction and Ca^{2+} signaling of small airways in mouse lung slices. Am J Respir Cell Mol Biol 38:524–531
28. Perez-Zoghbi JF, Sanderson MJ (2007) cGMP-mediated relaxation of bronchioles occur though inhibition of the Ca^{2+} oscillations without affecting the Ca^{2+} sensitivity. Am J Resp Crit Care Med 175:A524
29. Schlossmann J, Ammendola A, Ashman K, Zong X, Huber A, Neubauer G, Wang GX, Allescher HD, Korth M, Wilm M, Hofmann F, Ruth P (2000) Regulation of intracellular calcium by a signalling complex of IRAG, IP_3 receptor and cGMP kinase I β.Nature 404:197–201
30. Sneyd J, Tsaneva-Atanasova K, Yule DI, Thompson JL, Shuttleworth TJ (2004) Control of calcium oscillations by membrane fluxes. Proc Natl Acad Sci U S A 101:1392–1396
31. Putney JW, Bird GS (2008) Cytoplasmic calcium oscillations and store-operated calcium influx. J Physiol 586:3055–3059
32. Firth AL, Remillard CV, Yuan JX-J (2007) TRP channels in hypertension. Biochim Biophys Acta 1772:895–906
33. Ressmeyer AR, Bai Y, Delmotte P, Uy KF, Thistlethwaite P, Fraire A, Sato O, Ikebe M, Sanderson MJ (2009) Human airway contraction and formoterol-induced relaxation is determined by Ca^{2+} oscillations and Ca^{2+} sensitivity. Am J Respir Cell Mol Biol. Sep 18. [Epub ahead of print]
34. Bai Y, Edelmann M, Sanderson MJ (2009) The contribution of inositol 1,4,5-trisphosphate and ryanodine receptors to agonist-induced Ca(2+) signaling of airway smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 297:L347–L361
35. Delmotte P, Sanderson MJ (2009) Effects of formoterol on contraction and Ca^{2+} signaling of mouse airway smooth muscle cells. Am J Respir Cell Mol Biol. Jun 5. [Epub ahead of print]

Part II
TRP Channels in the Pulmonary Vasculature: Basics and New Findings

Introduction to TRP Channels: Structure, Function, and Regulation

Michael Y. Song and Jason X.-J. Yuan

Abstract Transient receptor potential or TRP families of ion channels demonstrate great diversity in activation and inhibition, and they are diverse in selectivity of ion conductance. TRP ion channels function as signal integrators through their ion conductance properties, and in some cases kinase activity. They mediate processes such as vision, taste, olfaction, hearing, touch, and thermo- and osmosensation. TRP cation channels function by mediating the flux of Na^+ and Ca^{2+} across the plasma membrane and into the cytoplasm. The influx of cations into the cytoplasm depolarizes cells and is necessary for action potentials in excitable cells such as neurons. In non-excitable cells, membrane depolarization by TRP) and-channels stimulates voltage- dependent channels (Ca^{2+}, K^+, Cl^- influences many cellular events, such as transcription, translation, contraction, and migration. TRP channels are important in human physiology, and mutations in TRP genes are associated with at least four diseases. Furthermore, altered expression, function, and/or regulation of TRP channels have been implicated in diseases such as pulmonary hypertension.

Keywords Canonical • Vanilliod • Melastatin • Ankyrin • Trpn=No mechanoreceptor potential • Trpp=Polycystin • Trpml=Mucolipin

1 Introduction

Transient receptor potential (TRP) cation channels mediate the flux of Na^+ and Ca^{2+} across the plasma membrane and into the cytoplasm.[1] TRP channels were first discovered due to a mutation in the *Drosophila* photoreceptor, which resulted in inhibited Ca^{2+} permeability and sensitivity to light.[2] The influx of cations into the cytoplasm depolarizes cells and is necessary for action potentials in excitable cells

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such as neurons.[3] In nonexcitable cells, membrane depolarization by TRP channels stimulates voltage-dependent channels (Ca^{2+}, K^+, Cl^-) and influences many cellular events, such as transcription, translation, contraction, and migration.[4] TRP channels and their regulation are fundamentally important in cellular function and disease.[1]

2 TRP Gene Expression

TRP genes are expressed in organisms from archaea to plants to animals.[5] In animals, TRP is expressed in brain, heart, lung, and other tissues.[5] Mammalian TRP channels possess a high degree of sequence homology, particularly in the putative functional regions.[6] The TRP superfamily of genes is categorized into two groups based on sequence and topological similarities.[6] Group 1 includes TRPC, TRPV, TRPM, TRPA, and TRPN. Group 2 includes TRPP and TRPML.

3 TRP Protein and Channel Function

TRP proteins form cation channels with varying selectivity to different cations.[7] TRP proteins are transmembrane proteins with six transmembrane domains with a pore domain wedged between the fifth and sixth transmembrane domains. The N- and C-terminal domains are intracellular and believed to be involved in regulation of TRP channel function and in channel assembly. It is believed that TRP channels are homo- or heterotetramers of TRP proteins, with each subunit contributing to selectivity of the ion-conducting pore.[8] Allosteric interactions between subunits are thought to contribute to gating of TRP channels; however, the location and structure of these gates are unknown. Amino acid sequences flanking the pore-forming regions of TRP proteins are strongly conserved across the various TRP channel families, highlighting their importance in pore formation or pore gating.[9]

4 TRP Channel Regulation

TRP channel function is regulated by (1) plasma membrane receptor activation, (2) ligand activation, (3) direct activation, and (4) indirect activation.[10] G protein-coupled receptors (GPCRs) and receptor tyrosine kinase act through diacylglycerol (DAG) to activate TRP channels on the plasma membrane.[11,12] These channels are termed *receptor-operated channels*. Furthermore, stimulation of these receptors depletes intracellular endoplasmic reticulum/sarcoplasmic reticulum (ER/SR) Ca^{2+} stores and leads to store-operated Ca^{2+} entry through TRP channels on the PM.[13] Various ligands can activate TRP channels. Ligand activation includes

activation by exogenous small molecules such as capsaicin, icilin, 2-Aminoethoxydiphenyl Borate, endogenous lipids such as DAG, phosphoinosidides, eicosanoids, purine nucleotides, ions such as Ca^{2+}, and Mg^{2+}, and the Ca^{2+}/Calmodulin complex.[14–16] TRP channel activity can be stimulated through direct activation. Examples of direct activation of TRP channels include temperature change,[17] mechanical stimulation,[18] and conformational coupling with other proteins such as STIM1 (stromal interacting molecule 1) or IP_3R (inositol 1,4,5-trisphosphate receptor). Indirect activation refers to transcriptional control or insertion of vesicles containing TRP proteins into the plasma membrane.

5 Summary of Mammalian TRPs (Fig. 6.1)

5.1 TRPC

TRPC1–7 are categorized into three categories based on sequence and functional characteristics.[5] TRPC1, 4, and 5 form one group. TRPC1 was the first mammalian TRP protein discovered. It is widely expressed in many tissues and thought to form heteromeric channels with TRPC4 and TRPC5.[19] TRPC4 and TRPC5 are believed to form homomeric channels. When expressed together, TRPC1, 4, and 5 form nonselective cation channels that are activated by G_q signaling through a phospholipase Cβ1 (PLCβ1) pathway.[20] Growth factor stimulates rapid translocation of TRPC5 into the plasma membrane from vesicles located near the plasma membrane.[21]

TRPC3, 6, and 7 have roughly 75% sequence homology and when coexpressed reconstitute nonselective, inward and outward rectifying cation channels.[10] These channels are activated by a receptor-mediated pathway involving DAG and are believed to be important in vascular and airway smooth muscle.[22] Channels formed by TRPC3 or TRPC6 are also regulated by N-linked glycosylation and Ca/CaM.[23] TRPC3 is activated by phosphorylation by PKG.[24] TRPC6 is phosphorylated by the Src family of tyrosine kinases.[25]

TRPC2 shares roughly 30% sequence homology with TRPC3/6/7.[26] TRPC2 full-length messenger RNA (mRNA) is expressed in mouse and rat tissues.[27] However, TRPC2 is a pseudogene in humans.[10]

5.2 TRPV

TRPV is subdivided into two groups composed of TRPV1–4 and TRPV5 and 6.[28] TRPV1 forms vanilloid receptor and noxious thermosensor cation channels that are outwardly rectifying in humans and mice.[29] Capsaicin-activated TRPV1 channels have roughly a 10:1 selectivity of Ca^{2+} over Na^+, with selectivity of $Ca^{2+} > Mg^{2+} > Na^+ = K^+ = Cs^+$.[30] Heat-activated TRPV1 channels have a 4:1 selectivity of Ca^{2+}

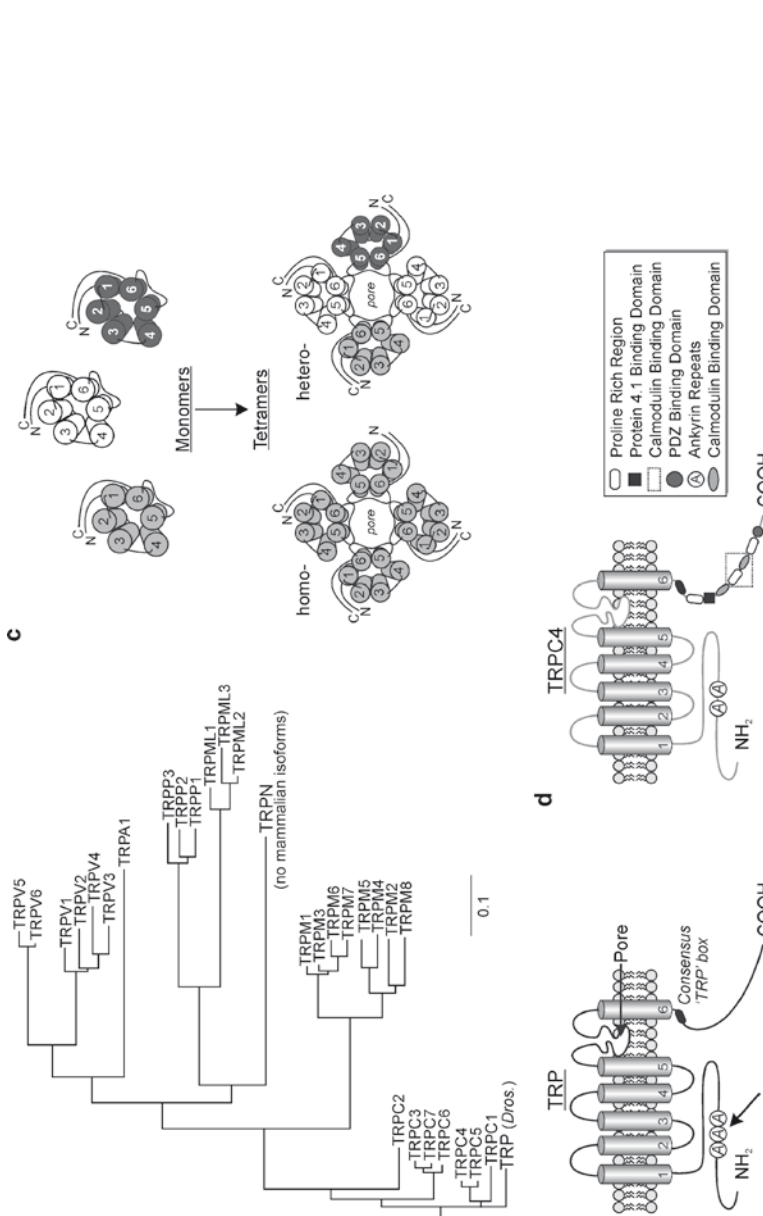

Fig. 6.1 Classification and structure of TRP channels. (**a**) Phylogenic tree of the seven known families of TRP proteins. The original TRP protein identified in *Drosophila melanogaster* is most closely related to TRP channels. (**b**) Two-dimensional representation of TRP channel structure. Six transmembrane domains (TM1-TM6), ankyrin repeats, pore region, and TRP box are shown and described further in the text. (**c**) Subunit arrangement of TRP monomers into functional homo- or heterotetrameric channels with a central ion-conducting pore. (**d**) Schematic representation of key regulatory sites for the TRPC4 protein. Regions and domains are described in the text

over Na^+.[31] TRPV1 is inactivated by phosphorylation by cyclic adenosine monophosphate (cAMP)-dependant protein kinase and pharmacologically by capsazepine, ruthenium red, iodoresiniferatoxin, (N-(4-tertiarybutylphenyl)-4-(3-cholorphyridin-2-yl)tetrahydropryazine-1(2H)-carbox-amide) (BCTC), and Phosphatidylinositol 4, 5-Bisphosphate (PIP_2). TRPV1 channels are activated by capsaicin (EC_{50} = 0.7 μ*M*), resiniferatoxin (EC_{50} = 40 n*M*), anandamide, heat (threshold of 43°C), extracellular protons, and ethanol.[32] TRPV1 is expressed in trigeminal and dorsal root ganglia, brain, and spinal cord.[10] TRPV1 channels function physiologically in nociception, inflammation, temperature sensation, and capsaicin detection.[33]

RPV2 possesses roughly 50% sequence homology to TRPV1 and is thought to function as a noxious heat thermosensor channel.[34] TRPV2 channels constitute an outwardly rectifying nonselective cation current with roughly 3:1 selectivity for Ca^{2+} over Na^+. TRPV2 channels are insensitive to capsaicin and activated by heat, with a threshold of 52°C. TRPV2 channels can be inhibited by ruthenium red, La^{3+}, and SKF-96365. TRPV2 is expressed in dorsal root ganglion neurons, brain, spinal cord, spleen, lung, vascular myocytes, and vascular smooth muscle.[31,34]

TRPV3 forms outwardly rectifying cation channels with roughly 10:1 selectivity of Ca^{2+} over Na^+. TRPV3 channels are activated by temperature at roughly 35°C and are believed to mediate sensation of warmth in skin. TRPV3 is inactivated by cooling and blocked by ruthenium red.[35]

TRPV4 forms outwardly rectifying cation channels with roughly 6:1 selectivity of Ca^{2+} over Na^+.[36] TRPV4 channels function as osmosensor channels, which mediate sensitivity to pressure and acidic nociception in nerve cells.[33] TRPV4 is activated by reduction in osmolarity, phorbol esters, arachidonic acid, and stretch.[37] TRPV4 is inactivated by the Ca/CaM complex and blocked by ruthenium red, gadolinium, and lanthanum. TRPV4 is expressed in brain, liver, kidney, fat, heart, testis, salivary gland, and trachea.[38]

TRPV5 forms constitutively active inwardly rectifying Ca^{2+} selective cation channels with 107:1 selectivity of Ca^{2+} over Na^+.[39] TRPV5 is expressed in intestine, kidney, and placenta.[40] However, its mechanisms of activation and inactivation are unclear. TRPV5 channels can be inhibited with ruthenium red and La^{3+}.[41]

TRPV6 also forms constitutively active inwardly rectifying Ca^{2+} selective cation channels with 130:1 selectivity of Ca^{2+} over Na^+.[42] TRPV6 is expressed in the intestine and in the kidneys.[40] However, its physiological function as well as mechanisms of activation and inactivation are unclear.[43] TRPV6 channels can be blocked by ruthenium red and La^{3+}.[43]

5.3 TRPM

TRPM is categorized into four groups: TRPM1/3, TRPM7/6, TRPM2/8, and TRPM4/5.[10] TRPM1, the first member of the TRPM family discovered, is a Ca^{2+}-permeable channel in the eye and melanocytes.[44] Downregulation of TRPM1 is a marker for metastasis in patients with melanoma.[45] TRPM1 is regulated by an

alternatively spliced form of TRPM1 and the transcription factor Microphthalmia-associated transcription factor (MITF).[45]

TRPM2 is a weakly voltage-sensing nonselective cation channel, which is expressed in the brain, placenta, lung, spinal cord, spleen, and lymphocytes.[46] TRPM2 is activated by increased cytoplasmic Ca^{2+} concentration, Nicotinamide adenine dinucleotide (β-NAD^+), and adenosine diphosphate (ADP)-ribose. TRPM2 is also activated by H_2O_2 and tumor necrosis factor α (TNF-α), suggesting that it may function as a redox sensor.[47] TRPM2 can be inactivated by flufenamic acid, clotrimazole, econazole, and Poly (ADP-Ribose) polymerase (PARP) inhibitors.[48]

TRPM3 is a constitutively active (enhanced by hypoosmolarity and sphingolipids) nonselective cation channel widely expressed in kidney and brain.[49] TRPM3 can be blocked by Gd^{3+} but is insensitive to ruthenium red.[7] Physiologically, TRPM3 is involved in Ca^{2+} absorption in renal collecting tubules.[49]

TRPM4 is a Ca^{2+}-activated Na^+ channel with roughly 100:1 greater selectivity to Na^+.[50] There are two splice variants of TRPM4: TRPM4a and TRPM4b. TRPM4a is inhibited by La^{3+} and Gd^{3+}, and TRPM4b is inhibited by free intracellular adenosine triphosphate (ATP), ADP, and spermine.[51] TRPM4 is regulated by calcium oscillations in activated T lymphocytes and causes myogenic cerebral artery vasoconstriction.[52]

TRPM5 forms outwardly rectifying nonselective, monovalent cation channels in eye, liver, lung stomach, and tongue.[53] TRPM5 is activated by cytoplasmic Ca^{2+}, GPCRs, and PLCβ2.[54] TRPM5 is believed to mediate tastes of sweet, bitter, and umami.[52]

TRPM6 functions as an outwardly rectifying nonselective cation channel and contains a protein kinase on its C-terminal domain.[55] TRPM6 is widely expressed in kidney and the gastrointestinal tract. Mutation of TRPM6 has been linked to human hypomagnesemia and secondary hypocalcemia.[56] TRPM6 channels can be blocked by ruthenium red.[55]

TRPM7 is similar to TRPM6 in that it forms cation channels and possesses kinase activity that autophosphorylates.[57] Furthermore, TRPM6 and TRPM7 can heteromulterimize into active ion channels.[58] TRPM7 is activated by PIP_2.[59] TRPM7 is also involved in cellular Mg^{2+} homeostasis.[60] TRPM7 can be blocked by Mg^{2+}, La^{3+}, and polyamines.[61]

TRPM8 channels are believed to be involved in cooling and menthol sensation. TRPM8 forms nonselective cation channels in sensory neurons of trigeminal and dorsal root ganglia and prostate epithelium.[62] TRPM8 channels are activated by cooling below about 22°C and menthol.[63] TRPM8 channels are inhibited by BCTC and capsazepine.[64]

5.4 TRPA

The TRPA "family" has only one member. TRPA1 has over a dozen ankyrin repeats near its N terminus. TRPA1 is a nonselective cation channel that is activated by membrane stretch, cytoskeletal perturbation, mustard oils, PLC-coupled GPCRs, and icilin.[65,66] TRPA1 channels are blocked by ruthenium red. TRPA1 is expressed in sensory neurons of trigeminal and dorsal root ganglia and the ear.[67,68] TRPA1 is believed to function as the putative sensor of wasabi.

5.5 TRPP

Mutation in TRPP1 causes autosomal dominant polycystic kidney disease.[69] TRPP1 forms nonselective cation channels in kidney, pancreas, heart, testis, and blood.[10] TRPP1 may be activated by mechanical stress and blocked by amiloride, Gd^{3+}, and La^{3+}. TRPP1 is involved in the development of mouse cardiac, skeletal, and renal cells and is important in kidney and liver cyst formation.[70]

TRPP2 forms nonselective cation channels in kidney, testis, and eye.[71] TRPP2 is activated by increased cytoplasmic calcium concentration.[72] TRPP2 is involved in kidney and retinal development.[73] TRPP3 forms nonselective cation channels.[10]

5.6 TRPML

The TRPML family of ion channels is likely restricted to intracellular vesicles.[74] TRPML1–3 form nonselective cation channels.[75] TRPML1 channels are widely expressed in heart kidney, testis, and blood and blocked by amiloride, Gd^{3+}, La^{3+}, and Ni^{2+}.[10] Not much is known about TRPML2 and TRPML3.

6 Conclusion

TRP families of ion channels demonstrate great diversity in activation and inhibition, and they are diverse in selectivity of ion conductance. TRP ion channels function as signal integrators through their ion conductance properties and in some cases kinase activity. They mediate vision, taste, olfaction, hearing, touch, and thermo- and osmosensation. TRP channels are important in human physiology, and mutations in TRP genes are associated with at least four diseases. Furthermore, altered expression, function, or regulation of TRP channels has been implicated in diseases such as pulmonary hypertension.

References

1. Berridge MJ, Bootman MD, Roderick HL (2003) Calcium signalling: dynamics, homeostasis and remodelling. Nat Rev Mol Cell Biol 4:517–529
2. Putney J (1977) Muscarinic, α-adrenergic and peptide receptors regulate the same calcium influx sites in the parotid gland. J Physiol 268:139–149
3. Tessier-Lavigne M, Goodman C (1996) The molecular biology of axon guidance. Science 274:1123–1133
4. Clapham D (1995) Calcium signaling. Cell 80:259
5. Clapham DE, Montell C, Schultz G, Julius D (2003) International Union of Pharmacology. XLIII. Compendium of voltage-gated ion channels: transient receptor potential channels. Pharmacol Rev 55:591–596
6. Montell C, Birnbaumer L, Flockerzi V et al (2002) A unified nomenclature for the superfamily of TRP cation channels. Mol Cell 9:229–231

7. Oberwinkler J, Lis A, Giehl KM, Flockerzi V, Philipp SE (2005) Alternative splicing switches the divalent cation selectivity of TRPM3 channels. J Biol Chem 280:22540–22548
8. Clapham DE, Julius D, Montell C, Schultz G (2005) International Union of Pharmacology. XLIX. Nomenclature and structure–function relationships of transient receptor potential channels. Pharmacol Rev 57:427–450
9. Nilius B, Talavera K, Owsianik G, Prenen J, Droogmans G, Voets T (2005) Gating of TRP channels: a voltage connection. J Physiol 567:35–44
10. Ramsey IS, Delling M, Clapham DE (2006) An introduction to TRP channels. Annu Rev Physiol 68:619–647
11. Okada T, Inoue R, Yamazaki K, Maeda A, Kurosaki T (1999) Molecular and functional characterization of a novel mouse transient receptor potential protein homologue TRP7. Ca^{2+}-permeable cation channel that is constitutively activated and enhanced by stimulation of G protein-coupled receptor. J Biol Chem 274:27359–27370
12. Hisatsune C, Kuroda Y, Nakamura K, Inoue T, Nakamura T (2004) Regulation of TRPC6 channel activity by tyrosine phosphorylation. J Biol Chem 279:18887–18894
13. Voets T, Prenen J, Fleig A et al (2001) CaT1 and the calcium release-activated calcium channel manifest distinct pore properties. J Biol Chem 276:47767–47770
14. Zhang J, Xia S, Block ER, Patel JM (2002) NO upregulation of a cyclic nucleotide-gated channel contributes to calcium elevation in endothelial cells. Am J Physiol Cell Physiol 283:C1080–C1089
15. Zhu M (2005) Multiple roles of calmodulin and other Ca^{2+}-binding proteins in the functional regulation of TRP channels. Pflügers Arch 451:105–115
16. Venkatachalam K, Zheng F, Gill DL (2003) Regulation of canonical transient receptor potential (TRPC) channel function by diacylglycerol and protein kinase C. J Biol Chem 278:29031–29041
17. Clapham DE (2003) TRP channels as cellular sensors. Nature 426:517–524
18. Maroto R, Raso A, Wood TG, Kurosky A, Martinac B, Hamill OP (2005) TRPC1 forms the stretch-activated cation channel in vertebrate cells. Nat Cell Biol 7:179–185
19. Wes PD, Chevesich J, Jeromin A, Rosenberg C, Stetten G, Montell C (1995) TRPC1, a human homolog of a *Drosophila* store-operated channel. Proc Natl Acad Sci U S A 92:9652–9656
20. Plant TD, Schaefer M (2005) Receptor-operated cation channels formed by TRPC4 and TRPC5. Naunyn Schmiedebergs Arch Pharmacol 371:266–276
21. Jung S, Mühle A, Schaefer M, Strotmann R, Schultz G, Plant TD (2003) Lanthanides potentiate TRPC5 currents by an action at extracellular sites close to the pore mouth. J Biol Chem 278:3562–3571
22. Hofmann T, Obukhov AG, Schaefer M, Harteneck C, Gudermann T, Schultz G (1999) Direct activation of human TRPC6 and TRPC3 channels by diacylglycerol. Nature 397:259–263
23. Dietrich A, Mederos y Schnitzler M, Emmel J, Kalwa H, Hofmann T, Gudermann T (2003) N-linked protein glycosylation is a major determinant for basal TRPC3 and TRPC6 channel activity. J Biol Chem 278:47842–47852
24. Kwan H, Huang Y, Yao X (2004) Regulation of canonical transient receptor potential isoform 3 (TRPC3) channel by protein kinase G. Proc Natl Acad Sci U S A 101:2625–2630
25. Vazquez G, Wedel BJ, Kawasaki BT, Bird GS, Putney JW Jr (2004) Obligatory role of Src kinase in the signaling mechanism for TRPC3 cation channels. J Biol Chem 279:40521–40528
26. Vannier B, Peyton M, Boulay G et al (1999) Mouse trp2, the homologue of the human trpc2 pseudogene, encodes mTrp2, a store depletion-activated capacitative Ca^{2+} entry channel. Proc Natl Acad Sci U S A 96:2060–2064
27. Lucas P, Ukhanov K, Leinders-Zufall T, Zufall F (2003) A diacylglycerol-gated cation channel in vomeronasal neuron dendrites is impaired in TRPC2 mutant mice: mechanism of pheromone transduction. Neuron 40:551–561
28. Niemeyer BA (2005) Structure–function analysis of TRPV channels. Naunyn Schmiedebergs Arch Pharmacol 371:285–294
29. Ross RA (2003) Anandamide and vanilloid TRPV1 receptors. Br J Pharmacol 140:790–801
30. Mohapatra DP, Nau C (2003) Desensitization of capsaicin-activated currents in the vanilloid receptor TRPV1 is decreased by the cyclic AMP-dependent protein kinase pathway. J Biol Chem 278:50080–50090

31. Woodbury CJ, Zwick M, Wang S et al (2004) Nociceptors lacking TRPV1 and TRPV2 have normal heat responses. J Neurosci 24:6410–6415
32. Hu HZ, Gu Q, Wang C et al (2004) 2-Aminoethoxydiphenyl borate is a common activator of TRPV1, TRPV2, and TRPV3. J Biol Chem 279:35741–35748
33. Vriens J, Watanabe H, Janssens A, Droogmans G, Voets T, Nilius B (2004) Cell swelling, heat, and chemical agonists use distinct pathways for the activation of the cation channel TRPV4. Proc Natl Acad Sci U S A 101:396–401
34. Muraki K, Iwata Y, Katanosaka Y, Ito T, Ohya S (2003) TRPV2 is a component of osmotically sensitive cation channels in murine aortic myocytes. Circ Res 93:829–838
35. Moqrich A, Hwang SW, Earley TJ et al (2005) Impaired thermosensation in mice lacking TRPV3, a heat and camphor sensor in the skin. Science 307:1468–1472
36. Watanabe H, Vriens J, Suh SH, Benham CD, Droogmans G, Nilius B (2002) Heat-evoked activation of TRPV4 channels in a HEK293 cell expression system and in native mouse aorta endothelial cells. J Biol Chem 277:47044–47051
37. Todaka H, Taniguchi J, Satoh J, Mizuno A, Suzuki M (2004) Warm temperature-sensitive transient receptor potential vanilloid 4 (TRPV4) plays an essential role in thermal hyperalgesia. J Biol Chem 279:35133–35138
38. Watanabe H, Vriens J, Prenen J, Droogmans G, Voets T, Nilius B (2003) Anandamide and arachidonic acid use epoxyeicosatrienoic acids to activate TRPV4 channels. Nature 424:434–438
39. Hoenderop JG, Voets T, Hoefs S et al (2003) Homo- and heterotetrameric architecture of the epithelial Ca^{2+} channels TRPV5 and TRPV6. EMBO J 22:776–785
40. Van Abel M, Hoenderop JG, Bindels RJ (2005) The epithelial calcium channels TRPV5 and TRPV6: regulation and implications for disease. Naunyn Schmiedebergs Arch Pharmacol 371:295–306
41. Chang Q, Gyftogianni E, van de Graaf SF et al (2004) Molecular determinants in TRPV5 channel assembly. J Biol Chem 279:54304–54311
42. Bödding M, Flockerzi V (2004) Ca^{2+} dependence of the Ca^{2+}-selective TRPV6 channel. J Biol Chem 279:36546–36552
43. Lambers TT, Weidema AF, Nilius B, Hoenderop JG, Bindels RJ (2004) Regulation of the mouse epithelial Ca^{2+} channel TRPV6 by the Ca^{2+}-sensor calmodulin. J Biol Chem 279:28855–28861
44. Schlingmann KP, Weber S, Peters M et al (2002) Hypomagnesemia with secondary hypocalcemia is caused by mutations in TRPM6, a new member of the TRPM gene family. Nat Genet 31:166–170
45. Fleig A, Penner R (2004) The TRPM ion channel subfamily: molecular, biophysical and functional features. Trends Pharmacol Sci 25:633–639
46. McHugh D, Flemming R, Xu SZ, Perraud AL, Beech DJ (2003) Critical intracellular Ca^{2+} dependence of transient receptor potential melastatin 2 (TRPM2) cation channel activation. J Biol Chem 278:11002–11006
47. Fonfria E, Marshall IC, Benham CD et al (2004) TRPM2 channel opening in response to oxidative stress is dependent on activation of poly(ADP-ribose) polymerase. Br J Pharmacol 143:186–192
48. Kolisek M, Beck A, Fleig A, Penner R (2005) Cyclic ADP-ribose and hydrogen peroxide synergize with ADP-ribose in the activation of TRPM2 channels. Mol Cell 18:61–69
49. Grimm C, Kraft R, Sauerbruch S, Schultz G, Harteneck C (2003) Molecular and functional characterization of the melastatin-related cation channel TRPM3. J Biol Chem 278:21493–21501
50. Launay P, Fleig A, Perraud AL, Scharenberg AM, Penner R, Kinet JP (2002) TRPM4 is a Ca^{2+}-activated nonselective cation channel mediating cell membrane depolarization. Cell 109:397–407
51. Nilius B, Prenen J, Voets T, Droogmans G (2004) Intracellular nucleotides and polyamines inhibit the Ca^{2+}-activated cation channel TRPM4b. Pflügers Arch 448:70–75
52. Ullrich ND, Voets T, Prenen J et al (2005) Comparison of functional properties of the Ca^{2+}-activated cation channels TRPM4 and TRPM5 from mice. Cell Calcium 37:267–278

53. Hofmann T, Chubanov V, Gudermann T, Montell C (2003) TRPM5 is a voltage-modulated and Ca^{2+}-activated monovalent selective cation channel. Curr Biol 13:1153–1158
54. Liu D, Liman ER (2003) Intracellular Ca^{2+} and the phospholipid PIP_2 regulate the taste transduction ion channel TRPM5. Proc Natl Acad Sci U S A 100:15160–15165
55. Voets T, Nilius B, Hoefs S et al (2004) TRPM6 forms the Mg^{2+} influx channel involved in intestinal and renal Mg^{2+} absorption. J Biol Chem 279:19–25
56. Walder RY, Landau D, Meyer P et al (2002) Mutation of TRPM6 causes familial hypomagnesemia with secondary hypocalcemia. Nat Genet 31:171–174
57. Ryazanova LV, Dorovkov MV, Ansari A, Ryazanov AG (2004) Characterization of the protein kinase activity of TRPM7/ChaK1, a protein kinase fused to the transient receptor potential ion channel. J Biol Chem 279:3708–3716
58. Matsushita M, Kozak JA, Shimizu Y et al (2005) Channel function is dissociated from the intrinsic kinase activity and autophosphorylation of TRPM7/ChaK1. J Biol Chem 280:20793–20803
59. Runnels LW, Yue L, Clapham D (2002) The TRPM7 channel is inactivated by PIP2 hydrolysis. Nat Cell Biol 4:329–336
60. Schmitz C, Perraud AL, Johnson CO et al (2003) Regulation of vertebrate cellular Mg^{2+} homeostasis by TRPM7. Cell 114:191–200
61. Dorovkov MV, Ryazanov AG (2004) Phosphorylation of annexin I by TRPM7 channel-kinase. J Biol Chem 279:50643–50646
62. Brauchi S, Orio P, Latorre R (2004) Clues to understanding cold sensation: thermodynamics and electrophysiological analysis of the cold receptor TRPM8. Proc Natl Acad Sci U S A 101:15494–15499
63. Weil A, Moore SE, Waite NJ, Randall A, Gunthorpe MJ (2005) Conservation of functional and pharmacological properties in the distantly related temperature sensors TRPV1 and TRPM8. Mol Pharmacol 68:518–527
64. Rohács T, Lopes CM, Michailidis I, Logothetis DE (2005) $PI_{4,5}P_2$ regulates the activation and desensitization of TRPM8 channels through the TRP domain. Nat Neurosci 8:626–634
65. Macpherson LJ, Geierstanger BH, Viswanath V, Bandell M, Eid SR (2005) The pungency of garlic: activation of TRPA1 and TRPV1 in response to allicin. Curr Biol 15:929
66. Corey DP, García-Añoveros J, Holt JR et al (2004) TRPA1 is a candidate for the mechanosensitive transduction channel of vertebrate hair cells. Nature 432:723–730
67. Bandell M, Story GM, Hwang SW et al (2004) Noxious cold ion channel TRPA1 is activated by pungent compounds and bradykinin. Neuron 41:849–857
68. Nagata K, Duggan A, Kumar G, García-Añoveros J (2005) Nociceptor and hair cell transducer properties of TRPA1, a channel for pain and hearing. J Neurosci 25:4052–4061
69. Menè P (2006) Transient receptor potential channels in the kidney: calcium signaling, transport and beyond. J Nephrol 19:21–29
70. Kwan HY, Huang Y, Yao X (2007) TRP channels in endothelial function and dysfunction. Biochim Biophys Acta 1772:907–914
71. Köttgen M, Buchholz B, Garcia-Gonzalez MA et al (2008) TRPP2 and TRPV4 form a polymodal sensory channel complex. J Cell Biol 182:437–447
72. Köttgen M, Benzing T, Simmen T et al (2005) Trafficking of TRPP2 by PACS proteins represents a novel mechanism of ion channel regulation. EMBO J 24:705–716
73. Wu G, D'Agati V, Cai Y et al (1998) Somatic inactivation of Pkd2 results in polycystic kidney disease. Cell 93:177–188
74. Song Y, Dayalu R, Matthews SA, Scharenberg AM (2006) TRPML cation channels regulate the specialized lysosomal compartment of vertebrate B-lymphocytes. Eur J Cell Biol 85:1253–1264
75. Qian F, Noben-Trauth K (2005) Cellular and molecular function of mucolipins (TRPML) and polycystin 2 (TRPP2). Pflügers Arch 451:277–285

Physiological Functions of Transient Receptor Potential Channels in Pulmonary Arterial Smooth Muscle Cells

Xiao-Ru Yang, Mo-Jun Lin, and James S. K. Sham

Abstract The transient receptor potential (TRP) gene superfamily, which consists of 7 subfamilies with at least 28 mammalian homologues, is known to encode a wide variety of cation channels with diverse biophysical properties, activation mechanisms, and physiological functions. Recent studies have identified multiple TRP channel subtypes, belonging to the canonical (TRPC), melastatin-related (TRPM), and vanilloid-related (TRPV) subfamilies, in pulmonary arterial smooth muscle cells (PASMCs). They operate as specific Ca^{2+} pathways responsive to stimuli, including Ca^{2+} store depletion, receptor activation, reactive oxygen species, growth factors, and mechanical stress. Increasing evidence suggests that these channels play crucial roles in agonist-induced pulmonary vasoconstriction, hypoxic pulmonary vasoconstriction, smooth muscle cell proliferation, vascular remodeling, and pulmonary arterial hypertension. This chapter highlighted and discussed these putative physiological functions of TRP channels in pulmonary vasculatures. Since Ca^{2+} ions regulate many cellular processes via specific Ca^{2+} signals, future investigations of these novel channels will likely uncover more important regulatory mechanisms of pulmonary vascular functions in health and in disease states.

Keywords TRP channels • store-operated calcium channels • receptor operated calcium channels • hypoxia • pulmonary hypertension

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1 Introduction

In vascular smooth muscle cells (VSMCs), the Ca^{2+} ion serves as a multifunctional messenger responsible for numerous cellular functions, ranging from muscle contraction to gene expression. Depending on the type of agonist and physiological stimulation, $[Ca^{2+}]_i$ can be elevated by Ca^{2+} influx through numerous Ca^{2+} pathways on the plasma membrane, including voltage-gated Ca^{2+} channels, receptor- and store-operated Ca^{2+} channels, nonselective cation channels (NSCCs), and Na^+–Ca^{2+} exchangers, as well as by Ca^{2+} release from the inositol 1,4,5-trisphosphate (IP_3) receptor, ryanodine receptor, and nicotinic acid adenine dinucleotide phosphate (NAADP)-gated intracellular Ca^{2+} stores. Voltage-dependent Ca^{2+} pathways in VSMCs have been well characterized, but the molecular identities and physiological properties of various voltage-independent Ca^{2+} pathways have long been enigmatic. Recent evidence suggests that the mammalian homologues of the *Drosophila* transient receptor potential (TRP) protein,[1] which encode a large repertoire of cation channels, are responsible for many voltage-independent Ca^{2+} pathways in VSMCs.

The TRP superfamily is divided into two groups of a total of seven subfamilies. Group 1 consists of the classical/canonical (TRPC), melastatin-related (TRPM), vanilloid-related (TRPV), ankyrin-related (TRPA), and no mechanoreceptor potential C NOMPC (TRPN) subfamilies; Group 2 includes the polycystin-related (TRPP) and mucolipin-related (TRPML) subfamilies.[2] All TRP proteins share the common features of having six transmembrane domains, a pore-forming loop between the fifth and sixth transmembrane segments, and the highly conserved TRP domains. But, they display remarkable diversity in physiological functions, such as cation selectivity and activation mechanisms. They play critical roles in the response to most major external stimuli, including light, sound, chemicals, temperature, and touch. To date, 28 mammalian TRP homologues have been found in a wide variety of cells and tissues, and at least 10 TRPs have been identified as functional channels in VSMCs (Table 7.1). They are implicated in many different vascular functions, such as myogenic response, agonist-induced vasoconstriction, Ca^{2+} and Mg^{2+} homeostasis, VSMC proliferation, and vascular remodeling.[3]

2 Expression of TRP Channels in Pulmonary Artery Smooth Muscle Cells

The classical/canonical TRPC subfamily, which is comprised of seven voltage-independent NSCCs, is the best-studied TRP family in pulmonary artery smooth muscle cells (PASMCs). Multiple TRPC messenger RNA (mRNA) transcripts and proteins have been identified. A survey of the literature showed that TRPC1 and TRPC6 are the two major TRPC channels expressed in rat, mouse, and human pulmonary arteries (PAs) and PASMCs (Table 7.2). TRPC3 and TRPC4 also were frequently detected, but their expressions varied depending on species and cell preparation (freshly isolated or cultured), whereas TRPC5 and TRPC7 were generally absent. In contrast, expression

Table 7.1 Functional TRP channels identified in vascular smooth muscles

Isoform	Distribution, P: pulmonary; S: systemic	Selectivity (P_{Ca}/P_{Na})	Activator/regulator	Putative physiological functions
TRPC1	P and S	Nonselective	Store depletion, vconformation coupling	Store-operated Ca^{2+} channel, agonist-induced vasoconstriction, VSMC and PASMC proliferation, mechanosensitive cation channel, hypoxic pulmonary hypertension
TRPC3	P and S	1.6	DAG, store depletion, tyrosine phosphorylation	SOCC/ROCC, agonist-induced vasoconstriction
TRPC4	P and S	7	Store depletion	SOCC
TRPC6	P and S	5	DAG, Phosphatidylinositol bisphosphate (PIP_2), tyrosine phosphorylation	ROCC, agonist-induced vasoconstriction, PASMC proliferation, acute hypoxic pulmonary vasoconstriction, myogenic response, idiopathic pulmonary hypertension
TRPV2	P and S	3	Cell stretch, temperature (>52°C)	Myogenic response, mechanosensitive cation channel
TRPV4	P and S	6	Hypoosmolarity, cell stretching, arachidonic acid, and metabolites epoxyeicosatrienoic acid (EETs), temperature (>27°C), 4α-PDD, tyrosine phosphorylation	Mechanosensitive cation channels, endothelium derived hyperpolarizing factor (EDHF)-dependent vasorelaxation, hypoxic pulmonary hypertension
TRPM4	P and S	Monovalent	intracellular Ca^{2+}, PKC, PIP_2, voltage	Myogenic response
TRPM7	P and S	Divalent	Mg^{2+}, ATP, angiotensin II, H^+, PIP_2	Mg^{2+} homeostasis and cell proliferation
TRPM8	P and S	3.3	Cold, menthol, pH, PIP_2	
TRPP2	S	Non-selective	Intracellular Ca^{2+}	Vascular integrity, mechanosensitive channels

of TRPC channels is different in canine PAs. Reverse-transcription polymerase chain reaction (RT-PCR) detected TRPC4, TRPC6, and TRPC7 mRNAs in canine main PAs, with TRPC4 the major expressed isoform.[4] A study showed that TRPC1 and TRPC6 levels are higher in distal than in proximal PAs of rats,[5] suggesting heterogeneity of TRPC expression exists in different locations along the pulmonary vascular tree.

Table 7.2 Expression of TRP channels in PASMCs

Species	Sample	C1	C2	C3	C4	C5	C6	C7	References
Human	Lung tissue, PA, cultured PASMCs	CCC		C	CC	–	CCC	–	17, 18, 21, 39, 46
Mouse	Cultured precapillary PASMCs	AAA	–/?	–/?	–/?	–/?	AAA	–/?	19
	PA and PASMCs	CC						CC	44
Rat	Main PA	CCC		C	C	A	CCC		47
	Cultured main PASMCs	AAA	AA	–	AA	A	AAA		25
	Proximal and distal PA, PASMCs	CCC	–	–	CC	–	CCC	–	5, 48
	Intralobar PA and PASMCs	CCC	–	CC	–	–	CCC	–	16
	Intralobar PA	BBB		BB	–	–	BB		49
	Intralobar PA			BB			BB		50
Dog	Main PASMCs	–	–	–	AAA	–	AA	A	4

A mRNA only; *B* protein only; *C* mRNA and protein

Compared to TRPC channels, information on other TRP subfamilies in the pulmonary vasculature is scant. We previously performed a survey on the expression of TRPM and TRPV channels in deendothelialized rat intralobar PAs and aorta.[6] The mRNA of TRPM2, TRPM3, TRPM4, TRPM7, and TRPM8 of the TRPM family and TRPV1, TRPV2, TRPV3, and TRPV4 of the TRPV family were detected in both PAs and aorta. The ranks of relative expression evaluated by quantitative real-time RT-PCR were TRPV4 > TRPV2 > TRPV1 >> TRPV3, and TRPM8 > TRPM4 > TRPM7 > TRPM3 > TRPM2 > TRPM5. Expression of TRPM2, TRPM8, TRPV1, and TRPV4 proteins in PAs was also verified by Western blot. Moreover, the TRPM8 agonist menthol or the TRPV4 agonist 4α-phorbol 12,13-didecanoate (4α-PDD) evoked a Ca^{2+} response in PASMCs. These responses could be abolished by the removal of Ca^{2+} or application of Ni^{2+} but were unaffected by nifedipine. These results indicate that multiple TRPM and TRPV channels are expressed, and at least TRPM8 and TRPV4 channels are functional Ca^{2+} influx pathways in PASMCs. Expression of other TRP subfamilies (TRPA, TRPP, and TRML) has not been reported in pulmonary vasculature.

3 Physiological Functions of TRP Channels in PASMCs

3.1 Store-Operated and Receptor-Operated Ca^{2+} Entry

Store-operated Ca^{2+} entry (SOCE) is defined as the capacitative Ca^{2+} entry activated by the depletion of intracellular Ca^{2+} stores.[7] Several mechanisms, including the diffusible calcium influx factor (CIF), exocytosis, and conformational coupling,

have been proposed. Studies have established that the stromal interacting molecule 1 (STIM1), a transmembrane protein with an N-terminal EF-hand Ca^{2+}-binding domain, acts as a Ca^{2+} sensor of endoplasmic reticulum (ER) or sarcoplasmic reticulum (SR) Ca^{2+} content.[8] Depletion of ER/SR Ca^{2+} results in rearrangement of STIM1 in the form of punctae underneath the plasma membrane and subsequent activation of Store-operated cation channel (SOCC). Moreover, the homologue STIM2 may operate as another Ca^{2+} sensor capable of detecting a small decrease in ER/SR $[Ca^{2+}]$ and triggering SOCE for feedback regulation of basal cytosolic and ER Ca^{2+} levels.[9] In vascular smooth muscle, SOCE is mostly as nonselective cation pathways, in contrast to the highly Ca^{2+}-selective Ca^{2+} release-activated current (I_{CRAC}) recorded in T lymphocytes.[10] Receptor-operated Ca^{2+} entry (ROCE) is usually defined loosely as voltage-independent Ca^{2+} entry that requires ligand binding to membrane receptors for activation. In fact, the definitions of SOCE and ROCE are not mutually exclusive, and the distinction between the two pathways sometimes is rather murky. For example, an agonist binds to a G_q protein-coupled receptor and activates phospholipase Cβ (PLC-β) to generate IP_3 and diacylglycerol (DAG), leading to dual activation of SOCE and ROCE.

Since their discovery, TRPC channels have been implicated as SOCC and Receptor-operated cation channels (ROCC) because they are nonselective Ca^{2+}-permeable channels and are activated by a PLC-dependent mechanism.[11] It is now clear that TRPC1, TRPC4, and TRPC5 represent a subgroup of TRPC channels activated by Ca^{2+} store depletion caused by inhibition of SR Ca^{2+}-ATPase (adenosine triphosphatase) using cyclopiazonic acid (CPA) or thapsigargin. TRPC3, TRPC6, and TRPC7 form another subgroup of channels participating in ROCE activated directly by DAG independent of protein kinase C (PKC). In a heterologous expression system, members of the TRPC1/4/5 or TRPC3/6/7 subgroup can coassemble directly to form heteromeric channels, but cross association does not occur between members of the two subgroups.[2,3,12] However, some studies showed that TRPC3, TRPC4, and TRPC5 can operate as both SOCC and ROCC.[13] Studies found that STIM1 not only can directly interact with TRPC1, TRPC4, and TRPC5 to activate SOCE[14] but also can mediate heteromerization of TRPC3 with TRPC1 and TRPC6 with TRPC4.[15] These new findings raise an important question of whether SOCE and ROCE are separate Ca^{2+} pathways or the same pathway due to heteromerization of various TRPC channels in native cells.

Our previous study provided evidence that SOCE and ROCE are independent Ca^{2+} pathways in rat intralobar PASMCs, where the putative store-operated TRPC1 and receptor-operated TRPC6 are predominantly expressed.[16] Direct activation of SOCE with thapsigargin and ROCE with the DAG analogue 1-oleoyl-2-acetyl-sn-glycerol (OAG) elicited distinctive cation entries that exhibited a 1,000-fold difference in their sensitivity to La^{3+} (IC_{50} of ~0.3 μ*M* for SOCE and ~300 μ*M* for ROCE). Small interfering RNA (siRNA) knockdown of TRPC1 and TRPC6 inhibited the thapsigargin- and OAG-activated cation entry, respectively. Furthermore, TRPC1 siRNA had no effect on OAG-induced cation entry, and TRPC6 siRNA did not alter the thapsigargin-induced response. These results indicate that thapsigargin-induced SOCE and OAG-mediated ROCE are mutually independent pathways,

with TRPC1 and TRPC6 the major determinants of SOCE and ROCE, respectively, in rat intralobar PASMCs.

The notion that TRPC1 is critical for SOCE is consistent with other observations that TRPC1-specific antisense oligonucleotides inhibited the TRPC1 expression and blocked SOCE in cultured human PASMCs,[17] and overexpression of TRPC1 in rat PAs enhanced SOCE-induced vasoconstriction.[18] Moreover, the importance of TRPC6 in ROCE is supported by the observation that OAG failed to activate ROCC in PASMCs of *trpc6*$^{-/-}$ mice.[19] However, a study in cultured rat main PASMCs showed that PDGF (platelet-derived growth factor) upregulated TRPC6 and enhanced SOCE during cell proliferation. Inhibition of TRPC6 with antisense oligonucleotides reduced the amplitude of SOCE and attenuated mitogen-mediated PASMC proliferation. Hence, TRPC6 might exhibit different properties in proliferating PASMCs, perhaps due to heteromerization with other TRPC subtypes through interactions with STIM1 or Orai1.[15,20] In addition, TRPC3 and TRPC4 have been reported to mediate SOCE in cultured human PASMCs,[21,22] but the TRPC subtypes responsible for ROCE have not been examined in human pulmonary myocytes.

3.2 *Agonist-Induced Pulmonary Vasoconstriction*

As mentioned, many vasoactive agonists and growth factors can exert their effects by binding to receptors coupled to G_q protein or receptor tyrosine kinases to activate PLC-β or PLC-γ, respectively, to generate IP_3 and DAG. IP_3 triggers Ca^{2+} release from IP_3-receptor-gated Ca^{2+} stores, leading to Ca^{2+} influx through SOCC, and DAG directly activates ROCC and modulates other effectors through PKC. In addition to the PLC-IP_3/DAG pathways, agonists and growth factors may activate receptor-operated TRP channels by direct tyrosine phosphorylation through Src family protein tyrosine kinases, including Src and Fyn.[23,24]

Vasoactive agonists, including endothelin I, angiotensin II, norepinephrine, PDGF, EGF (epidermal growth factor), and ATP (adenosine triphosphate) have been shown to activate SOCE or ROCE in VSMCs. It is generally assumed that TRPC channels, by mediating SOCE and ROCE, play significant roles in agonist-induced pulmonary vasoconstriction. However, despite evidence that SOCE is able to elicit pulmonary vasoconstriction[18,25] and phenylephrine-induced contractions of PAs could be inhibited partially by the nonselectively cation channel blockers SKF-96365 and Ni^{2+},[25,26] it is unclear which subtypes of TRPC channels are participating and what contributions TRPC channels have in a particular agonist-induced pulmonary vasoconstriction. In fact, our preliminary observations suggested that agonist-induced vasoconstriction was unabated in PAs of *trpc1*$^{-/-}$ and *trpc6*$^{-/-}$ mice (Ref. [27] and unpublished data), similar to a previous report on phenylephrine-induced vasoconstriction in aorta and mesenteric arteries of *trpc6*$^{-/-}$ mice.[28] It is likely that agonists activate vasoconstriction through redundant mechanisms; hence, deletion of one TRP channel subtype could be

effectively compensated by other Ca^{2+} pathways. Moreover, 5-hydroxytryptamine or serotonin (5-HT)-induced constriction of small, pressurized rat PAs is mediated through an arachidonic acid-sensitive Ca^{2+} influx,[29] which has properties similar to the TRPV4 channels.[30] These results highlight the fact that some agonists may induce ROCE and pulmonary vasoconstriction by activating NSCCs other than TRPC channels through PLC-independent signaling pathways.

3.3 Hypoxic Pulmonary Vasoconstriction

Acute reduction in alveolar O_2 tension causes reversible constriction of small resistant PAs. It serves as an adaptive mechanism for diverting blood flow from poorly ventilated to better ventilated regions of the lung to improve ventilation-perfusion matching. It has been proposed that hypoxia alters the redox state of PASMCs, leading to the inhibition of voltage-gated K^+ channels, membrane depolarization, activation of voltage-gated Ca^{2+} channels, $[Ca^{2+}]_i$ increase, and vasoconstriction.[31] Increasing evidence suggests that voltage-independent Ca^{2+} entry also plays a critical role in hypoxic pulmonary vasoconstriction (HPV). It was shown first in canine PAs that hypoxia activated a nisoldipine- and ryanodine-insensitive Ca^{2+} influx in the presence of thapsigargin or CPA[32] and later in small rat PAs that the initial phase (phase I) of HPV was carried by capacitative Ca^{2+} influx related to a thapsigargin-sensitive store. Phase II contraction was supported by Ca^{2+} influx through a separate voltage-independent pathway.[33] In isolated canine and rat PASMCs, hypoxia activated a dihydropyridine-insensitive Ca^{2+} influx, which had a pharmacological profile similar to SOCE.[34,35] Antagonists of SOCC/NSCC, including SKF-96365, Ni^{2+}, and La^{3+}, inhibited HPV in isolated perfused rat lungs at concentrations that inhibit vasoconstrictions induced by SOCE but not by KCl.[36]

A study showed that the expression levels of STIM1, TRPC1, TRPC4, and TRPC6 were higher in distal than proximal PAs of rats. Enhanced expression of these proteins was associated with a higher magnitude of CPA-induced SOCE and hypoxia-induced Ca^{2+} response in distal PASMCs.[5] Moreover, knockdown of the SR Ca^{2+} sensor STIM1 with siRNA in rat PASMCs abolished SOCE and hypoxia-induced Ca^{2+} response.[37] Collectively speaking, evidence at the organ, tissue, and cell levels suggests unequivocally that SOCCs/NSCCs participate in HPV, even though the specific TRP channel has not been determined. These observations together with other studies lead to an alternative hypothesis that HPV activates Ca^{2+} release from ryanodine receptor-gated Ca^{2+} stores, causing activation of SOCE.

Direct evidence of a critical role for TRP channels in HPV emerged from the study of *trpc6*$^{-/-}$ mice.[19] The evidence includes the following: The acute phase of HPV was completely absent in isolated perfused lungs of *trpc6*$^{-/-}$ mice; the hypoxia-induced increase in $[Ca^{2+}]_i$, nonselective cation influx, and membrane currents were missing in Endothelin-1 (ET-1)-primed *trpc6*$^{-/-}$ microvascular PASMCs; and hypoxia-induced Ca^{2+} response was rescued by expressing TRPC6 in *trpc6*$^{-/-}$ PASMCs.

It is intriguing that the hypoxia-induced activation of TRPC6 channels required priming of PASMCs with ET-1 and was mediated by DAG accumulation. These observations are congruent with the widely accepted knowledge that priming of isolated perfused lung, PAs, or PASMCs with an agonist facilitates robust hypoxic responses, but the DAG-mediated activation of TRPC6 indicates a crucial contribution of ROCE instead of the previously suggested SOCE in HPV. It is unclear whether the discrepancy depends on animal species or the presence of priming. It will be important to evaluate the DAG/TRPC6 mechanism in agonist-primed PASMCs of rats and other species for a better understanding of ROCE involvement in HPV.

The TRPC channels may contribute to HPV by providing Ca^{2+} for direct activation of calmodulin/myosin light chain kinase/actin-myosin interactions and by causing membrane depolarization to activate Ca^{2+} influx through L-type Ca^{2+} channels. This is consistent with the fact that both antagonists of SOCE/NSCC and L-type Ca^{2+} channels attenuated independently the hypoxia-induced Ca^{2+} response in PASMCs and HPV in isolated perfused lungs.[34,36] Moreover, hypoxia-induced activation of TRPC channels may lead to local accumulation of Na^{+} ions, facilitating Ca^{2+} influx through reverse Na^{+}–Ca^{2+} exchange. However, this possibility is still controversial and requires further investigation.

3.4 PASMC Growth and Proliferation

Mitogen-induced proliferation of PASMCs requires elevation of $[Ca^{2+}]_i$ to activate Ca^{2+}-dependent signaling pathways and Ca^{2+}-sensitive transcription factors. Based on a series of articles published by Yuan and associates, it is now established that PASMC proliferation is associated with augmented SOCE mediated by TRPC channel upregulation.[38] Depending on species, mitogens, and physiological states, most of the TRPC subtypes expressed in PASMCs, including TRPC1, TRPC3, TRPC4, and TRPC6, have been implicated as a mediator for cell proliferation. In human PASMCs, cell proliferation induced by serum and growth factors was associated with increased resting $[Ca^{2+}]_i$, enhanced capacitative Ca^{2+} entry, and upregulation of TRPC1.[17,39] Downregulation of TRPC1 using antisense oligonucleotide, removal of extracellular Ca^{2+}, and application of Ni^{2+} all inhibited the enhancement of cell proliferation induced by serum and growth factors, suggesting Ca^{2+} influx via the upregulated TRPC1 mediates PASMC growth. In another study, incubation of human PASMCs with ATP increased phosphorylation of cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB), TRPC4 expression, SOCE, and cell proliferation. Transfection of a CREB mutant abolished ATP-induced TRPC4 upregulation, and introduction of a siRNA against TRPC4 attenuated SOCE and cell proliferation activated by ATP. These data suggested that ATP exerts its mitogenic effect via CREB-dependent upregulation of TRPC4 channels. Furthermore, PDGF stimulates Signal transducer

and activator of transcription 3 (STAT3) phosphorylation, leading to the upregulation of c-Jun, which activates the transcription of TRPC6, resulting in enhanced SOCE and PASMC proliferation.[40,41]

It is interesting to note that the mentioned studies involved different mitogens, signaling pathways, and TRPC channels; yet they all found enhancement in SOCE and cell proliferation. The simplest explanation is that an enhanced SOCE, irrespective of the TRPC subtype involved, supports the mitogen-induced PASMC proliferation in a nondiscriminatory manner. However, Ca^{2+} signals carried by distinctive Ca^{2+} channels/pathways are known to preferentially regulate specific Ca^{2+}-sensitive transcription factors for different physiological processes, according to the signal amplitude and frequency as well as the spatial association with their effectors.[42] Indeed, PGDF-induced PASMC proliferation was unaffected by the L-type Ca^{2+} channel inhibitor nifedipine but was inhibited by the SOCE/NSCC inhibitor SKF-96365, suggesting a specific contribution of SOCE-dependent signaling pathways.[41] Hence, it is tempting to speculate that various mitogens might regulate different TRPC subtypes to modulate specific Ca^{2+}-dependent processes at various stages in the cell cycle.

3.5 Pulmonary Arterial Hypertension

Pulmonary arterial hypertension (PAH), both idiopathic (IPAH) and secondary PAH, involves numerous interacting factors that lead to massive vascular remodeling, increase in vasomotor tone, and alterations in vascular reactivity. Vascular remodeling is characterized by pronounced medial and adventitial thickening due to PASMC proliferation and migration, recruitment and differentiation of progenitor cells, and synthesis of extracellular matrix. Increase in vasomotor tone is evident by the acute reduction in pulmonary arterial pressure (Ppa) in response to vasodilators, such as prostacyclin, nitric oxide, phosphodiesterase V inhibitors, K^+ channel opener, and Rho kinase inhibitors. Alterations in vascular reactivity are manifested by the enhanced responsiveness to agonists, such as endothelin 1, serotonin, and angiotensin II, and the reduced relaxation to various vasodilators.

3.5.1 Chronic Hypoxia-Induced Pulmonary Hypertension

All the salient characteristics of PAH can be either directly or indirectly related to alterations of Ca^{2+} homeostasis in PASMCs. It has been shown in the chronic hypoxic rat model that major changes in ionic balance occur in PASMCs, including membrane depolarization, reduction in K_V currents, and increase in resting $[Ca^{2+}]_i$. The increase in resting $[Ca^{2+}]_i$ in PASMCs is mainly due to Ca^{2+} influx through voltage-independent Ca^{2+} channels because removal of extracellular Ca^{2+} reduces resting $[Ca^{2+}]_i$ to the level of normoxic PASMCs, but inhibition of the voltage-gated Ca^{2+} channel with nifedipine has little effect.[16,43] In the search of alternative

Ca^{2+} entry pathways for the disturbance of resting $[Ca^{2+}]_i$, we found that the expression of TRPC channels in PAs is greatly altered in rats exposed to 10% O_2 for 4 weeks.[16] TRPC1 and TRPC6 mRNA and protein levels were more than doubled, while TRPC3 was unchanged in hypoxic PAs. Ca^{2+} measurements and Mn^{2+}-quenching experiments showed that both thapsigargin-induced SOCE and OAG-induced ROCE were enhanced proportionally in hypoxic PASMCs. More importantly, inhibition of SOCE with a low concentration of La^{3+} (10 μ*M*) caused a reduction in basal $[Ca^{2+}]_i$ similar to the removal of extracellular Ca^{2+}, but increased La^{3+} concentration to inhibit ROCE failed to cause additional decline in $[Ca^{2+}]_i$. Similar results were obtained in parallel experiments using PA rings to evaluate basal vascular tone. Since TRPC1 is responsible for SOCE in rat PASMCs,[16] these results suggested that the upregulation of TRPC1 and SOCE contribute to the elevated $[Ca^{2+}]_i$ in PASMCs and the increase in basal vascular tone of PAs of chronic hypoxic rats.

A subsequent study further extended our findings showing that TRPC1 and TRPC6 upregulation also occurred in cultured PASMCs incubated under 4% O_2 for 60 h, and in nonhypoxic PASMCs overexpressing hypoxia-inducible factor 1α (HIF-1α).[44] The increase of TRPC expression was absent when mice of partial HIF-1α deficiency were exposed to hypoxia. These results clearly indicate that the upregulation of TRPC channels is a direct effect of hypoxia on PASMCs mediated by HIF-1α.

However, several important questions remain to be answered. Are TRPC1 and TRPC6 essential for the development of hypoxic PAH? Are they required specifically for vascular remodeling, increased Ppa, and enhanced vasoconstriction to agonists in PAH? It had been reported that the elevation of right ventricular systolic pressure (RVSP), right heart hypertrophy, and pulmonary vascular remodeling were unaltered in chronic hypoxic *trpc6*$^{-/-}$ mice.[19] This argued against an indispensable role for TRPC6 in chronic hypoxia-induced PAH. In contrast, our preliminary study found that hypoxia-induced PAH was significantly attenuated in *trpc1*$^{-/-}$ mice.[27] This further supports the idea that the store-operated TRPC1 channels participate in the development of hypoxic PAH.

We recently have extended our investigation on the roles of TRPM and TRPV channels in chronic hypoxic PAH.[45] Preliminary experiments showed that among the six subtypes of TRPV and eight subtypes of TRPM, the mechanosensitive TRPV4 was the only channel that was upregulated in PAs of rats exposed to hypoxia. Upregulation of TRPV4 was associated with enhanced Ca^{2+} response induced by the TRPV4 agonist 4α-PDD and hypotonicity in hypoxic PASMCs. Significant myogenic tone, sensitive to the TRPV blocker ruthenium red, was also observed in pressurized pulmonary microvessels of chronic hypoxic but not normoxic rats. Moreover, the severity of PAH was significantly attenuated in *trpv4*$^{-/-}$ mice exposed to hypoxia. These results suggest that the mechanosensitive TRPV4 channels are upregulated in PASMCs during chronic hypoxia and play significant roles in the development of myogenic tone and PAH. A schematic is presented in Fig. 7.1 to depict our present understandings on the participation of TRPC and TRPV channels in chronic hypoxia-induced PAH.

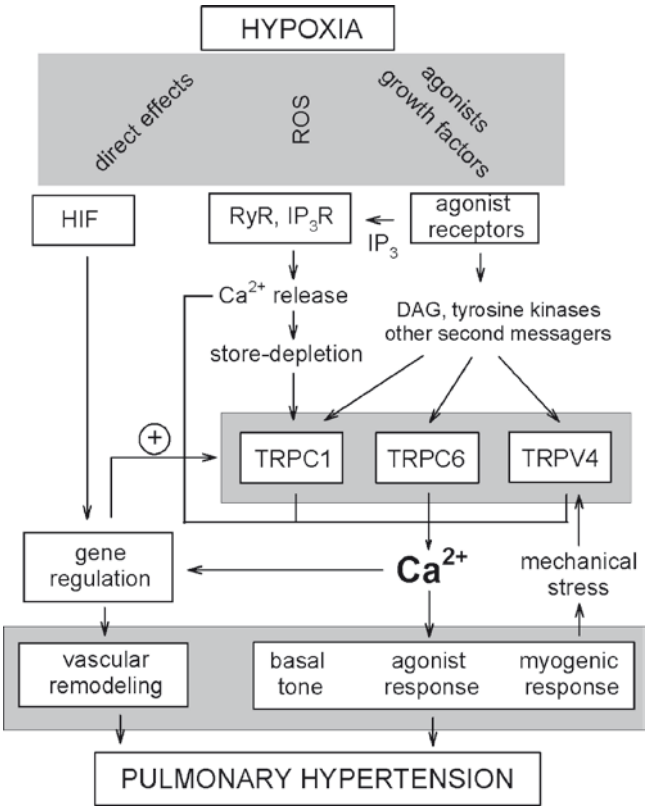

Fig. 7.1 Schematic diagram depicting the involvement of TRP channels in chronic hypoxic PAH. Hypoxia activates release of ROS (reactive oxygen species), agonists, and growth factors, leading to SOCE, ROCE, and Non-selective cation entry (NSCE) through TRPC1, TRPC6, and TRPV4 channels, respectively. Increase in intracellular $[Ca^{2+}]_i$ enhances basal vascular tone and agonist-induced responses to cause pulmonary vasoconstriction. Increase in Ppa activates mechanosensitive TRPV4 channels to elicit myogenic response. Furthermore, HIF and other Ca^{2+}-dependent transcriptional factors mediate gene transcription to upregulate TRPC1, TRPC6, and TRPV4 expression and promote cell proliferation and vascular remodeling

3.5.2 Idiopathic Pulmonary Arterial Hypertension

In addition to chronic hypoxic PAH, a link has also been established between TRPC and IPAH. Elevated levels of TRPC6 and TRPC3 mRNA and protein were found in cultured PASMCs isolated from patients with IPAH but not in PASMCs of patients with secondary PAH or those who were not hypertensive.[46] The enhanced TRPC expression appears to be responsible for the higher activity of SOCE and cell proliferation in IPAH PASMCs[21,41,46] because downregulation of TRPC6 by siRNA blocked the accelerated proliferation of these cells.[46] The underlying cause for the enhanced TRPC expression in IPAH PASMCs is unclear. It is unrelated to mitogenic

or autocrine factors released during PAH because elevated TRPC expression was observed in cells after multiple passages. However, it could be a specific phenotype of a population of PASMCs or myofibroblasts that are selectively expanded or recruited during the development of IPAH. It is important to note that the ET receptor antagonist bosentan suppressed TRPC6 expression and proliferation of IPAH PASMCs in the absence of agonist[41]; and PASMCs from normal subjects and patients with IPAH showed divergence in response to cAMP-dependent regulation on TRPC3 expression and SOCE.[21] These observations suggest that aberrations in the constitutive activities of endogenous receptors and transcriptional pathways may contribute to the enhanced TRPC expression in IPAH PASMCs.

4 Conclusion

Ever since the discovery of the first TRP channel 20 years ago,[1] TRP channels have continuously amazed the scientific community by their incredibly diverse complexity in structural interactions, modes of activation, molecular regulations, and physiological functions. Novel discoveries about these channels have been reported in a daily basis (>400 papers in 2008). In comparison, research on TRP channels in pulmonary vasculature is still in its infancy. The functions and regulations of most TRP channels of PASMCs, especially members of TRPV, TRPM, TRPA, TRPP, and TRPML subfamilies, have not been explored. Since Ca^{2+} ions regulate multiple cellular processes via specific Ca^{2+} signals from diverse pathways, future investigations of these unexplored TRP channels will likely uncover important regulatory pathways for pulmonary vascular functions in health and in disease states.

References

1. Montell C, Rubin GM (1989) Molecular characterization of the *Drosophila* trp locus: a putative integral membrane protein required for phototransduction. Neuron 2:1313–1323
2. Venkatachalam K, Montell C (2007) TRP channels. Annu Rev Biochem 76:387–417
3. Inoue R, Jensen LJ, Shi J et al (2006) Transient receptor potential channels in cardiovascular function and disease. Circ Res 99:119–131
4. Walker RL, Hume JR, Horowitz B (2001) Differential expression and alternative splicing of TRP channel genes in smooth muscles. Am J Physiol Cell Physiol 280:C1184–C1192
5. Lu W, Wang J, Shimoda LA, Sylvester JT (2008) Differences in STIM1 and TRPC expression in proximal and distal pulmonary arterial smooth muscle are associated with differences in Ca^{2+} responses to hypoxia. Am J Physiol Lung Cell Mol Physiol 295:L104–L113
6. Yang XR, Lin MJ, McIntosh LS, Sham JSK (2006) Functional expression of transient receptor potential melastatin- and vanilloid-related channels in pulmonary arterial and aortic smooth muscle. Am J Physiol Lung Cell Mol Physiol 290:L1267–L1276
7. Putney JW Jr, Broad LM, Braun FJ, Lievremont JP, Bird GS (2001) Mechanisms of capacitative calcium entry. J Cell Sci 114:2223–2229
8. Roos J, DiGregorio PJ, Yeromin AV et al (2005) STIM1, an essential and conserved component of store-operated Ca^{2+} channel function. J Cell Biol 169:435–445

9. Brandman O, Liou J, Park WS, Meyer T (2007) STIM2 is a feedback regulator that stabilizes basal cytosolic and endoplasmic reticulum Ca^{2+} levels. Cell 131:1327–1339
10. McDonald TV, Premack BA, Gardner P (1993) Flash photolysis of caged inositol 1,4,5-trisphosphate activates plasma membrane calcium current in human T cells. J Biol Chem 268:3889–3896
11. Abramowitz J, Birnbaumer L (2009) Physiology and pathophysiology of canonical transient receptor potential channels. FASEB J 23:297–328
12. Hofmann T, Schaefer M, Schultz G, Gudermann T (2002) Subunit composition of mammalian transient receptor potential channels in living cells. Proc Natl Acad Sci U S A 99:7461–7466
13. Schaefer M, Plant TD, Obukhov AG, Hofmann T, Gudermann T, Schultz G (2000) Receptor-mediated regulation of the nonselective cation channels TRPC4 and TRPC5. J Biol Chem 275:17517–17526
14. Huang GN, Zeng W, Kim JY et al (2006) STIM1 carboxyl-terminus activates native SOC, I_{CRAC} and TRPC1 channels. Nat Cell Biol 8:1003–1010
15. Yuan JP, Zeng W, Huang GN, Worley PF, Muallem S (2007) STIM1 heteromultimerizes TRPC channels to determine their function as store-operated channels. Nat Cell Biol 9:636–645
16. Lin MJ, Leung GP, Zhang WM et al (2004) Chronic hypoxia-induced upregulation of store-operated and receptor-operated Ca^{2+} channels in pulmonary arterial smooth muscle cells: a novel mechanism of hypoxic pulmonary hypertension. Circ Res 95:496–505
17. Sweeney M, Yu Y, Platoshyn O, Zhang S, McDaniel SS, Yuan JX-J (2002) Inhibition of endogenous TRP1 decreases capacitative Ca^{2+} entry and attenuates pulmonary artery smooth muscle cell proliferation. Am J Physiol Lung Cell Mol Physiol 283:L144–L155
18. Kunichika N, Yu Y, Remillard CV, Platoshyn O, Zhang S, Yuan JX-J (2004) Overexpression of TRPC1 enhances pulmonary vasoconstriction induced by capacitative Ca^{2+} entry. Am J Physiol Lung Cell Mol Physiol 287:L962–L969
19. Weissmann N, Dietrich A, Fuchs B et al (2006) Classical transient receptor potential channel 6 (TRPC6) is essential for hypoxic pulmonary vasoconstriction and alveolar gas exchange. Proc Natl Acad Sci U S A 103:19093–19098
20. Liao Y, Erxleben C, Abramowitz J et al (2008) Functional interactions among Orai1, TRPCs, and STIM1 suggest a STIM-regulated heteromeric Orai/TRPC model for SOCE/I_{CRAC} channels. Proc Natl Acad Sci U S A 105:2895–2900
21. Zhang S, Patel HH, Murray F et al (2007) Pulmonary artery smooth muscle cells from normal subjects and IPAH patients show divergent cAMP-mediated effects on TRPC expression and capacitative Ca^{2+} entry. Am J Physiol Lung Cell Mol Physiol 292:L1202–L1210
22. Zhang S, Remillard CV, Fantozzi I, Yuan JX-J (2004) ATP-induced mitogenesis is mediated by cyclic AMP response element-binding protein-enhanced TRPC4 expression and activity in human pulmonary artery smooth muscle cells. Am J Physiol Cell Physiol 287:C1192–C1201
23. Hisatsune C, Kuroda Y, Nakamura K et al (2004) Regulation of TRPC6 channel activity by tyrosine phosphorylation. J Biol Chem 279:18887–18894
24. Kawasaki BT, Liao Y, Birnbaumer L (2006) Role of Src in C3 transient receptor potential channel function and evidence for a heterogeneous makeup of receptor- and store-operated Ca^{2+} entry channels. Proc Natl Acad Sci U S A 103:335–340
25. McDaniel SS, Platoshyn O, Wang J et al (2001) Capacitative Ca^{2+} entry in agonist-induced pulmonary vasoconstriction. Am J Physiol Lung Cell Mol Physiol 280:L870–L880
26. Doi S, Damron DS, Horibe M, Murray PA (2000) Capacitative Ca^{2+} entry and tyrosine kinase activation in canine pulmonary arterial smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 278:L118–L130
27. Yang XR, Cao YN, Birnbaumer L, Sham JSK (2008) TRPC1 channels contributes to hypoxic pulmonary hypertension and right heart hypertrophy: evidence from TRPC1 knockout mice. Am J Respir Crit Care Med 177:A534
28. Dietrich A, Mederos YSM, Gollasch M et al (2005) Increased vascular smooth muscle contractility in TRPC6$^{-/-}$ mice. Mol Cell Biol 25:6980–6989
29. Guibert C, Marthan R, Savineau JP (2004) 5-HT induces an arachidonic acid-sensitive calcium influx in rat small intrapulmonary artery. Am J Physiol Lung Cell Mol Physiol 286:L1228–L1236

30. Ducret T, Guibert C, Marthan R, Savineau JP (2008) Serotonin-induced activation of TRPV4-like current in rat intrapulmonary arterial smooth muscle cells. Cell Calcium 43:315–323
31. Archer SL, Huang J, Henry T, Peterson D, Weir EK (1993) A redox-based O_2 sensor in rat pulmonary vasculature. Circ Res 73:1100–1112
32. Jabr RI, Toland H, Gelband CH, Wang XX, Hume JR (1997) Prominent role of intracellular Ca^{2+} release in hypoxic vasoconstriction of canine pulmonary artery. Br J Pharmacol 122:21–30
33. Robertson TP, Hague D, Aaronson PI, Ward JP (2000) Voltage-independent calcium entry in hypoxic pulmonary vasoconstriction of intrapulmonary arteries of the rat. J Physiol 525:669–680
34. Wang J, Shimoda LA, Weigand L, Wang W, Sun D, Sylvester JT (2005) Acute hypoxia increases intracellular [Ca^{2+}] in pulmonary arterial smooth muscle by enhancing capacitative Ca^{2+} entry. Am J Physiol Lung Cell Mol Physiol 288:L1059–L1069
35. Ng LC, Wilson SM, Hume JR (2005) Mobilization of sarcoplasmic reticulum stores by hypoxia leads to consequent activation of capacitative Ca^{2+} entry in isolated canine pulmonary arterial smooth muscle cells. J Physiol 563:409–419
36. Weigand L, Foxson J, Wang J, Shimoda LA, Sylvester JT (2005) Inhibition of hypoxic pulmonary vasoconstriction by antagonists of store-operated Ca^{2+} and nonselective cation channels. Am J Physiol Lung Cell Mol Physiol 289:L5–L13
37. Lu W, Wang J, Shimoda LA, Sylvester JT (2008) Knockdown of stromal interaction molecule 1 (STIM-1) decreases store-operated calcium entry (SOCE) and attenuates hypoxic calcium response in pulmonary artery smooth muscle cells (PASMC). FASEB J 22:1213.4
38. Landsberg JW, Yuan JX-J (2004) Calcium and TRP channels in pulmonary vascular smooth muscle cell proliferation. News Physiol Sci 19:44–50
39. Golovina VA, Platoshyn O, Bailey CL et al (2001) Upregulated TRP and enhanced capacitative Ca^{2+} entry in human pulmonary artery myocytes during proliferation. Am J Physiol Heart Circ Physiol 280:H746–H755
40. Yu Y, Sweeney M, Zhang S et al (2003) PDGF stimulates pulmonary vascular smooth muscle cell proliferation by upregulating TRPC6 expression. Am J Physiol Cell Physiol 284:C316–C330
41. Kunichika N, Landsberg JW, Yu Y et al (2004) Bosentan inhibits transient receptor potential channel expression in pulmonary vascular myocytes. Am J Respir Crit Care Med 170:1101–1107
42. Dolmetsch R (2003) Excitation–transcription coupling: signaling by ion channels to the nucleus. Sci STKE 2003:PE4
43. Shimoda LA, Sham JSK, Shimoda TH, Sylvester JT (2000) L-type Ca^{2+} channels, resting $[Ca^{2+}]_i$, and ET-1-induced responses in chronically hypoxic pulmonary myocytes. Am J Physiol Lung Cell Mol Physiol 279:L884–L894
44. Wang J, Weigand L, Lu W, Sylvester JT, Semenza GL, Shimoda LA (2006) Hypoxia inducible factor 1 mediates hypoxia-induced TRPC expression and elevated intracellular Ca^{2+} in pulmonary arterial smooth muscle cells. Circ Res 98:1528–1537
45. Yang XR, Hughes JM, Cao YN, Flavahan NA, Liedtke W, Sham JSK (2008) Upregulation of TRPV4 channels in pulmonary arteries (PAs) contribute to chronic hypoxia induced myogenic tone and pulmonary hypertension. FASEB J 22:1213.5 (abstract)
46. Yu Y, Fantozzi I, Remillard CV et al (2004) Enhanced expression of transient abstract receptor potential channels in idiopathic pulmonary arterial hypertension. Proc Natl Acad Sci U S A 101:13861–13866
47. Ng LC, Gurney AM (2001) Store-operated channels mediate Ca^{2+} influx and contraction in rat pulmonary artery. Circ Res 89:923–929
48. Wang J, Shimoda LA, Sylvester JT (2004) Capacitative calcium entry and TRPC channel proteins are expressed in rat distal pulmonary arterial smooth muscle. Am J Physiol Lung Cell Mol Physiol 286:L848–L858
49. McElroy SP, Gurney AM, Drummond RM (2008) Pharmacological profile of store-operated Ca^{2+} entry in intrapulmonary artery smooth muscle cells. Eur J Pharmacol 584:10–20
50. Rodat L, Savineau JP, Marthan R, Guibert C (2007) Effect of chronic hypoxia on voltage-independent calcium influx activated by 5-HT in rat intrapulmonary arteries. Pflügers Arch 454:41–51

The Contribution of TRPC1 and STIM1 to Capacitative Ca^{2+} Entry in Pulmonary Artery

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Abstract Capacitative calcium entry (CCE) through store-operated channels (SOCs) has been shown to contribute to the rise in intracellular calcium concentration ($[Ca^{2+}]_i$) and mediate pulmonary artery smooth muscle contraction. CCE is activated as a result of depletion of intracellular Ca^{2+} stores but there is a great deal of controversy surrounding the underlying signal that active CCE and the molecular makeup of SOCs. The discovery of canonical subgroup of transient receptor potential channels (TRPC) and recent identification of stromal-interacting molecule 1 (STIM1) protein have opened a door to the study of the identity of SOCs and the signal that activates these channels. Among all the TRPC channels, TRPC1 is widely studied in many cell types and shown to be part of SOCs components, whereas STIM1 protein is found to act as a Ca^{2+} sensor in the intracellular Ca^{2+} stores and activates SOCs. However, there is very little evidence for the roles of TRPC1 and STIM1 in the contribution of CCE in pulmonary artery. This chapter outlines the roles of TRPC1 and STIM1 in pulmonary artery smooth muscle cells and discusses our recent findings that TRPC1 and STIM1 are functionally interact with each other to mediate CCE in these cells. We also propose a model for the molecular makeup of SOCs formed by TRPC1 and STIM1 in pulmonary artery.

Keywords TRPC1 • STIM1 • capacitative calcium entry • store-operated channels • pulmonary artery

1 Introduction

Capacitative Ca^{2+} entry (CCE) or store-operated Ca^{2+} entry was first proposed by Putney in 1986: Depletion of intracellular Ca^{2+} stores leads to the activation of Ca^{2+} entry from the extracellular space to reload the stores.[1] The stores serve as a

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capacitor, and much as in an electrical circuit, a capacitor must be charged before current can flow through it.[1] CCE can be activated by any procedure that causes depletion of Ca^{2+} from the intracellular Ca^{2+} stores. This includes agonists activating receptors coupled to the inositol 1,4,5-trisphosphate (IP_3) signaling pathway, intracellular dialysis of high concentrations of Ca^{2+} chelators Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) or 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), thereby preventing store refilling by chelating Ca^{2+} that leaks from the stores or using agents that inhibit the sarcoplasmic reticulum (SR)/endoplasmic reticulum Ca^{2+}-ATPase (adenosine triphosphatase) (SERCA), such as cyclopiazonic acid (CPA) or thapsigargin.[2] The concept of CCE was first confirmed by electrophysiological evidence in mast cells, in which store depletion caused activation of a Ca^{2+} current, so-called Ca^{2+}-release activated Ca^{2+} current (I_{CRAC}).[3] I_{CRAC} is voltage independent, inwardly rectifying, and highly selective to Ca^{2+} and has a very low unitary conductance of 0.02 pS. It is now accepted that I_{CRAC} is a predominant Ca^{2+} entry pathway in nonexcitable cells, but it is not the only store-operated current.[2] Many studies have reported a variety of store-operated currents with a range of channel conductances (>1 pS), along with varying Ca^{2+} selectivity in both nonexcitable and excitable cells.[2]

Unlike nonexcitable cells, Ca^{2+} entry in excitable cells such as vascular smooth muscle cells is generally accomplished by voltage-operated Ca^{2+} channels (VOCCs) or receptor-operated channels (ROCs). However, increasing evidence has shown that Ca^{2+} entry through store-operated nonselective cation channels (SOCs) contributes to the rise in $[Ca^{2+}]_i$ and responsible for vascular smooth muscle contraction.[4] To date, there are only a few studies that showed the recording of store-operated currents in vascular smooth muscle cells. In cell-attached patches of cultured aorta smooth muscle cells, a 3-pS cation-conducting channel activated by thapsigargin or the cell-permeant Ca^{2+} chelator BAPTA-AM (acetoxymethyl ester) was reported by Trepakova et al.[5] In cell-attached patches of cultured human pulmonary artery smooth muscle cells (PASMCs), Golovina et al.[6] reported a 5.4-pS Ca^{2+}-permeable channel that was activated by CPA. In rabbit portal vein cells, CPA, caffeine, BAPTA-AM, or calmodulin antagonist W-7 activated SOCs with a single-channel conductance of 2.1 pS.[7] Following this observation, Saleh et al.[8,9] also recorded 2.6- and 1.9-pS SOC in coronary artery and mesenteric artery smooth muscle cells, respectively. It is not clear whether the single-channel events described in these studies were indeed due to SOCs as opposed to another Ca^{2+} entry pathway because Ca^{2+} release from the stores may activate other nonselective cation channels; even in cells loaded with BAPTA-AM, it has not always been shown that sufficient BAPTA has accumulated in the cytosol to suppress the rise in intracellular Ca^{2+} following store depletion.[2] Perhaps the more convincing evidence for the existence of SOCs in vascular smooth muscle cells comes from the whole-cell recording of rat PASMCs[10] and human saphenous vein cells.[11] In the former study, a CPA-induced inward current was recorded when cells were internally dialyzed with 10 m*M* EGTA or 10 m*M* BAPTA. The latter study showed that thapsigargin activated lanthanum-sensitive currents in cells dialyzed with 40 m*M* EGTA. However, the single-channel con-

ductance for SOCs has not been reported in these studies. Thus, the electrophysiological evidence for SOCs in vascular smooth muscle cells remains to be elucidated.

2 Molecular Composition and Molecular Signals that Underlie Store-Operated Channels in Vascular Smooth Muscle

Over the past decade, CCE has gained a significant amount of attention in vascular smooth muscle research.[5–15] However, the molecular composition of SOCs and the intracellular signals that activate these channels remain unclear. The discovery of mammalian homologues of *Drosophila* transient receptor potential (TRP) gene that encode 28 nonselective cation channel proteins with varying permeability to Ca^{2+} has led to a plethora of studies on the possible role of TRP channels underlying SOCs and ROCs. Since 1995, increasing evidence indicated members of the canonical subgroup of transient receptor potential nonselective cation channel (TRPC) constitute tetramers of both ROCs and SOCs.[2,16,17] Based on the structural and functional similarities, members of the TRPC family are divided into four subgroups: TRPC1, TRPC2, TRPC3/6/7, and TRPC4/5. These TRPC members have all been suggested as components of SOCs, but there is also evidence that they are components of ROCs.[16] This could be explained by the findings that different TRPC subunits can associate to form heterotetramers in different cell types. In general, TRPC1, 4, and 5 are sensitive to store depletion and interact with each other to function as SOCs, whereas TRPC3, 6, and 7 can interact with each other and function as ROCs that are gated by G protein-phospholipase C and diacylglycerol.[16] TRPC2 is a pseudogene in humans.

Several studies have confirmed the existence of TRPC channels in various vascular preparations.[4,17] However, there is little evidence that TRPC channels function as SOCs in vascular smooth muscle cells. Using inhibitory antibodies, antisense, and small-interfering RNA (siRNA) methods, several studies have presented evidence for TRPC1 as an essential component for SOCs in vascular smooth muscle cells from the aorta,[18,19] cerebral artery,[20] mesenteric artery,[8] coronary artery,[9,21] and portal vein.[7,9] Interestingly, TRPC1 and TRPC5 have been shown to colocalize and associate with one another in the rabbit pial arteriole,[22] and antibodies directed against TRPC1 and TRPC5 were shown to inhibit store-operated currents in mesenteric artery,[9] suggesting that TRPC1/TRPC5 may form heterotetramers in vascular smooth muscle. TRPC1, 5, and 6 were suggested to form SOCs in rabbit coronary artery, and TRPC1, 5, and 7 were suggested to form SOCs in rabbit portal vein.[9] This finding goes against the original principle that TRPC1, 4, or 5 and TRPC3, 6, or 7 can only interact with each other within the group to form heterotetramers and function as ion channels.[23,24] However, it has also been proposed that functioning cation channels may be composed of subunits from both of these groups.[25–27] Thus, comparison of the proposed heterotetrameric structures in expression systems with native SOCs is required to precisely identify the molecular makeup of endogenous SOCs.

While the molecular identity of SOCs is incomplete, the molecular signals that activate these channels also remain unresolved. Many studies have been performed since 1990 to examine the molecular signals that activate SOCs.[2] These include (1) the generation of a "Ca^{2+} influx factor" from the SR after store depletion, which induces activation of Ca^{2+}-independent phospholipase A_2, leading to the activation of SOCs; (2) conformational coupling of the SR IP_3 receptors with SOCs on the cell membrane; and (3) fusion of vesicles containing SOCs in the cell membrane, leading to the increase in channel number in the membrane. However, none of these hypotheses is unequivocally accepted for the activation of SOCs.[2] The discovery of STIM1 (stromal interacting molecule 1) has opened a new direction toward the search for a molecular intermediate involved in the activation of SOCs. STIM1 was found to act as a sensor within the stores[28,29] and may play a role in the plasma membrane[29,30] to activate I_{CRAC}. To date, there is little information on the role of STIM1 in vascular smooth muscle cells. STIM1 messenger RNA (mRNA) was shown to be expressed in cultured human coronary artery smooth muscle cells,[31] mouse aorta smooth muscle cells,[32] and human saphenous vein cells,[11] and siRNA targeting STIM1 resulted in reduction of Ca^{2+} entry and whole-cell current activated by CPA or thapsigargin.[11,31,33] These results suggest an important role of STIM1 in mediating CCE in vascular smooth muscle cells. Interestingly, In human embryonic kidney 293 (HEK293) cells, STIM1 was found to bind to TRPC1, TRPC4, and TRPC5 and directly regulate these channels, whereas the regulation of TRPC3 and TRPC6 by STIM1 was mediated by STIM1-dependent heteromultimerization of TRPC3 with TRPC1 and TRPC6 with TRPC4.[34] Therefore, the notion that STIM1 interacts with various TRPC channels to mediate CCE cells has emerged as an important focus in vascular research.

3 Role of TRPC1 as an Essential Component for Store-Operated Channels in Pulmonary Artery

The TRPC channels, except TRPC2 have been found in pulmonary vascular preparations.[10,14,35–37] However, there is little evidence for the functional significance of these channels in pulmonary vascular research. Because of the relatively abundant expression of TRPC1 in PASMCs, the functional role of TRPC1 has been widely studied compared to other TRPC channels. There is increasing evidence that TRPC1 functions as SOCs in pulmonary arteries. In human PASMCs, CCE is enhanced in proliferative cells,[38] and this is associated with an increase in TRPC1 expression in these cells.[6] In addition, TRPC1 gene expression and CCE were significantly reduced in human PASMCs treated with TRPC1 antisense.[39] Overexpression of human TRPC1 in rat pulmonary arteries enhanced the contractile responses to CPA.[40] Knockdown of TRPC1 protein with siRNA inhibited cation influx caused by thapsigargin in rat PASMCs,[41] further supports that TRPC1 is an important molecular candidate for SOCs in PASMCs. Our study in mouse PASMCs also suggested that TRPC1 is an essential component of SOCs in these cells.[42] We found that CPA caused an increase in nifedipine-insensitive transient and sustained

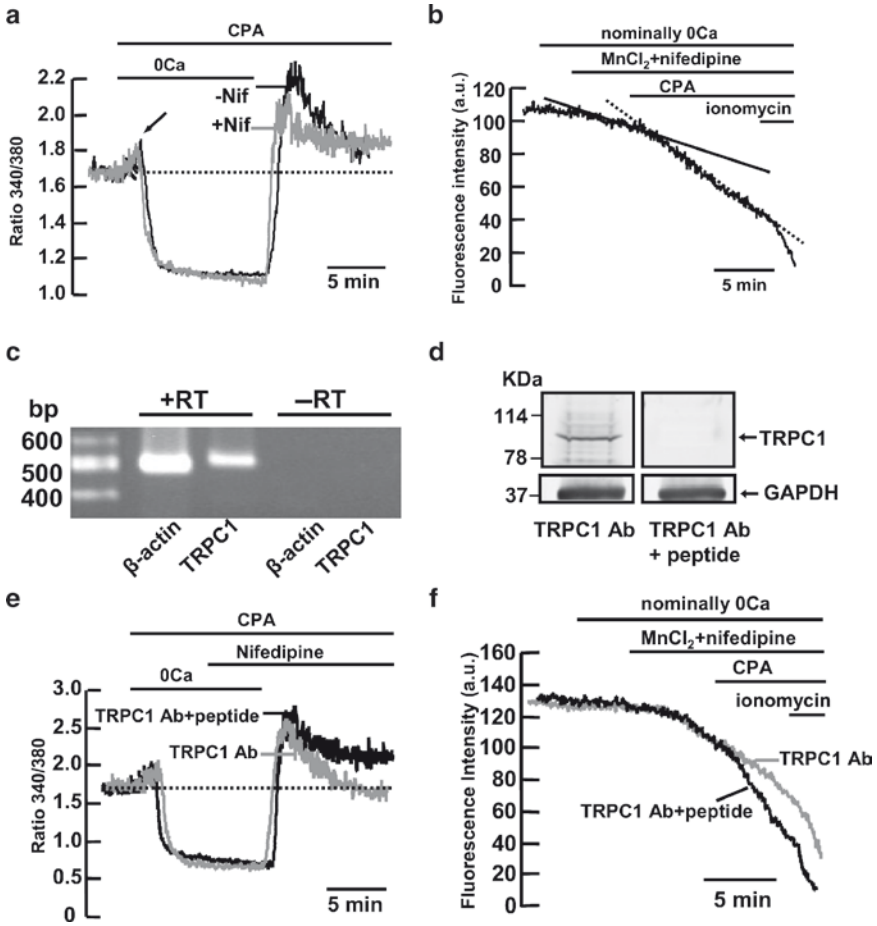

Fig. 8.1 TRPC1 mediates CCE in mouse PASMCs. (**a**) When applied in Ca^{2+}-free solution, depletion of intracellular stores with 10 μ*M* CPA transiently elevated the fura-2 fluorescence ratio, indicating Ca^{2+} release from the intracellular stores (*arrow*). Readdition of 2 m*M* Ca^{2+} in the continued presence of CPA caused a transient followed by a sustained increase in the fluorescence ratio. The transient but not the sustained component was reduced by 10 μ*M* nifedipine (*Nif*). (**b**) Depletion of intracellular Ca^{2+} stores with 10 μ*M* CPA increased the rate of Mn^{2+} quench of fura-2 fluorescence in the presence of 10 μ*M* nifedipine. (**c**) RT-PCR (reverse-transcription polymerase chain reaction) products from cultured mouse PASMCs amplified using primers for mouse TRPC1 (516 bp) and β-actin (498 bp). (**d**) TRPC1 protein and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were detected in cultured mouse PASMCs using Western blot analysis. A negative control was performed by preincubating TRPC1 antibody with the antigen peptide. (**e**) TRPC1 antibody (1:100) inhibited the CPA-induced sustained but not transient increase in fura-2 fluorescence ratio in the presence of 10 μ*M* nifedipine. (**f**) TRPC1 antibody (1:100) inhibited the increase in Mn^{2+} quench of fura-2 fluorescence caused by 10 μ*M* CPA in the presence of 10 μ*M* nifedipine. Modified with permission[42]

rise in $[Ca^{2+}]_i$ and an increase in Mn^{2+} quench of fura-2 fluorescence (Fig. 8.1). These increases in $[Ca^{2+}]_i$ and Mn^{2+} quench rate were due to CCE because they were activated by store depletion and blocked by SKF-96365, Ni^{2+}, La^{3+}, and Gd^{3+},[42]

a characteristic property of SOCs in many tissues, including pulmonary arteries.[10,12,14,15,36] We have also shown the expression of endogenous TRPC1 mRNA and protein in mouse PASMCs. Furthermore, the increase in dihydropyridine-insensitive sustained rise in $[Ca^{2+}]_i$ and the increase in Mn^{2+} quench rate caused by CPA were both inhibited by antibody raised against an extracellular epitope of TRPC1, suggesting an important role of TRPC1 in the contribution of CCE in mouse PASMCs.

4 STIM1 Mediates Capacitative Ca^{2+} Entry in Pulmonary Artery Smooth Muscle

STIM1 mRNA and protein were found to express in rat PASMCs.[37] However, the functional role of STIM1 in the activation of CCE in PASMCs remains unknown. We have studied the functional role of STIM1 in mouse PASMCs using the siRNA approach and overexpression methods.[42] We have shown that endogenous STIM1 mRNA and protein express in mouse PASMCs, and the expression level of STIM1 protein was reduced by siRNA knockdown of STIM1 mRNA (Fig. 8.2). We found that siRNA knockdown of STIM1 protein reduced the dihydropyridine-insensitive transient and sustained rise in $[Ca^{2+}]_i$ and cation influx activated by store depletion, and these responses to store depletion were enhanced in cells overexpressing STIM1. These data provide the first functional evidence that endogenous STIM1 contributes to CCE in PASMCs.

5 Functional Interaction of TRPC1 and STIM1 in Pulmonary Artery Smooth Muscle

So far, there is little evidence for the functional interaction between TRPC1 and STIM1 in vascular smooth muscle cells. Although TRPC1 was shown to be an essential component of SOCs in PASMCs,[39] aorta,[18,19] cerebral artery,[20] mesenteric artery,[8] and coronary artery[21] smooth muscle cells and STIM1 was found to mediate CCE in human airway smooth muscle cells,[33] cultured human coronary artery cells,[31] and mouse aorta smooth muscle cells,[32] none of these studies showed that STIM1 is functionally linked to TRPC1 in mediating CCE in the same cell. Only one study suggested STIM1 may interact with TRPC1, which was reported by Li et al.[11] in cultured human saphenous vein cells. They showed that thapsigargin-induced rise in $[Ca^{2+}]_i$ was inhibited by STIM1 antibody and STIM1 siRNA. On the other hand, they also showed that thapsigargin-induced rise in $[Ca^{2+}]_i$ was inhibited by TRPC1 antibody. However, they did not test whether STIM1 is functionally associated with TRPC1. To address this question, we have studied the effects of TRPC1 antibody in cells overexpressing STIM1.[42] We found that

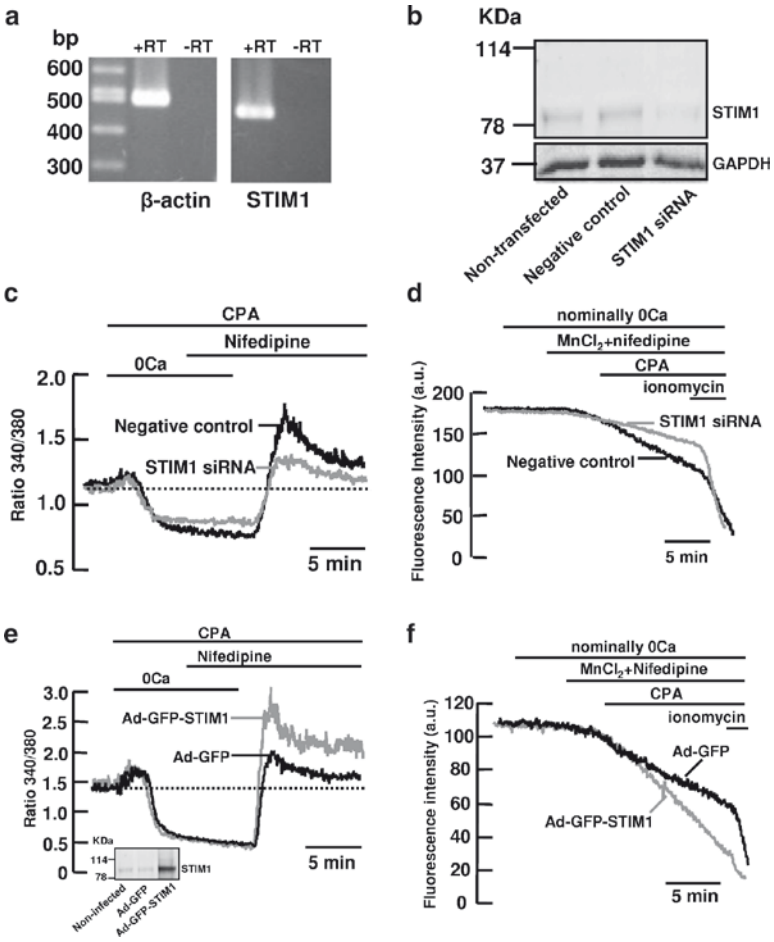

Fig. 8.2 STIM1 mediates CCE in mouse PASMCs. (**a**) RT-PCR products from cultured mouse PASMCs amplified using primers for mouse STIM1 (473 bp) and β-actin (498 bp). (**b**) STIM1 protein and GAPDH were detected in mouse PASMCs (nontransfected) and in PASMCs transfected with 200 n*M* scrambled siRNA (negative control). The expression of STIM1 but not GAPDH was reduced significantly in cells transfected with 200 n*M* STIM1 siRNA. (**c**) siRNA knockdown of STIM1 reduced the CPA-induced transient and sustained increase in fura-2 fluorescence ratio in the presence of 10 μ*M* nifedipine. (**d**) siRNA knockdown of STIM1 reduced the increase in Mn^{2+} quench of fura-2 fluorescence caused by 10 μ*M* CPA in the presence of 10 μ*M* nifedipine. (**e**) Overexpression of STIM1 enhanced the increase in CPA-induced transient and the sustained rise in fura-2 fluorescence ratio in the presence of 10 μ*M* nifedipine. *Inset* STIM1 protein and GAPDH were detected in noninfected mouse PASMCs and in PASMCs infected with adenovirus containing green fluorescent protein (Ad-GFP). The expression of STIM1 increased markedly in cells infected with STIM1-GFP-adenovirus (Ad-GFP-STIM1). (**f**) Overexpression of STIM1 enhanced the increase in Mn^{2+} quench of fura-2 fluorescence caused by 10 μ*M* CPA in the presence of 10 μ*M* nifedipine. Modified with permission[42]

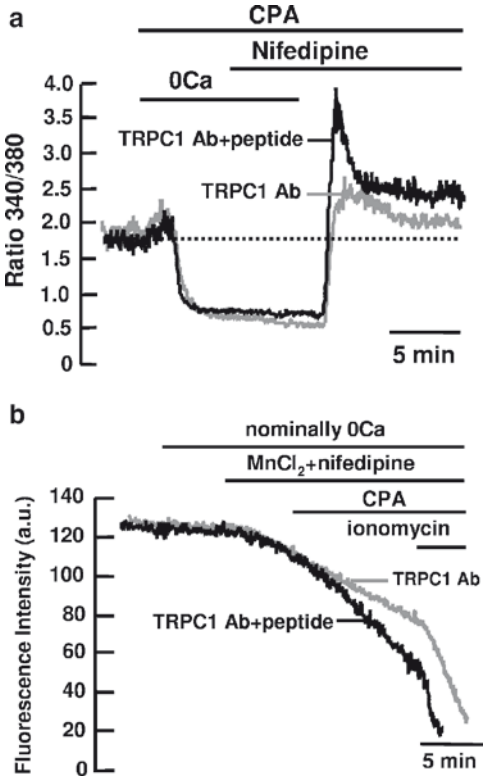

Fig. 8.3 STIM1 associates with TRPC1 to mediate CCE in mouse PASMCs. (**a**) In cultured mouse PASMCs overexpressed with STIM1, TRPC1 antibody (1:100) reduced the CPA-induced transient and sustained increase in the fura-2 fluorescence ratio in the presence of 10 μ*M* nifedipine. (**b**) In cultured mouse PASMCs overexpressing STIM1, TRPC1 antibody (1:100) inhibited the increase in Mn^{2+} quench of fura-2 fluorescence caused by 10 μ*M* CPA in the presence of 10 μM nifedipine. Modified with permission[42]

overexpression of STIM1 resulted in an increase in the dihydropyridine-insensitive transient and sustained rise in $[Ca^{2+}]_i$ and the Mn^{2+} quench rate caused by CPA (Fig. 8.3). These responses were reduced in cells treated with TRPC1 antibody, suggesting a functional association of STIM1 and TRPC1 to mediate CCE in mouse PASMCs.

More interestingly, we found that STIM1 coimmunoprecipitates TRPC1, and the precipitation level of TRPC1 was increased during store depletion (Fig. 8.4). Therefore, SOCs may consist of a molecular complex composed of TRPC1 and STIM1 in mouse PASMCs, and when the intracellular Ca^{2+} stores are depleted, STIM1 that resides in the cytosol may be recruited to the cell membrane and interacts with more TRPC1 to enhance CCE. This molecular complex has not previously been described in any vascular smooth muscle preparation, including PASMCs. Although STIM1 has been shown to coimmunoprecipitate with TRPC1 in cultured

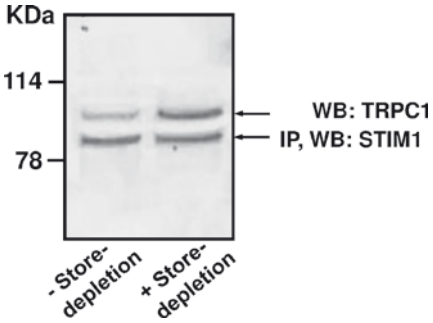

Fig. 8.4 TRPC1 interacts with STIM1 to form SOCs in mouse PASMCs. STIM1 coimmunoprecipitates TRPC1 in cultured mouse PASMCs in the absence and presence of store depletion. STIM1 was first immunoprecipitated (IP) with EXBIO STIM1 antibody (10 μg), and the blot was subsequently probed with BD Biosciences STIM1 antibody (WB, 1:100). The blot was then probed for co-IP of TRPC1 expression using TRPC1 antibody (WB, 1:100, Alomone). Modified with permission[42]

human saphenous vein cells, the possibility that the association between TRPC1 and STIM1 maybe increased after store depletion in these cells was not examined.[11]

6 Summary and Conclusions

Our study in mouse PASMCs confirmed a novel functional interaction between STIM1 and TRPC1, in which TRPC1 mediates CCE through activation of STIM1.[42] This is supported by the evidence that endogenous TRPC1 (Fig. 8.1) and STIM1 (Fig. 8.2) mediate CCE in mouse PASMCs. Overexpression of STIM1 increased CCE, and this increase in CCE was significantly reduced by TRPC1 antibody (Fig. 8.3). Moreover, STIM1 coimmunoprecipitates TRPC1, and store depletion enhances the association of STIM1 and TRPC1 in mouse PASMCs (Fig. 8.4). These data provide strong evidence for a functional link between STIM1 and TRPC1 to mediate CCE.

Another interesting finding is that the dihydropyridine-insensitive transient rise in $[Ca^{2+}]_i$ caused by CPA was not affected by TRPC1 antibody but was significantly reduced in PASMCs transfected with STIM1 siRNA (Figs. 8.1 and 8.2). Therefore, it is likely that other TRPC channels may heteromultimerize with TRPC1 and STIM1 to function as SOCs in mouse PASMCs. It is also possible that the dihydropyridine-insensitive transient rise in $[Ca^{2+}]_i$ may be mediated by Orai1, which has been shown to be a pore-forming subunit of the calcium release-activated calcium (CRAC) channel in nonexcitable cells.[43,44] Coexpression of Orai1 and STIM1 was found to cause a significant gain in CRAC channel function, suggesting that STIM1 interacts with Orai1 to cause CCE.[45,46] On the other hand, overexpression of Orai1 in HEK cells was found to interact with the store depletion-insensitive channels TRPC3 and TRPC6 and confer store depletion sensitivity to these channels.[47] Furthermore, TRPC1 was shown to form a complex with STIM1 and Orai1 to

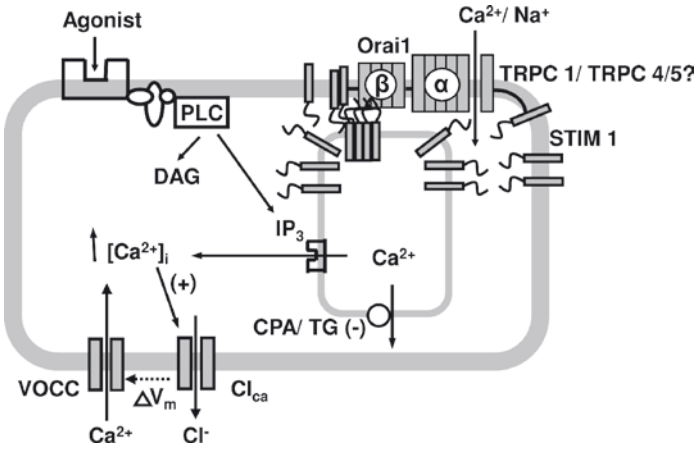

Fig. 8.5 A plausible model for molecular makeup of store-operated channels activated by STIM1 in mouse PASMCs. *Abbreviations*: *Clca* Ca^{2+}-activated chloride channel; *CPA* cyclopiazonic acid; *DAG* diacylglycerol; *IP_3* inositol 1,4,5-trisphosphate; *PLC* phospholipase C; *TG* thapsigargin; *VOCC* voltage-operated Ca^{2+} channel

activate SOCs in human salivary gland cells.[48] Thus, future studies on whether other TRPC channels or Orai1 interact with STIM1 and mediate the dihydropyridine-insensitive transient rise in $[Ca^{2+}]_i$ in mouse PASMCs are warranted.

Based on our study in mouse PASMCs and other studies in nonexcitable cells,[42,45,48] we have proposed a model for the molecular makeup of SOCs activated by STIM1 and other Ca^{2+} entry pathways activated by store depletion in PASMCs (Fig. 8.5). Depletion of intracellular Ca^{2+} stores causes Ca^{2+} release from the SR, activation of the Ca^{2+}-activated chloride channel, leading to chloride efflux and membrane depolarization, which causes activation of VOCCs. Store depletion also causes activation of SOCs, leading to Na^+ and Ca^{2+} entry. When the stores are depleted, STIM1 in the SR senses the depletion of Ca^{2+}, coalesces, and moves to the cell membrane to interact with STIM1 that resides in the membrane. The STIM1 complex then activates the opening of SOCs in the cell membrane. SOCs may be composed of α- and β-subunits. TRPC1 channels possess six transmembrane-spanning regions, which may serve as the α-subunit, either as a homotetramer or a heterotetramer with other TRPC channels. Orai1 is composed of four transmembrane-spanning regions that may serve as the β-subunit. Orai1 may act as a transducer of STIM1 signals, leading to the activation of nonselective cation TRPC1 channels and Ca^{2+} and Na^+ entry.

To date, the study of CCE has become one of the important areas for pulmonary vascular research because of its implication in hypoxic pulmonary vasoconstriction (HPV). Pulmonary vasoconstriction in response to hypoxia is an important protective mechanism that diverts blood flow away from hypoxic alveoli into better-ventilated regions of the lung. This acute hypoxic pressor response is a unique physiological process that distinguishes pulmonary from the systemic circulation, which usually dilates in response to hypoxia. Evidence that hypoxia causes a sustained rise in

$[Ca^{2+}]_i$ through activation of CCE in PASMCs,[15,37] intact pulmonary arteries,[49] and isolated lungs[50] confirms a significant role of CCE in HPV. Interestingly, expression of TRPC1 and TRPC6 was significantly elevated in chronic hypoxia, and TRPC1 was found to mediate CCE in rat PASMCs,[41] suggesting a potential role of the TRPC1 channel in HPV. As discussed in this chapter, STIM1 was found to interact with TRPC1 to mediate CCE in mouse PASMCs.[42] This may serve as an important model for future studies of the mechanisms underlying HPV.

Acknowledgments Our work was supported by National Institutes of Health grants HL 49254 (Joseph R. Hume), P20RR15581 from the National Center for Research Resources (Joseph R. Hume), and an American Heart Association Scientist Development Grant (Lih Chyuan Ng).

References

1. Putney JW Jr (1986) A model for receptor-regulated calcium entry. Cell Calcium 7:1–12
2. Parekh AB, Putney JW Jr (2005) Store-operated calcium channels. Physiol Rev 85:757–810
3. Hoth M, Penner R (1992) Depletion of intracellular calcium stores activates a calcium current in mast cells. Nature 355:353–356
4. Leung FP, Yung LM, Yao X, Laher I, Huang Y (2007) Store-operated calcium entry in vascular smooth muscle. Br J Pharmacol 153:846–857
5. Trepakova ES, Gericke M, Hirakawa Y, Weisbrod RM, CohenRA, Bolotina VM (2001) The properties of a native cation channel activated by Ca^{2+} store depletion in vascular smooth muscle cells. J Biol Chem 276:7782–7790
6. Golovina VA, Platoshyn O, Bailey CL et al (2001) Upregulated TRP and enhanced capacitative Ca^{2+} entry in human pulmonary artery myocytes during proliferation. Am J Physiol Heart Circ Physiol 280:H746–H755
7. Albert AP, Large WA (2002) A Ca^{2+}-permeable non-selective cation channel activated by depletion of internal Ca^{2+} stores in single rabbit portal vein myocytes. J Physiol 538:717–728
8. Saleh SN, Albert AP, Peppiatt CM, Large WA (2006) Angiotensin II activates two cation conductances with distinct TRPC1 and TRPC6 channel properties in rabbit mesenteric artery myocytes. J Physiol 577:479–495
9. Saleh SN, Albert AP, Peppiatt-Wildman CM, Large WA (2008) Diverse properties of store-operated TRPC channels activated by protein kinase C in vascular myocytes. J Physiol 586:2463–2476
10. Ng LC, Gurney AM (2001) Store-operated channels mediate Ca^{2+} influx and contraction in rat pulmonary artery. Circ Res 89:923–929
11. Li J, Sukumar P, Milligan CJ et al (2008) Interactions, functions, and independence of plasma membrane STIM1 and TRPC1 in vascular smooth muscle cells. Circ Res 103:e97–e104
12. Wilson SM, Mason HS, Smith GD et al (2002) Comparative capacitative calcium entry mechanisms in canine pulmonary and renal arterial smooth muscle cells. J Physiol 543:917–931
13. Weirich J, Dumont L, Fleckenstein-Grun G (2005) Contribution of capacitative and non-capacitative Ca^{2+}-entry to M_3-receptor-mediated contraction of porcine coronary smooth muscle. Cell Calcium 38:457–467
14. McElroy SP, Gurney AM, Drummond RM (2008) Pharmacological profile of store-operated Ca^{2+} entry in intrapulmonary artery smooth muscle cells. Eur J Pharmacol 584:10–20
15. Ng LC, Kyle BD, Lennox AR, Shen XM, Hatton WJ, Hume JR (2008) Cell culture alters Ca^{2+} entry pathways activated by store-depletion or hypoxia in canine pulmonary arterial smooth muscle cells. Am J Physiol Cell Physiol 294:C313–C323
16. Pedersen SF, Owsianik G, Nilius B (2005) TRP channels: an overview. Cell Calcium 38:233–252

17. Albert AP, Saleh SN, Peppiatt-Wildman CM, Large WA (2007) Multiple activation mechanisms of store-operated TRPC channels in smooth muscle cells. J Physiol 583:25–36
18. Xu SZ, Beech DJ (2001) TrpC1 is a membrane-spanning subunit of store-operated Ca^{2+} channels in native vascular smooth muscle cells. Circ Res 88:84–87
19. Brueggemann LI, Markun DR, Henderson KK, Cribbs LL, Byron KL (2006) Pharmacological and electrophysiological characterization of store-operated currents and capacitative Ca^{2+} entry in vascular smooth muscle cells. J Pharmacol Exp Ther 317:488–499
20. Bergdahl A, Gomez MF, Wihlborg AK et al (2005) Plasticity of TRPC expression in arterial smooth muscle: correlation with store-operated Ca^{2+} entry. Am J Physiol Cell Physiol 288:C872–C880
21. Takahashi Y, Watanabe H, Murakami M et al (2007) Involvement of transient receptor potential canonical 1 (TRPC1) in angiotensin II-induced vascular smooth muscle cell hypertrophy. Atherosclerosis 195:287–926
22. Xu SZ, Boulay G, Flemming R, Beech DJ (2006) E3-targeted anti-TRPC5 antibody inhibits store-operated calcium entry in freshly isolated pial arterioles. Am J Physiol Heart Circ Physiol 291:H2653–H2669
23. Goel M, Sinkins WG, Schilling WP (2002) Selective association of TRPC channel subunits in rat brain synaptosomes. J Biol Chem 277:48303–48310
24. Hofmann T, Schaefer M, Schultz G, Gudermann T (2002) Subunit composition of mammalian transient receptor potential channels in living cells. Proc Natl Acad Sci U S A 99:7461–7466
25. Strübing C, Krapivinsky G, Krapivinsky L, Clapham DE (2003) Formation of novel TRPC channels by complex subunit interactions in embryonic brain. J Biol Chem 278:39014–39019
26. Liu X, Bandyopadhyay BC, Singh BB, Groschner K, Ambudkar IS (2005) Molecular analysis of a store-operated and 2-acetyl-*sn*-glycerol-sensitive non-selective cation channel. Heteromeric assembly of TRPC1–TRPC3. J Biol Chem 280:21600–21606
27. Poteser M, Graziani A, Rosker C et al (2006) TRPC3 and TRPC4 associate to form redox-sensitive cation channel. Evidence for expression of native TRPC3–TRPC4 heterotetrameric channels in endothelial cells. J Biol Chem 281:13588–13595
28. Roos J, DiGregorio PJ, Yeromin AV et al (2005) STIM1, an essential and conserved component of store-operated Ca^{2+} channel function. J Cell Biol 169:435–445
29. Zhang SL, Yu Y, Roos J et al (2005) STIM1 is a Ca^{2+} sensor that activates CRAC channels and migrates from the Ca^{2+} store to the plasma membrane. Nature 437:902–905
30. Spassova MA, Soboloff J, He LP, Xu W, Dziadek MA, Gill DL (2006) STIM1 has a plasma membrane role in the activation of store-operated Ca^{2+} channels. Proc Natl Acad Sci U S A 103:4040–4045
31. Takahashi Y, Watanabe H, Murakami M et al (2007) Functional role of stromal interaction molecule 1 (STIM1) in vascular smooth muscle cells. Biochem Biophys Res Commun 361:934–940
32. Dietrich A, Kalwa H, Storch U et al (2007) Pressure-induced and store-operated cation influx in vascular smooth muscle cells is independent of TRPC1. Pflügers Arch 455:465–477
33. Peel SE, Liu B, Hall IP (2006) A key role for STIM1 in store operated calcium channel activation in airway smooth muscle. Respir Res 7:119–126
34. Yuan JP, Zeng W, Huang GN, Worley PF, Muallem S (2007) STIM1 heteromultimerizes TRPC channels to determine their function as store-operated channels. Nat Cell Biol 9:636–645
35. Walker RL, Hume JR, Horowitz B (2001) Differential expression and alternative splicing of TRP channel genes in smooth muscles. Am J Physiol Cell Physiol 280:C1184–C1192
36. Wang J, Shimoda LA, Sylvester JT (2003) Capacitative calcium entry and TRPC channel proteins are expressed in rat distal pulmonary arterial smooth muscle. Am J Physiol Lung Cell Mol Physiol 286:L848–L858
37. Lu W, Wang J, Shimoda LA, Sylvester JT (2008) Differences in STIM1 and TRPC expression in proximal and distal pulmonary arterial smooth muscle are associated with differences in Ca^{2+} responses to hypoxia. Am J Physiol Lung Cell Mol Physiol 295:L104–L113

38. Golovina VA (1999) Cell proliferation is associated with enhanced capacitative Ca^{2+} entry in human arterial myocytes. Am J Physiol Cell Physiol 277:C343–C349
39. Sweeney M, Yu Y, Platoshyn O, Zhang S, McDaniel SS, Yuan JX-J (2002) Inhibition of endogenous TRP1 decreases capacitative Ca^{2+} entry and attenuates pulmonary artery smooth muscle cell proliferation. Am J Physiol Lung Cell Mol Physiol 283:L144–L155
40. Kunichika N, Yu Y, Remillard CV, Platoshyn O, Zhang S, Yuan JX-J (2004) Overexpression of TRPCs enhances pulmonary vasoconstriction induced by capacitative Ca^{2+} entry. Am J Physiol Lung Cell Mol Physiol 287:L962–L969
41. Lin MJ, Leung GP, Zhang WM et al (2004) Chronic hypoxia-induced upregulation of store-operated and receptor-operated Ca^{2+} channels in pulmonary arterial smooth muscle cells: a novel mechanism of hypoxic pulmonary hypertension. Circ Res 95:496–505
42. Ng LC, McCormack MD, Airey JA et al (2009) TRPC1 and STIM1 mediate capacitative calcium entry in mouse pulmonary artery smooth muscle cells. J Physiol 587:2429–2442
43. Feske S, Gwack Y, Prakriya M et al (2006) A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function. Nature 441:179–185
44. Prakriya M, Feske S, Gwack Y, Srikanth S, Rao A, Hogan PG (2006) Orai1 is an essential pore subunit of the CRAC channel. Nature 443:230–233
45. Soboloff J, Spassova MA, Tang XD, Hewavitharana T, Xu W, Gill DL (2006) Orai1 and STIM reconstitute store-operated calcium channel function. J Biol Chem 281:20661–20665
46. Mercer JC, Dehaven WI, Smyth JT et al (2006) Large store-operated calcium selective currents due to co-expression of Orai1 or Orai2 with the intracellular calcium sensor. Stim1. J Biol Chem 281:24979–24990
47. Liao Y, Erxleben C, Yildirim E, Abramowitz J, Armstrong DL, Birnbaumer L (2007) Orai proteins interact with TRPC channels and confer responsiveness to store depletion. Proc Natl Acad Sci U S A 104:4682–4687
48. Cheng KT, Liu X, Ong HL, Ambudkar IS (2008) Functional requirement for Orai1 in store-operated TRPC1-STIM1 channels. J Biol Chem 283:12935–12940
49. Robertson TP, Hague D, Aaronson PI, Ward JP (2000) Voltage-independent calcium entry in hypoxic pulmonary vasoconstriction of intrapulmonary arteries of the rat. J Physiol 525:669–680
50. Weigand L, Foxson J, Wang J, Shimoda LA, Sylvester JT (2005) Inhibition of hypoxic pulmonary vasoconstriction by antagonists of store-operated Ca^{2+} and nonselective cation channels. Am J Physiol Lung Cell Mol Physiol 289:L5–L13

Store-Operated Calcium Entry Channels in Pulmonary Endothelium: The Emerging Story of TRPCS and Orai1

Donna L. Cioffi, Christina Barry, and Troy Stevens

Abstract Cells of diverse origin utilize shifts in cytosolic calcium concentrations as intracellular signals to elicit physiological responses. In endothelium, inflammatory first messengers increase cytosolic calcium as a signal to disrupt cell-cell borders and produce inter-cellular gaps. Calcium influx across the plasma membrane is required to initiate barrier disruption, although the calcium entry mechanism responsible for this effect remains poorly understood. This chapter highlights recent efforts to define the molecular anatomy of the ion channel responsible for triggering endothelial cell gap formation. Resolving the identity and function of this calcium channel will pave the way for new anti-inflammatory therapeutic targets.

Keywords Acute lung injury • Vascular barrier dysfunction • Endothelial cells • transient receptor potential channel • lung microvascular endothelium

1 Introduction

Lung endothelium forms a semipermeable barrier that restricts water, solute, and macromolecular access to the interstitium, which is important in optimizing gas exchange. Inflammation disrupts this barrier function, causing accumulation of a protein-rich fluid in interstitial and alveolar compartments that compromises gas

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exchange and contributes to the genesis of acute respiratory distress syndrome. A unifying finding is that multiple different inflammatory agents promote calcium influx across the endothelial cell plasma membrane. This calcium influx triggers cytoskeletal reorganization that initiates intercellular gap formation and increases permeability. However, endothelial cells express many different ion channels, bringing into question exactly which channel provides the calcium source that reorganizes the cytoskeleton and induces gap formation necessary to increase permeability. Findings indicate that transient receptor proteins (TRPs) within the canonical family (TRPC) contribute subunits of an ion channel that provides the calcium source needed to disrupt cell-cell adhesion and induce endothelial cell gaps. Indeed, we have found that endothelial cells, both in vitro and in vivo, express an endogenous channel that possesses TRPC1, TRPC3, and TRPC4 subunits, a so-called TRPC1/3/4 channel, which importantly regulates endothelial cell barrier function. This chapter reviews the calcium channels that are expressed in lung endothelium and addresses regulation and function of the TRPC1/3/4 channel.

2 Fidelity of Calcium Signals in Endothelium

Many neurohumoral inflammatory mediators bind membrane receptors and promote calcium influx across the plasma membrane, resulting in increased endothelial cell permeability.[1–3] However, the molecular composition of the calcium channels responsible for increased endothelial cell permeability is poorly understood. It is remarkable to consider that as recently as 1990, not a single endothelial cell calcium channel was known.[4] We presently know of at least six different endothelial cell calcium channels, including the TRPC1/3/4 channel that is the focus of this chapter, an Orai1-containing channel,[5] a TRPC3/6-containing channel,[6–11] a T-type calcium channel,[12–14] a TRPV4 (TRP channel of the vanilloid subfamily)-containing channel,[6,15–21] and a cyclic nucleotide gated channel.[22–27] While we recognize that endothelial cells express a diversity of calcium channels, the molecular anatomy, regulation, and physiological function of these channels represent important areas of ongoing investigation.

Study of calcium channel diversity has led to new and unexpected insight regarding the unique behaviors of endothelium along the pulmonary vascular tree. For example, the T-type calcium channel is expressed only in lung capillary endothelium and is not found in extraalveolar pulmonary artery or vein endothelium.[14] Activation of this channel is essential for P-selectin surface expression but does not increase endothelial cell permeability (Songwei Wu, personal communication). The TRPV4 channel is also expressed predominantly in lung capillary endothelium,[17] but in contrast to the T-type calcium channel, its activation increases capillary endothelial cell permeability and does not influence P-selectin surface expression[90]. Thus, T-type and TRPV4 calcium channels are expressed in the same vascular segment, and while activation of each of these channels increases endothelial cell calcium, the T channel calcium signal translocates P-selectin from the cytosol to the plasma membrane, whereas the TRPV4 calcium signal increases endothelial cell permeability.

Like the TRPV4 channel, activation of the TRPC1/3/4 channel increases endothelial cell permeability.[28–32] However, two critical differences have been noted regarding the activation and function of these channels. First, the TRPC1/3/4 channel is expressed in pulmonary artery, capillary, and vein endothelium; when the TRPC1/3/4 channel is activated, it increases permeability in all three vascular segments,[28–32] whereas TRPV4 increases permeability primarily in the capillary segment.[17] Second, TRPC1/3/4 activation induces interendothelial cell gaps, both in vivo and in vitro, resulting in a paracellular pathway for fluid and macromolecular permeability,[31,32] as originally described by Majno and Palade in 1961.[33,34] In contrast, TRPV4 activation does not induce interendothelial cell gap formation; rather, calcium influx through TRPV4 decreases cell-matrix tethering and results in endothelial cell sluffing.[17] Thus, TRPC1/3/4 and TRPV4 channels provide calcium influx pathways that regulate quite different cell functions.

3 Activation of Store-Operated Calcium Entry Increases Endothelial Cell Permeability

Formation of interendothelial cell gaps is now a well-recognized cause of tissue edema. Pioneering studies by Majno and Palade[33,34] first revealed that neurohumoral inflammatory mediators induce interendothelial cell gaps in postcapillary venules of the systemic circulation, although the cellular basis of this observation was not known. Since the time of these original observations, many G_q-linked calcium agonists, such as bradykinin, histamine, thrombin, and platelet-activating factor, have been shown to activate membrane calcium channels and promote calcium influx that induces gap formation in both systemic and pulmonary endothelium.[1] G_q-linked agonists hydrolyze phosphatidyl inositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol, both of which are important intracellular signals. $InsP_3$ releases calcium from the endoplasmic reticulum, and the transiently depleted calcium store triggers calcium entry across the cell membrane through so-called store-operated calcium entry channels, as originally described by Putney.[35] The activation of store-operated calcium entry serves two functions; it initiates physiologically important intracellular responses, and it replenishes the depleted calcium store. The endoplasmic reticulum calcium-filling state is therefore inversely related to how much calcium enters the cell from across the plasma membrane. In contrast to this mechanism of calcium entry, diacylglycerol activates another calcium entry pathway, generally referred to as receptor-operated calcium entry. Our specific focus has been to resolve the molecular anatomy of store-operated calcium entry pathways and not receptor-operated calcium entry pathways as considerable evidence demonstrated that activation of store-operated calcium entry induces interendothelial cell gaps and increases endothelial cell permeability.

Store-operated calcium entry channels can be directly activated by agents that deplete endoplasmic reticulum calcium.[35] Endoplasmic reticulum calcium can be reduced by chelating intracellular calcium, using the calcium chelators *N*,*N*,*N*′,*N*′-tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN) and 1,2-bis(o-aminophenoxy)

ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), by photoactivation of caged $InsP_3$ or by inhibiting the sarcoplasmic, endoplasmic reticulum calcium adenosine triphosphatase (ATPase) with either thapsigargin or cyclopiazonic acid. Both in vitro and in vivo, thapsigargin (and cyclopiazonic acid) induces pulmonary endothelial cell gaps and increases lung permeability; this increase in permeability is abolished when calcium entry through store-operated calcium entry channels is inhibited.[36,37] Moreover, photoactivation of caged $InsP_3$ is sufficient to induce interendothelial cell gap formation in cultured endothelial cells. Thus, activation of store-operated calcium entry, using physiologically relevant agonists (e.g., thrombin, platelet-activating factor), thapsigargin, or photoactivation of caged $InsP_3$, induces endothelial cell gap formation and increases permeability.

Thapsigargin promotes a slowly developing and sustained increase in endothelial cell cytosolic calcium (Fig. 9.1). To better understand whether thapsigargin activates one or more calcium influx pathways, we examined the cationic permeation characteristics of store-operated calcium channels in live cells. Thapsigargin activated at least two separate pathways in endothelial cells. One store-operated calcium entry mechanism was calcium nonselective, meaning that both monovalent and divalent cations permeated the channel pore.[27] A second store-operated calcium entry mechanism was calcium selective, meaning that calcium permeated the channel pore with preference over other divalent cations.[28–32] As we examined the biophysical nature of these two distinct store-operated calcium entry pathways and their physiological function, we came to learn that it was the calcium-selective channel that provides the calcium source necessary to induce interendothelial cell gaps.[31,32]

Calcium nonselective and selective store-operated calcium entry pathways can be systematically studied using whole-cell electrophysiology approaches. Endothelial cells possess a store-operated nonselective current that is activated by thapsigargin.[27,30] This current is a large, linear current that possesses a reversal potential of 0 mV and conducts various mono- and divalent cations. Endothelial cells also possess a store-operated calcium-selective current that is activated by thapsigargin, cyclopiazonic acid, $InsP_3$, intracellular BAPTA, and TPEN; this calcium-selective current is activated when cytosolic calcium is buffered by ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′*N*′-tetraacetic acid (EGTA), indicating it is calcium store depletion, and not a rise in cytosolic calcium, that is responsible for current activation.[28–32] This current is small (1–1.5 pA/pF) and inwardly rectifying, with a reversal potential of approximately -40 mV (Fig. 9.2). The fundamental biophysical properties of this current, called I_{SOC}, are similar in pulmonary artery and capillary endothelial cells, although mechanisms controlling channel activation differ among cell types.[31,32]

We were curious to know whether the store-operated nonselective and calcium-selective currents represent different ion channels with unique activation properties or whether they reflect the same ion channel with different permeation characteristics. As we sought experimental approaches to distinguish nonselective from calcium-selective store-operated calcium entry pathways, we became intrigued by studies in platelets incriminating the actin cytoskeleton in the activation of store-operated calcium entry.[38–40] Actin is not thought to directly interact with transmembrane proteins, such as ion channels. Rather, the spectrin membrane skeleton cross-links actin imme-

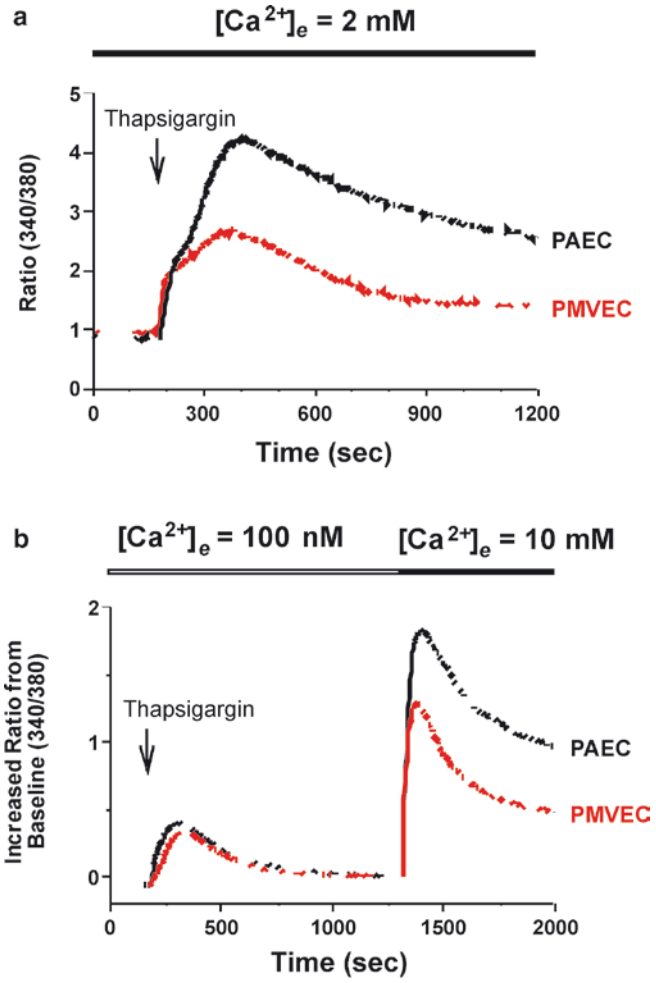

Fig. 9.1 Thapsigargin activates store-operated calcium entry channels and increases cytosolic calcium. (**a**) Confluent monolayers of pulmonary artery endothelial cells (PAEC) and pulmonary microvascular endothelial cells (PMVEC) endothelial cells were loaded with the calcium indicator fura-2. Thapsigargin (1 μ*M*) was applied at the indicated time, and cytosolic calcium concentrations were monitored. Thapsigargin induces a slowly developing and sustained rise in cytosolic calcium in endothelial cells, although the calcium rise is greater in PAECs than it is in PMVECs. (**b**) Thapsigargin inhibits the sarcoplasmic, endoplasmic calcium ATPase, causing depletion of stored calcium. Such depletion of endoplasmic reticulum calcium opens store-operated calcium entry channels on the plasma membrane, resulting in calcium influx into the cell. These separate phases can be distinguished using a recalcification protocol in which thapsigargin is first applied to fura-2-loaded cells in low extracellular calcium. A transient rise in cytosolic calcium is observed as calcium is released from the endoplasmic reticulum. As extracellular calcium is replenished, it enters the cell through open store-operated calcium entry channels. For experimental details, see Ref. [31]

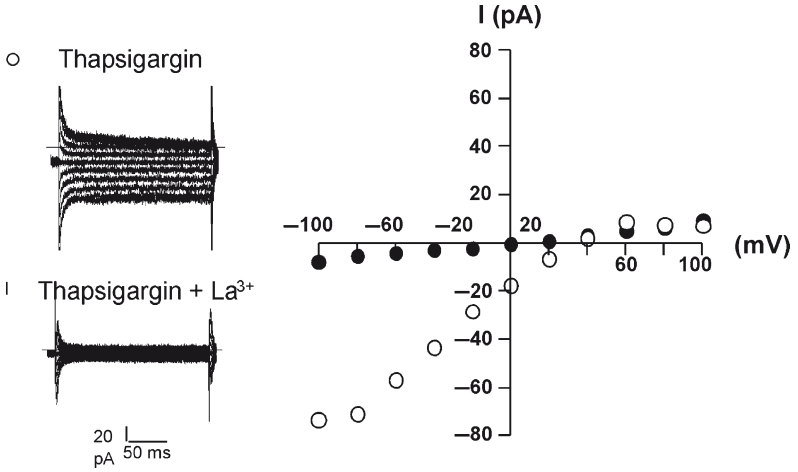

Fig. 9.2 Thapsigargin activates a calcium-selective store-operated current in endothelial cells, referred to as I_{SOC}. Macroscopic currents were resolved in single pulmonary artery endothelial cells. Thapsigargin (1 μ*M*) was applied in the patch pipet in the whole-cell configuration. The holding potential was 0 mV, and a ramp protocol was performed from –100 to +100 mV. Thapsigargin activates a small inward calcium current at negative voltages, with a reversal potential that ranges between +30 and +40 mV. This current is abolished by lanthanum perfusion. For experimental details, see Refs. [28–32]

diately adjacent to the inner leaflet of the phospholipid bilayer.[20,41,42] Spectrin binds to protein 4.1 and ankyrin, and it is these spectrin-bound proteins that directly interact with transmembrane proteins.[43] We selectively disrupted the spectrin-actin and the spectrin-protein 4.1 interactions, and observed that only disruption of spectrin from protein 4.1 inhibited I_{SOC} activation.[30] Indeed, disrupting the spectrin-protein 4.1 interaction had no effect on the thapsigargin-activated nonselective current, and disrupting the spectrin-actin association had no impact on activation of either the I_{SOC} or the nonselective current. These findings provided evidence that channels with different molecular compositions account for the calcium-selective (e.g., I_{SOC}) and nonselective currents. We were interested in resolving the molecular composition of the I_{SOC} channel as functional studies indicated that activation of this current is essential for inter-endothelial cell gap formation.

4 TRPC Proteins Form the I_{SOC} Channel

Evidence that the I_{SOC} channel, and not the nonselective channel, interacts with protein 4.1 suggested that protein 4.1 could be used as "bait" to resolve the channel's molecular identity. We therefore began to screen for channel proteins that interact with protein 4.1. There was precedence for this idea in the spectrin field as the spectrin-binding protein ankyrin was known to interact with spectrin and fast

sodium channels in nodes of Ranvier.[44–51] Discovery of TRPC proteins revealed potential candidates for store-operated calcium entry pathways.[35] However, not all TRPC proteins are store operated; in some instances, these proteins form receptor-operated channels. Store-operated channels must be activated by depletion of endoplasmic reticulum calcium, must not require increased cytosolic calcium for their activation, and must not require diacylglycerol or its analogue OAG (1-oleoyl-2-acetyl-sn-glycerol) for activation. Based on these criteria, TRPC1, TRPC4, and TRPC5 proteins can form store-operated calcium entry channels. Interestingly, sequence alignment of all TRPC isoforms revealed that TRPC4 possesses a putative protein 4.1-binding domain (Fig. 9.3). We were excited to find that the protein 4.1-binding domain on TRPC4 was adjacent to the channel pore,[28] suggesting that a protein 4.1-TRPC4 interaction may be critical for channel activation.

Work from the Flockerzi and Nilius groups indicated that TRPC4 contributes to the molecular identity of the I_{SOC} channel.[52] Indeed, TRPC4 knockdown using small-interfering RNA (siRNA) prevented thapsigargin from activating I_{SOC}, and in endothelial cells isolated from TRPC4 deficient mice, I_{SOC} similarly could not be activated. We examined whether protein 4.1 binds to TRPC4 and regulates channel activation using two related approaches (Fig. 9.3).[28] In the first approach, the protein 4.1-binding domain was deleted from TRPC4 and the chimera expressed in endothelium. The chimeric protein was appropriately expressed and targeted to the membrane. The chimeric TRPC4 protein did not interact with protein 4.1, and in cells expressing this chimera, thapsigargin was unable to activate I_{SOC}. In the second approach, a competitive peptide was generated to target the protein 4.1-binding domain on TRPC4. Introduction of the competitive peptide into endothelium inhibited protein 4.1 binding to the endogenously expressed channel and prevented thapsigargin from activating I_{SOC}. These data, taken together with those of the Flockerzi and Nilius groups,[52] indicated that the protein 4.1-TRPC4 interaction is essential for I_{SOC} activation.

Studies establishing the pivotal role for TRPC4 in store-operated calcium entry, and in I_{SOC} activation more specifically, represented an exciting advance. Yet, molecular cloning studies and hydropathy plots faithfully projected that functional channels would be comprised of four TRPC subunits.[53] Thus, it was not clear whether the I_{SOC} channel was encoded by four TRPC4 subunits or by TRPC4 and some combination of other TRPC proteins. Indeed, siRNA inhibition of TRPC1 reduced the magnitude of the I_{SOC}, suggesting it may contribute a subunit,[29] and in heterologous expression studies, TRPC1 was shown to interact with TRPC4 as an essential step in channel membrane insertion.[54] Moreover, biochemical approaches documented interaction between TRPC1 and TRPC4.[55,56] These collective findings were taken as evidence that TRPC1 and TRPC4 interact and together contribute to the molecular basis of the I_{SOC} channel.

Physiological studies further supported the assertion that TRPC1 and TRPC4 interact as each protein has been implicated in control of endothelial cell permeability. In key studies published by the Malik and Tiruppathi groups, TRPC4-deficient mice were protected from thrombin (or protease-activated receptor ligand)-induced permeability.[57,58] Moreover, inflammatory mediators increase TRPC1 expression, and the upregulated expression of TRPC1 potentiates store-operated calcium entry

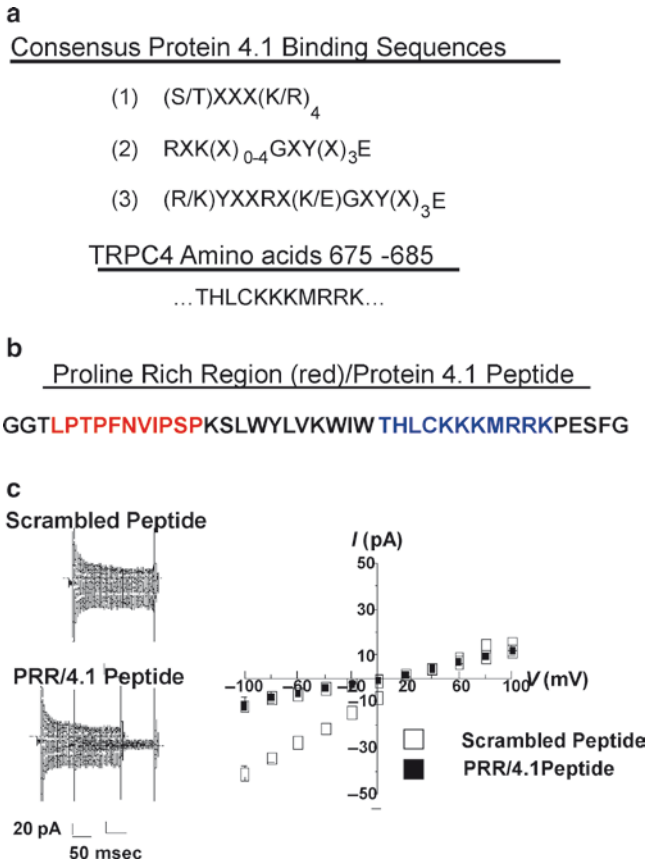

Fig. 9.3 TRPC4 possesses a conserved protein 4.1-binding sequence that is critical for I_{SOC} activation. (**a**) Consensus protein 4.1-binding sequences have been identified in MAGUKs (membrane-associated guanylate kinases), glycophorin C, and other transmembrane proteins.[88,89] A conserved protein 4.1-binding sequence was identified on TRPC4 between amino acids 675 and 685. (**b**) A peptide with sequence corresponding to the protein 4.1-binding domain was generated. (**c**) This peptide, which encompassed the proline-rich region and adjacent protein 4.1-binding domain, was introduced into endothelial cells, and the thapsigargin-induced I_{SOC} was measured. Introduction of the competitive peptide abolished I_{SOC} activation. For experimental details, see Ref. [28]

responses.[59] Perhaps most compelling for our work, however, were studies undertaken by the Townsley group, in which an aortocaval fistula was placed in rats to generate a model of congestive heart failure.[60–62] In their studies, thapsigargin was applied to wild-type, sham-operated, and heart failure animals to determine how development of heart failure has an impact on pulmonary endothelial cell barrier function. Whereas thapsigargin increased permeability in wild-type and sham-operated animals, it was without effect in heart failure animals. Immunohistochemical analysis revealed that development of heart failure was accompanied by downregulation of three TRPC proteins (TRPC1, TRPC3, and TRPC4) (Fig. 9.4).

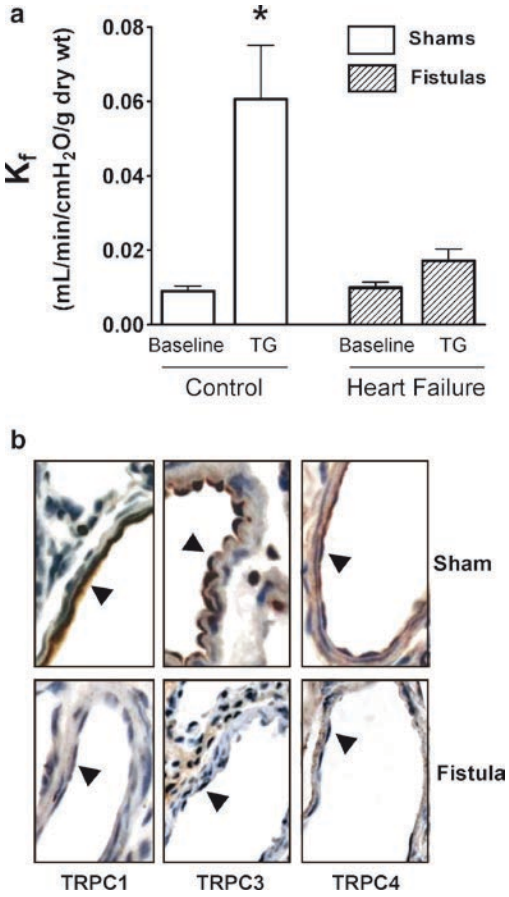

Fig. 9.4 Animals with heart failure do not respond to thapsigargin with an increase in lung endothelial cell permeability. (**a**) Aortocaval fistulas were placed in rats to induce heart failure. Heart and lungs were isolated en bloc, and permeability responses to thapsigargin were measured. Whereas thapsigargin increased permeability in sham-operated animals, animals with heart failure failed to respond to thapsigargin with an increase in permeability. (**b**) This absence of permeability response was accompanied by the selective downregulation of TRPC1, TRPC3, and TRPC4 proteins in lung endothelium. *Arrowheads* denote endothelium. For experimental details, see Ref. [62]

We were struck by the significance of this observation and wondered whether the downregulation of TRPC1, TRPC3, and TRPC4 was coincidental, representing the simultaneous downregulation of multiple channels, or whether all three proteins were a part of a common channel, perhaps the I_{SOC} channel. It was clear that determining the oligomeric state (number of subunits in an endogenous channel) and stoichiometry (how many of each subunit is in the channel) of the endogenously expressed I_{SOC} channel would be essential if we were to identify how inflammatory agonists induce endothelial cell gap formation and increase permeability.

Our group has spent considerable effort resolving both the oligomeric state and stoichiometry of the endogenous I_{SOC} channel in pulmonary endothelium. We have discovered that the observation by Townsley and colleagues that TRPC1, TRPC3, and TRPC4 are each downregulated in endothelium following heart failure is not coincidental. We utilized protein 4.1 as bait to enrich for the endogenously expressed channel and then developed a novel Förster resonance energy transfer (FRET) approach in collaboration with Drs. Claudette St. Croix and Bruce Pitt (at the Center for Biologic Imaging, University of Pittsburgh) to resolve subunit stoichiometry (D.L. Cioffi and T. Stevens, unpublished data). Our findings indicated that each of these proteins contributes subunits to the endogenous I_{SOC} channel; the I_{SOC} channel is comprised of one TRPC1, one TRPC3, and two TRPC4 subunits. Moreover, in these studies we utilized membranes derived from pulmonary artery endothelial cells, pulmonary microvascular endothelial cells, and caveolin-rich fractions isolated from the intact pulmonary circulation. In each case, both in vitro and in vivo, the channel's oligomeric state and stoichiometry were the same. These findings represent the first evidence for an endogenously expressed TRPC channel stoichiometry, the I_{SOC} channel, which is directly incriminated in endothelial cell gap formation and increased permeability.

5 Orai Proteins and Their Relationship to TRPC Channels

Our emerging data indicate that TRPC1/3/4 proteins coalesce to form a channel in which four proteins, each with six transmembrane domains, are required to generate an ion pore. This heterotetramer complex resembles the voltage-gated T-type calcium channel, with the exception that in the latter case, the four repeats of six transmembrane-spanning domains are all contained within a single gene product, the α_1 subunit.[63,64] Indeed, we were struck by the anatomic similarity between TRPC1/3/4 and T-type calcium channel topologies (Fig. 9.5). A more detailed examination of voltage-gated calcium channel organization revealed that its channel complex includes an associated subunit, the γ subunit, that interacts with the pore-forming α_1 subunit and contributes to channel activation and ionic permeation. If such an ancillary protein is critical to channel ionic permeation and if TRPC1/3/4 and voltage-gated channels possess a conserved anatomy, then it is likely that the TRPC1/3/4 channel interacts with another subunit that influences its calcium selectivity as well. Indeed, Orai1 may fulfill the role of a regulatory subunit of the TRPC1/3/4 channel.

The T-type calcium channel's γ subunit is a four-transmembrane-spanning domain protein with amino and carboxy termini that reside in the cytosol.[63,64] Functional interaction between the α_1 pore-forming subunit and the γ subunit requires a conserved sequence [(G/A/S) XXX (G/A/S)] in the γ subunit's first transmembrane-spanning domain (Fig. 9.5). This sequence is not required for α_1–γ subunit binding but is required for the γ subunit to control calcium permeation through the α_1 pore. We therefore screened for protein domains with homology to this sequence and were intrigued to find that a protein incriminated in store-operated

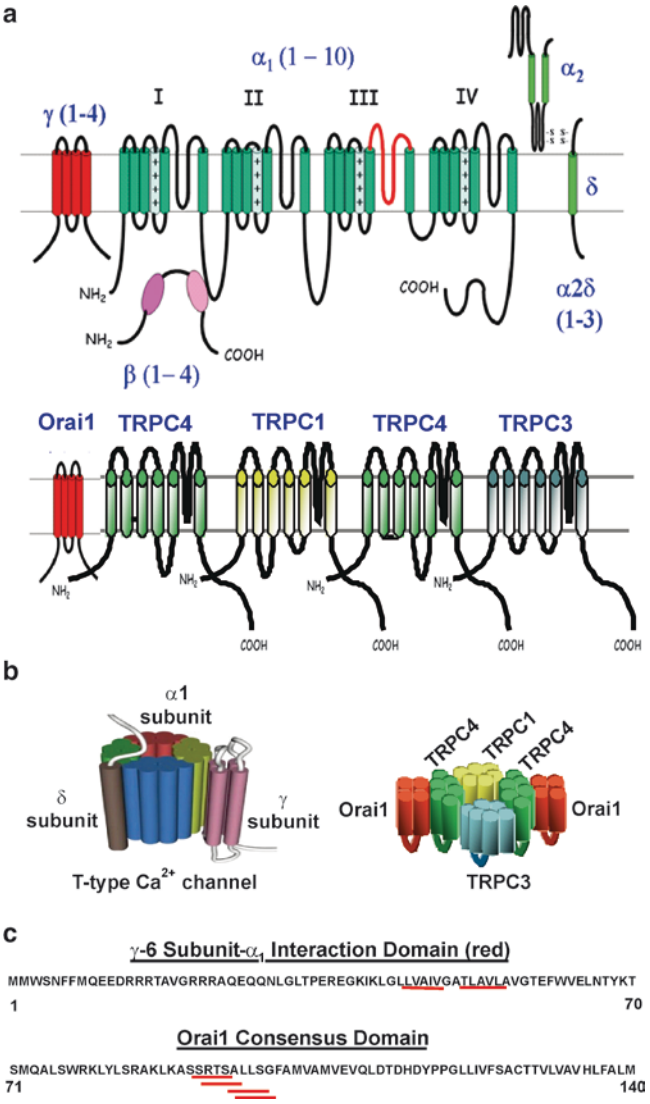

Fig. 9.5 The TRPC1/3/4 channel topology is reminiscent of the voltage-gated T-type calcium channel. (**a**) Schematic of the T-type calcium channel (*top*) and TRPC1/3/4 channel (*bottom*) illustrating a pore-forming unit formed from four cassettes of six transmembrane-spanning domains.[64] Similar to the γ subunit of the T-type calcium channel, Orai1 is a four-transmembrane-spanning domain protein that may interact with the pore-forming unit. (**b**) Schematic is shown of the three-dimensional T-type calcium channel and TRPC1/3/4 channel arrangements.[63] Channel interaction with the γ subunit (for the T-type calcium channel – *top*) and Orai1 (for the TRPC1/3/4 channel – *bottom*) is highlighted. (**c**) Functional interaction between the γ and α1 subunits occurs through a conserved sequence in the first transmembrane-spanning domain of the γ subunit. A similar interaction domain is resolved in Orai1 (see *underlined* regions)

calcium entry, orai1, was enriched with this conserved motif (Fig. 9.5). Orai1 is a four-transmembrane-spanning domain protein with amino and carboxy termini residing in the cytosol, just like the γ subunit. Thus, these findings suggest that Orai1 could interact with the TRPC1/3/4 channel and influence its calcium permeation.

The role that Orai1 plays in store-operated calcium entry is debated, with some evidence that it forms a channel pore and with other evidence that it interacts with channel proteins, such as TRPCs. Orai1 was first identified in 2006 using unbiased genetic approaches to identify putative channels that contribute to store-operated calcium entry.[65–67] In one approach, a modified linkage analysis with single-nucleotide polymorphism arrays was used to screen genes in patients with hereditary severe combined immunodeficiency (SCID) syndrome.[67] Whereas thapsigargin typically activates a highly calcium-selective current in T cells, referred to as the calcium release-activated calcium current, or I_{CRAC}, T cells from SCID patients lack the thapsigargin-activated I_{CRAC}. Genetic analysis revealed a missense mutation in exon 1 of the human ORAI1 coding sequence that resulted in mutation of a conserved arginine residue to tryptophan, a R91W mutation. Expression of wild-type Orai1 in T cells derived from SCID patients rescued the thapsigargin-activated I_{CRAC}, providing direct evidence that Orai1 fulfills an essential role in the I_{CRAC}. In a second approach, a genomewide RNA interference screen in cells from *Drosophila* was used to detect proteins that inhibit store-operated calcium influx.[65,66] Using this approach, separate groups identified Orai1 as a protein necessary for activation of the I_{CRAC}, again providing strong evidence that Orai1 is an essential component of the store-operated calcium entry channel.

From this pioneering work came a series of studies specifically addressing whether Orai1, and its related proteins Orai2 and Orai3, form a channel pore.[68,69] The I_{CRAC} is highly calcium selective, as are voltage-gated calcium channels, which utilize glutamate residues in the pore region to coordinate calcium binding. Analysis of the putative Orai1 pore revealed glutamate residues on the extracellular surface, at E106 in the first transmembrane domain, and at E190 in the third transmembrane domain. Several negatively charged aspartate residues were also found in the second transmembrane domain that may bind and sieve calcium as it passes through the pore. Replacing the conserved glutamate in the first transmembrane domain with glutamine (E106Q; substitution that cannot bind calcium) prevented thapsigargin from activating the I_{CRAC}. Replacing either the glutamate in the third transmembrane-spanning domain with glutamine (E190Q) or substituting aspartate to alanine (D110A and D112A) changed calcium selectivity and allowed for the channel to nonselectively conduct cations, including monovalent cations such as sodium and divalent cations such as barium and strontium. Thus, Orai1 may form an ion pore responsible for the I_{CRAC}.

While these studies suggested that Orai1 contributes to a calcium channel pore, channel oligomeric state and stoichiometry had not been determined. To address whether Orai1 forms a tetramer in living cells, it was expressed as a fusion protein in tandem with reporter fluorophores.[70] Once expression and membrane localization were confirmed, FRET was performed. FRET studies revealed that Orai1 organizes into tetramers in the plasma membrane. To assess whether four Orai1

subunits can generate a functional channel, four individual subunits were preassembled and expressed in cells typically deficient of the I_{CRAC}.[71] Expression of this entire tetrameric channel complex reconstituted the thapsigargin-activated I_{CRAC}, providing evidence not only that a functional channel is comprised of orai1 but also that four such subunits in tandem generate a pore-forming channel.

Despite evidence that Orai1 can form a channel, it remains unclear regarding whether it comprises the pore-forming channel accounting for the I_{CRAC} in vivo. For example, thapsigargin does not activate I_{CRAC} in all cells that express Orai1,[72–75] as discussed by Ambudkar and colleagues.[76] Moreover, the Birnbaumer[77,78] and Ambudkar[76,79] groups have independently noted that the anatomy of Orai1 is unusual for a calcium channel and, as discussed, recognized that it is more reminiscent of channel ancillary proteins, such as the γ subunit of the T channel. Both Birnbaumer[77,78,80] and Ambudkar[76,79,81] groups have proposed that Orai1 is an essential subunit of TRPC channels and is required for TRPC proteins to sense calcium store depletion and hence to be "store operated." At present, this central issue remains unresolved. It is not inconceivable that Orai1 forms the channel responsible for the I_{CRAC} and associates with TRPC channels to confer their sensitivity to store depletion. Indeed, Trebak and colleagues[5] suggested that thapsigargin activates both I_{CRAC} and I_{SOC} in endothelial cells, and that Orai1 is responsible for the I_{CRAC} in these cells, while TRPC4 contributes to I_{SOC}. Our preliminary evidence supports the idea that Orai1 interacts with the TPRC1/3/4 channel. Further work is therefore required to rigorously vet the role that Orai1 plays in calcium signaling, both as a channel and as a putative TRPC channel subunit.

6 Protein 4.1 Is an Essential Determinant of TRPC1/3/4 Activation

It is important to consider whether Orai1 contributes to activation of the TRPC1/3/4 channel. For many years, the mechanisms responsible for sensing a decrease in endoplasmic reticulum calcium were poorly understood. Identification of stromal interacting molecule-1 (STIM1) provided insight into this mechanism as STIM1 is a transmembrane protein possessing an EF hand, which is a calcium binding motif, on the luminal side of the endoplasmic reticulum.[82–85] Thus, as endoplasmic reticulum calcium decreases, it is sensed by STIM1, resulting in the punctate colocalization of STIM1 with Orai1. STIM1 and Orai1 coexpression potentiates thapsigargin activation of store-operated calcium entry.[86,87] These findings are taken to suggest that calcium store depletion results in association between the endoplasmic reticulum and calcium entry channels, necessary for channel activation.

If TRPC proteins function as store-operated calcium entry channels, and if STIM1 is necessary for activation of store-operated calcium entry, then STIM1 may interact with TRPC proteins following depletion of endoplasmic reticulum calcium. Several groups have now confirmed that this is true as STIM1 reportedly interacts with TRPC1 following thapsigargin treatment.[76–81] Interestingly, it appears that the

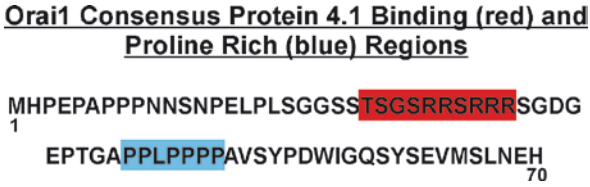

Fig. 9.6 Orai1 possesses a conserved protein 4.1-binding domain and proline-rich region in its amino-terminal domain. Similar to TRPC4, Orai1 possesses a conserved protein 4.1-binding domain and proline-rich region. At present, the function of these domains, and their relation to TRPC4-protein 4.1 binding, is unknown

interaction between STIM1 and TRPC1 is not direct but requires the coassociation of Orai1 with TRPC1. Indeed, Orai1 may be necessary for TRPC1 proteins to fulfill the criteria of a "store-operated" channel, again suggesting that Orai1 is an ancillary protein to the pore-forming TRPC channel.

While we know from preliminary work in our lab that the TRPC1/3/4 channel interacts with Orai1, we also know that TRPC1/3/4 activation requires a constitutive interaction between this channel complex and protein 4.1 and between protein 4.1 and spectrin; indeed, disruption of either of these protein-protein interactions prevents thapsigargin from activating the I_{SOC}. We began to question whether there is a relationship between the requirement for protein 4.1 to interact with the channel complex and Orai1. Remarkably, sequence alignment revealed that the Orai1 amino terminus possesses a conserved protein 4.1-binding domain immediately upstream of a proline-rich region (Fig. 9.6). It is not presently clear whether this protein 4.1-binding domain is important for the orai1-TRPC1/3/4 channel association, whether it contributes to TRPC1/3/4 channel activation, or whether it is necessary for the channel to be store operated. Thus, a principal goal of future studies must be to determine how the protein 4.1-binding domain on Orai1 contributes to TRPC1/3/4 channel activation.

7 Summary

It is an exciting time to study calcium-dependent signal transduction in endothelium. Since the mid-1990's, great strides have been made, with new protein candidates rapidly emerging as putative calcium channels. In most cases, molecular anatomy of calcium channel architecture is lacking and represents an essential goal for future work. Moreover, establishing the physiological role of calcium channels in endothelium is critically important. The TRPC1/3/4 channel provides a calcium source that disrupts cell-cell adhesion and increases endothelial cell permeability. Studies seeking to better resolve the molecular anatomy of this channel, its associated subunits, and its mode of activation will be critically important steps in refining our understanding of how the vasculature responds to inflammatory cues.

References

1. Mehta D, Malik AB (2006) Signaling mechanisms regulating endothelial permeability. Physiol Rev 86:279–367
2. Moore TM, Chetham PM, Kelly JJ, Stevens T (1998) Signal transduction and regulation of lung endothelial cell permeability. Interaction between calcium and cAMP. Am J Physiol 275:L203–L22
3. Vandenbroucke E, Mehta D, Minshall R, Malik AB (2008) Regulation of endothelial junctional permeability. Ann N Y Acad Sci 1123:134–145
4. Nilius B, Droogmans G (2001) Ion channels and their functional role in vascular endothelium. Physiol Rev 81:1415–1459
5. Abdullaev IF, Bisaillon JM, Potier M, Gonzalez JC, Motiani RK, Trebak M (2008) Stim1 and Orai1 mediate CRAC currents and store-operated calcium entry important for endothelial cell proliferation. Circ Res 103:1289–1299
6. Nilius B, Droogmans G, Wondergem R (2003) Transient receptor potential channels in endothelium: solving the calcium entry puzzle? Endothelium 10:5–15
7. Kwan HY, Huang Y, Yao X (2004) Regulation of canonical transient receptor potential isoform 3 (TRPC3) channel by protein kinase G. Proc Natl Acad Sci U S A 101:2625–2630
8. Poteser M, Graziani A, Rosker C et al (2006) TRPC3 and TRPC4 associate to form a redox-sensitive cation channel. Evidence for expression of native TRPC3–TRPC4 heteromeric channels in endothelial cells. J Biol Chem 281:13588–13595
9. Dietrich A, Kalwa H, Fuchs B, Grimminger F, Weissmann N, Gudermann T (2007) In vivo TRPC functions in the cardiopulmonary vasculature. Cell Calcium 42:233–244
10. Watanabe H, Murakami M, Ohba T, Takahashi Y, Ito H (2008) TRP channel and cardiovascular disease. Pharmacol Ther 118:337–351
11. Singh I, Knezevic N, Ahmmed GU, Kini V, Malik AB, Mehta D (2007) Gα q-TRPC6-mediated Ca^{2+} entry induces RhoA activation and resultant endothelial cell shape change in response to thrombin. J Biol Chem 282:7833–7843
12. Zhou C, Chen H, Lu F et al (2007) Cav3.1 α1G controls von Willebrand factor secretion in rat pulmonary microvascular endothelial cells. Am J Physiol Lung Cell Mol Physiol 292:L833–L844
13. Zhou C, Wu S (2006) T-type calcium channels in pulmonary vascular endothelium. Microcirculation 13:645–656
14. Wu S, Haynes J Jr, Taylor JT et al (2003) Cav3.1 α1G T-type Ca^{2+} channels mediate vaso-occlusion of sickled erythrocytes in lung microcirculation. Circ Res 93:346–353
15. Earley S, Heppner TJ, Nelson MT, Brayden JE (2005) TRPV4 forms a novel Ca^{2+} signaling complex with ryanodine receptors and BK_{Ca} channels. Circ Res 97:1270–1279
16. Kotlikoff MI (2005) EDHF redux: EETs, TRPV4, and Ca^{2+} sparks. Circ Res 97:1209–1210
17. Alvarez DF, King JA, Weber D, Addison E, Liedtke W, Townsley MI (2006) Transient receptor potential vanilloid 4-mediated disruption of the alveolar septal barrier: a novel mechanism of acute lung injury. Circ Res 99:988–995
18. Marrelli SP, O'Neil RG, Brown RC, Bryan RM Jr (2007) PLA2 and TRPV4 channels regulate endothelial calcium in cerebral arteries. Am J Physiol Heart Circ Physiol 292:H1390–H397
19. Hartmannsgruber V, Heyken WT, Kacik M et al (2007) Arterial response to shear stress critically depends on endothelial TRPV4 expression. PLoS One 2:e827
20. Cioffi DL, Lowe K, Alvarez DF, Barry C, Stevens T (2009) TRPing on the lung endothelium. Calcium channels that regulate barrier function. Antioxid Redox Signal 11:765–776
21. Zhang DX, Mendoza SA, Bubolz AH et al (2009) Transient receptor potential vanilloid type 4-deficient mice exhibit impaired endothelium-dependent relaxation induced by acetylcholine in vitro and in vivo. Hypertension 53:532–538
22. Yao X, Leung PS, Kwan HY, Wong TP, Fong MW (1999) Rod-type cyclic nucleotide-gated cation channel is expressed in vascular endothelium and vascular smooth muscle cells. Cardiovasc Res 41:282–290

23. Zhang J, Xia SL, Block ER, Patel JM (2002) NO upregulation of a cyclic nucleotide-gated channel contributes to calcium elevation in endothelial cells. Am J Physiol Cell Physiol 283:C1080–C1089
24. Cheng KT, Chan FL, Huang Y, Chan WY, Yao X (2003) Expression of olfactory-type cyclic nucleotide-gated channel (CNGA2) in vascular tissues. Histochem Cell Biol 120:475–481
25. Cheng KT, Leung YK, Shen B et al (2008) CNGA2 channels mediate adenosine-induced Ca^{2+} influx in vascular endothelial cells. Arterioscler Thromb Vasc Biol 28:913–918
26. Shen B, Cheng KT, Leung YK et al (2008) Epinephrine-induced Ca^{2+} influx in vascular endothelial cells is mediated by CNGA2 channels. J Mol Cell Cardiol 45:437–445
27. Wu S, Moore TM, Brough GH et al (2000) Cyclic nucleotide-gated channels mediate membrane depolarization following activation of store-operated calcium entry in endothelial cells. J Biol Chem 275:18887–18896
28. Cioffi DL, Wu S, Alexeyev M, Goodman SR, Zhu MX, Stevens T (2005) Activation of the endothelial store-operated I_{SOC} Ca^{2+} channel requires interaction of protein 4.1 with TRPC4. Circ Res 97:1164–1172
29. Brough GH, Wu S, Cioffi D et al (2001) Contribution of endogenously expressed Trp1 to a Ca^{2+}-selective, store-operated Ca^{2+} entry pathway. FASEB J 15:1727–1738
30. Wu S, Sangerman J, Li M, Brough GH, Goodman SR, Stevens T (2001) Essential control of an endothelial cell I_{SOC} by the spectrin membrane skeleton. J Cell Biol 154:1225–1233
31. Wu S, Cioffi EA, Alvarez D et al (2005) Essential role of a Ca^{2+}-selective, store-operated current (I_{SOC}) in endothelial cell permeability: determinants of the vascular leak site. Circ Res 96:856–863
32. Wu S, Chen H, Alexeyev MF et al (2007) Microtubule motors regulate I_{SOC} activation necessary to increase endothelial cell permeability. J Biol Chem 282:34801–34808
33. Majno G, Palade GE (1961) Studies on inflammation. 1. The effect of histamine and serotonin on vascular permeability: an electron microscopic study. J Biophys Biochem Cytol 11:571–605
34. Majno G, Palade GE, Schoefl GI (1961) Studies on inflammation. II. The site of action of histamine and serotonin along the vascular tree: a topographic study. J Biophys Biochem Cytol 11:607–626
35. Parekh AB, Putney JW Jr (2005) Store-operated calcium channels. Physiol Rev 85:757–810
36. Chetham PM, Babal P, Bridges JP, Moore TM, Stevens T (1999) Segmental regulation of pulmonary vascular permeability by store-operated Ca^{2+} entry. Am J Physiol 276:L41–L50
37. Chetham PM, Guldemeester HA, Mons N et al (1997) Ca^{2+}-inhibitable adenylyl cyclase and pulmonary microvascular permeability. Am J Physiol 273:L22–L30
38. Rosado JA, Jenner S, Sage SOA (2000) Role for the actin cytoskeleton in the initiation and maintenance of store-mediated calcium entry in human platelets. Evidence for conformational coupling. J Biol Chem 275:7527–7533
39. Rosado JA, Sage SO (2000) The actin cytoskeleton in store-mediated calcium entry. J Physiol 526:221–229
40. Rosado JA, Sage SO (2000) A role for the actin cytoskeleton in the initiation and maintenance of store-mediated calcium entry in human platelets. Trends Cardiovasc Med 10:327–332
41. Prasain N, Stevens T (2009) The actin cytoskeleton in endothelial cell phenotypes. Microvasc Res 77:53–63
42. Cioffi DL, Stevens T (2006) Regulation of endothelial cell barrier function by store-operated calcium entry. Microcirculation 13:709–723
43. De Matteis MA, Morrow JS (2000) Spectrin tethers and mesh in the biosynthetic pathway. J Cell Sci 113:2331–2343
44. Davis JQ, Lambert S, Bennett V (1996) Molecular composition of the node of Ranvier: identification of ankyrin-binding cell adhesion molecules neurofascin (mucin$^+$/third FNIII domain$^-$) and NrCAM at nodal axon segments. J Cell Biol 135:1355–1367
45. Joe EH, Angelides K (1992) Clustering of voltage-dependent sodium channels on axons depends on Schwann cell contact. Nature 356:333–335
46. Wood SJ, Slater CR (1998) β-Spectrin is colocalized with both voltage-gated sodium channels and ankyrinG at the adult rat neuromuscular junction. J Cell Biol 140:675–684

47. Lambert S, Davis JQ, Bennett V (1997) Morphogenesis of the node of Ranvier: co-clusters of ankyrin and ankyrin-binding integral proteins define early developmental intermediates. J Neurosci 17:7025–7036
48. Komada M, Soriano P (2002) βIV-Spectrin regulates sodium channel clustering through ankyrin-G at axon initial segments and nodes of Ranvier. J Cell Biol 156:337–348
49. Van Wart A, Boiko T, Trimmer JS, Matthews G (2005) Novel clustering of sodium channel Nav1.1 with ankyrin-G and neurofascin at discrete sites in the inner plexiform layer of the retina. Mol Cell Neurosci 28:661–673
50. Voas MG, Lyons DA, Naylor SG, Arana N, Rasband MN, Talbot WS (2007) αII-Spectrin is essential for assembly of the nodes of Ranvier in myelinated axons. Curr Biol 17:562–568
51. Kosaka T, Komada M, Kosaka K (2008) Sodium channel cluster, βIV-Spectrin and ankyrinG positive "hot spots" on dendritic segments of parvalbumin-containing neurons and some other neurons in the mouse and rat main olfactory bulbs. Neurosci Res 62:176–186
52. Freichel M, Suh SH, Pfeifer A et al (2001) Lack of an endothelial store-operated Ca^{2+} current impairs agonist-dependent vasorelaxation in $TRP4^{-/-}$ mice. Nat Cell Biol 3:121–127
53. Birnbaumer L, Zhu X, Jiang M et al (1996) On the molecular basis and regulation of cellular capacitative calcium entry: roles for Trp proteins. Proc Natl Acad Sci U S A 93:15195–15202
54. Hofmann T, Schaefer M, Schultz G, Gudermann T (2002) Subunit composition of mammalian transient receptor potential channels in living cells. Proc Natl Acad Sci U S A 99:7461–7466
55. Goel M, Sinkins WG, Schilling WP (2002) Selective association of TRPC channel subunits in rat brain synaptosomes. J Biol Chem 277:48303–48310
56. Strübing C, Krapivinsky G, Krapivinsky L, Clapham DE (2003) Formation of novel TRPC channels by complex subunit interactions in embryonic brain. J Biol Chem 278:39014–39019
57. Tiruppathi C, Ahmmed GU, Vogel SM, Malik AB (2006) Ca^{2+} signaling, TRP channels, and endothelial permeability. Microcirculation 13:693–708
58. Tiruppathi C, Freichel M, Vogel SM et al (2002) Impairment of store-operated Ca^{2+} entry in $TRPC4^{-/-}$ mice interferes with increase in lung microvascular permeability. Circ Res 91:70–76
59. Paria BC, Malik AB, Kwiatek AM et al (2003) Tumor necrosis factor-alpha induces nuclear factor-κB-dependent TRPC1 expression in endothelial cells. J Biol Chem 278:37195–37203
60. Townsley MI, King JA, Alvarez DF (2006) Ca^{2+} channels and pulmonary endothelial permeability: insights from study of intact lung and chronic pulmonary hypertension. Microcirculation 13:725–739
61. Alvarez DF, Gjerde EA, Townsley MI (2004) Role of EETs in regulation of endothelial permeability in rat lung. Am J Physiol Lung Cell Mol Physiol 286:L445–L451
62. Alvarez DF, King JA, Townsley MI (2005) Resistance to store depletion-induced endothelial injury in rat lung after chronic heart failure. Am J Respir Crit Care Med 172:1153–1160
63. Schreiber HM, Kannan S (2004) Regulatory role of E-NTPase/E-NTPDase in Ca^{2+}/Mg^{2+} transport via gated channel. Theor Biol Med Model 1:3
64. Millar ID, Bruce J, Brown PD (2007) Ion channel diversity, channel expression and function in the choroid plexuses. Cerebrospinal Fluid Res 4:8
65. Vig M, Peinelt C, Beck A et al (2006) CRACM1 is a plasma membrane protein essential for store-operated Ca^{2+} entry. Science 312:1220–1223
66. Zhang SL, Yeromin AV, Zhang XH et al (2006) Genome-wide RNAi screen of Ca^{2+} influx identifies genes that regulate Ca^{2+} release-activated Ca^{2+} channel activity. Proc Natl Acad Sci U S A 103:9357–9362
67. Feske S, Gwack Y, Prakriya M et al (2006) A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function. Nature 441:179–185
68. Prakriya M, Feske S, Gwack Y, Srikanth S, Rao A, Hogan PG (2006) Orai1 is an essential pore subunit of the CRAC channel. Nature 443:230–233
69. Vig M, Beck A, Billingsley JM et al (2006) CRACM1 multimers form the ion-selective pore of the CRAC channel. Curr Biol 16:2073–2079
70. Ji W, Xu P, Li Z et al (2008) Functional stoichiometry of the unitary calcium-release-activated calcium channel. Proc Natl Acad Sci U S A 105:13668–13673

71. Mignen O, Thompson JL, Shuttleworth TJ (2008) Orai1 subunit stoichiometry of the mammalian CRAC channel pore. J Physiol 586:419–425
72. Wissenbach U, Philipp SE, Gross SA, Cavalie A, Flockerzi V (2007) Primary structure, chromosomal localization and expression in immune cells of the murine ORAI and STIM genes. Cell Calcium 42:439–446
73. DeHaven WI, Smyth JT, Boyles RR, Putney JW Jr (2007) Calcium inhibition and calcium potentiation of Orai1, Orai2, and Orai3 calcium release-activated calcium channels. J Biol Chem 282:17548–17556
74. Gross SA, Wissenbach U, Philipp SE, Freichel M, Cavalie A, Flockerzi V (2007) Murine ORAI2 splice variants form functional Ca^{2+} release-activated Ca^{2+} (CRAC) channels. J Biol Chem 282:19375–19384
75. Vig M, DeHaven WI, Bird GS et al (2008) Defective mast cell effector functions in mice lacking the CRACM1 pore subunit of store-operated calcium release-activated calcium channels. Nat Immunol 9:89–96
76. Cheng KT, Liu X, Ong HL, Ambudkar IS (2008) Functional requirement for Orai1 in store-operated TRPC1-STIM1 channels. J Biol Chem 283:12935–12940
77. Liao Y, Erxleben C, Yildirim E, Abramowitz J, Armstrong DL, Birnbaumer L (2007) Orai proteins interact with TRPC channels and confer responsiveness to store depletion. Proc Natl Acad Sci U S A 104:4682–4687
78. Liao Y, Erxleben C, Abramowitz J et al (2008) Functional interactions among Orai1, TRPCs, and STIM1 suggest a STIM-regulated heteromeric Orai/TRPC model for SOCE/I_{CRAC} channels. Proc Natl Acad Sci U S A 105:2895–2900
79. Ong HL, Cheng KT, Liu X et al (2007) Dynamic assembly of TRPC1-STIM1-Orai1 ternary complex is involved in store-operated calcium influx. Evidence for similarities in store-operated and calcium release-activated calcium channel components. J Biol Chem 282:9105–9116
80. Liao Y, Plummer NW, George MD, Abramowitz J, Zhu MX, Birnbaumer L (2009) A role for Orai in TRPC-mediated Ca^{2+} entry suggests that a TRPC:Orai complex may mediate store and receptor operated Ca^{2+} entry. Proc Natl Acad Sci U S A 106:3202–3206
81. Ambudkar IS, Ong HL, Liu X, Bandyopadhyay BC, Cheng KT (2007) TRPC1: the link between functionally distinct store-operated calcium channels. Cell Calcium 42:213–223
82. Luik RM, Wu MM, Buchanan J, Lewis RS (2006) The elementary unit of store-operated Ca^{2+} entry: local activation of CRAC channels by STIM1 at ER-plasma membrane junctions. J Cell Biol 174:815–825
83. Roos J, DiGregorio PJ, Yeromin AV et al (2005) STIM1, an essential and conserved component of store-operated Ca^{2+} channel function. J Cell Biol 169:435–445
84. Putney JW Jr (2005) Capacitative calcium entry: sensing the calcium stores. J Cell Biol 169:381–382
85. Wu MM, Buchanan J, Luik RM, Lewis RS (2006) Ca^{2+} store depletion causes STIM1 to accumulate in ER regions closely associated with the plasma membrane. J Cell Biol 174:803–813
86. Mercer JC, Dehaven WI, Smyth JT et al (2006) Large store-operated calcium selective currents due to co-expression of Orai1 or Orai2 with the intracellular calcium sensor. Stim1. J Biol Chem 281:24979–24990
87. Soboloff J, Spassova MA, Tang XD, Hewavitharana T, Xu W, Gill DL (2006) Orai1 and STIM reconstitute store-operated calcium channel function. J Biol Chem 281:20661–20665
88. Hoover KB, Bryant PJ (2000) The genetics of the protein 4.1 family: organizers of the membrane and cytoskeleton. Curr Opin Cell Biol 12:229–234
89. Littleton JT, Bhat MA, Bellen HJ (1997) Deciphering the function of neurexins at cellular junctions. J Cell Biol 137:793–796
90. Wu S, Jian M-Y, Xu Y-C, Zhou C, Al-Mehdi A-B, Liedtke W, Shin H-S, Townsley MI (2009) Ca^{2+} entry via 1G and TRPV4 channels differentially regulates surface expression of P-selectin and barrier integrity in pulmonary capillary endothelium. Am J Physiol Lung Cell Mol Physiol 297:L650–L657; doi:10.1152/ajplung.00015.2009

TRPM2 Channel Regulates Endothelial Barrier Function

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Abstract Oxidative stress, through the production of oxygen metabolites such as hydrogen peroxide (H_2O_2), increases vascular endothelial permeability and plays a crucial role in several lung diseases. The transient receptor potential (melastatin) 2 (TRPM2) is an oxidant-sensitive, nonselective cation channel that is widely expressed in mammalian tissues, including the vascular endothelium. We have demonstrated the involvement of TRPM2 in mediating oxidant-induced calcium entry and endothelial hyperpermeability in cultured pulmonary artery endothelial cells. Here, we provide evidence that neutrophil activation-dependent increase in endothelial permeability and neutrophil extravasation requires TRPM2 in cultured endothelial cells. In addition, protein kinase Cα (PKCα) that rapidly colocalizes with the short (nonconducting) TRPM2 isoform after exposure to hydrogen peroxide positively regulates calcium entry through the functional TRPM2 channel. Thus, increase in lung microvessel permeability and neutrophil sequestration depends on the activation of endothelial TRPM2 by neutrophilic oxidants and on PKCα regulation of TRPM2 channel activity. Manipulating TRPM2 function in the endothelium may represent a novel strategy aimed to prevent oxidative stress-related vascular dysfunction.

Keywords Oxidative stress • vascular endothelial permeability

1 Introduction

Reactive oxygen species (ROS) generated at sites of inflammation and injury are important mediators of vascular endothelial barrier dysfunction and edema formation.[1–4] Although the pathophysiology of oxidative stress is not well understood at

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the molecular level, calcium (Ca^{2+}) entry into pulmonary endothelial cells is well recognized to promote gap formation and increase barrier permeability.[5–7] Among the members of the transient receptor potential (TRP) superfamily potentially responsible for oxidative damage in the endothelium,[8–10] we identified the critical role of transient receptor potential melastatin (TRPM) channel 2.[11] TRPM2 is a voltage-independent, calcium-permeable, nonselective cation channel ubiquitously expressed in various mammalian tissues, including the lungs. The channel opening is unique as its gating is induced by the binding of the intracellular second messenger adenosine diphosphoribose (ADP-ribose) to a nudix-box (NUDT9-H)[12–14] in its C terminus domain. The mode of action of oxidants, however, is a matter of debate. Hydrogen peroxide (H_2O_2) produced in the cytosol during oxidative stress[15] activates the nuclear and mitochondrial production of ADP-ribose[16,17] and may also result in the activation of poly-ADP-ribose polymerases (PARPs). PARP enzymes catalyze the breakdown of nicotinamide adenine dinucleotide (NAD) into nicotinamide and ADP-ribose,[18] which in turn opens TRPM2 channels. These channels thus constitute a signaling pathway by which oxidative stress can elicit Ca^{2+} entry and provoke the subsequent specific Ca^{2+}-dependent cellular reactions.[5,6]

Functional TRPM2 molecules are tetramers,[19] and subunit composition is a factor in regulation of the channel opening. In the human, a major transcript encoding the full-length functional TRPM2 (TRPM2-L) is expressed in various tissues, whereas several minor physiological splice variants are more specifically expressed: Among those, a short splicing variant of the TRPM2 gene that was first discovered in human bone marrow has an additional stop codon between exon 16 and exon 17, thus encoding only the first two transmembrane domains of TRPM2-L.[20] The resulting short-form protein (TRPM2-S), through an interaction with TRPM2-L, acts in a dominant-negative fashion to inhibit the formation of functional homotetrameric channels and suppresses TRPM2-S H_2O_2-induced Ca^{2+} influx through TRPM2-L.[11,20] TRPM2-S is indeed an important isoform of TRPM2 that may modulate channel activity and cell death induced by oxidative stress activation of TRPM2-L.[20]

TRPM2 plays a critical role in the mechanism of endothelial barrier disruption following oxidative stress. H_2O_2 at noncytolytic concentrations elicits marked Ca^{2+} influx via TRPM2 channels, which thereby signals increased endothelial permeability.[11] Our goal is to address the potentially crucial role of the endothelial cell plasma membrane TRPM2 channel activation in mediating the neutrophil activation-induced endothelial permeability, neutrophil extravasation, and the signaling mechanisms by which oxidant activation of TRPM2 induces channel opening, thereby allowing Ca^{2+} entry in endothelial cells. The present data suggest that protein kinase Cα (PKCα) regulates oxidant-induced Ca^{2+} entry and increase in endothelial barrier permeability by modulation of TRPM2 channel activation. Inhibition of endogenous PKCα expression and function in endothelial cells by RNA silencing or a pharmacological PKCα blocker significantly decreased H_2O_2-induced increase in intracellular Ca^{2+} and the resulting increase in endothelial permeability. TRPM2 is a potentially important pharmacological target for inhibiting the pathological increase in endothelial permeability.

2 Role of TRPM2 Channel in Mediating H_2O_2-Induced Ca^{2+} and Endothelial Hyperpermeability

2.1 TRP Channels in the Regulation of Lung Endothelial Barrier Function

The vascular endothelium regulates the passage of macromolecules and circulating cells from blood to tissues. Vascular inflammation induces endothelial cell contraction and cell shape changes that result in interendothelial gap formation.[4,21] Specifically, inflammatory mediators such as thrombin and oxidants increase Ca^{2+} permeability of endothelial cell membrane.[5–7] The resulting elevation of intracellular Ca^{2+} could contribute to barrier disruption since Ca^{2+} entry into endothelial cells is recognized to promote interendothelial gap formation. Studies have demonstrated the implication of TRP channels in the regulation of lung vascular permeability and in endothelial barrier dysfunction. The TRPC3/4 and TRPM2 act as endothelial redox sensors, and TRP1, TRPC4, TRPC6, TRPV4, and TRPM2 have been implicated in endothelial barrier dysfunction.[10] Ultimately, TRP channels will become important novel pharmacological targets for the treatment of human vascular diseases.

The depletion of stored Ca^{2+} in response to inflammatory agonists, including thrombin and histamine, activates Ca^{2+} entry response in human pulmonary artery endothelial (HPAE) cells. In the lung endothelial system, store depletion and ensuing activation of Ca^{2+} entry via TRPC1, TRPC4, and TRPC6 has been demonstrated to disrupt the barrier.[22–27] The role of TRPC4 in endothelial permeability increase was demonstrated by Tiruppathi's group in TRPC4$^{-/-}$ knockout mice, in which the lack of thrombin-induced Ca^{2+} entry through the channel interfered with increases in lung vascular permeability.[22] In addition to TRPC4, thrombin activation of protease-activated receptor 1 induces Ca^{2+} entry through store-operated channel TRPC1 in endothelial cells.[23] Specifically, the TRPC1-mediated increase in endothelial permeability may be associated with Rho activation or PKCα phosphorylation.[26] G_q-TRPC6-mediated[24] and TRPC1/4-mediated Ca^{2+} entry[27] were proposed to induce the resultant changes in endothelial cell shape.

2.2 TRPM2 Regulates H_2O_2-Induced Ca^{2+} Entry in Endothelial Cells

We identified another TRP channel, the TRPM2, as responsible for signaling increase in endothelial permeability. The vascular endothelium is a major target of oxidant stress. Oxidants generated at sites of inflammation and injury (i.e., by activation of neutrophils adherent to endothelial cells during sepsis) induced increase in vascular permeability.[1–4] Oxidants increase Ca^{2+} permeability of cell membrane,

which is well known to mediate both long-term and acute endothelial responses to oxidative stress. The first evidence for the involvement of TRP channels in oxidant-induced endothelial injury was proposed by Groschner's group.[28,29] The same group of investigators[29] described a mechanism by which oxidants induced association of the TRPC3 and TRPC4 subunits to form a redox-sensitive cation channel and induce Na^+ and Ca^{2+} entry into porcine aortic endothelial cells through mechanisms that are dependent on phospholipase C. There is, however, growing evidence suggesting the contribution of TRPM2 in signaling oxidant-induced vascular endothelial injury.[10,11]

TRPM2 has been identified in the heart vessel and pulmonary artery endothelium, where it acts as an oxidant sensor and may play a key role in the activation of leukocytes, vascular endothelial permeability, and injury.[10,11] In HPAE cells, we indeed established the role of TRPM2 in mediating the effects of oxidants on the endothelium by mechanisms that involve production of the second messenger ADP-ribose.[11] Using a Ca^{2+} "add-back" protocol, we have shown that H_2O_2-induced Ca^{2+} entry in endothelial cells occurs entirely via TRPM2 channels. In Ca^{2+}-free medium, addition of a sublytic concentration of H_2O_2 (100 μM) released no intracellular Ca^{2+}, whereas Ca^{2+} repletion in the continued presence of H_2O_2 elicited a Ca^{2+} transient (Fig. 10.1), which represents the Ca^{2+} entry stimulated by H_2O_2. Ca^{2+} entry was nearly abolished by TRPM2 silencing and considerably reduced (>65%) by a treatment with a pharmacological inhibitor of PARP to prevent ADP-ribose agonist formation, either the 3,4-dihydro-5-[4-(1-piperidinyl)butoxyl]-1(2H)-isoquinolinone (DPQ; 100 nM) or the 3-aminobenzamide (3-AB; 1 μM) (Fig. 10.1). These observations are consistent with known properties of TRPM2 channel, which is essentially activated by the generated intracellular ligand ADP-ribose. In addition, overexpression of TRPM2-S isoform or a treatment of the same cells with a specific TRPM2-blocking antibody also suppressed Ca^{2+} entry through TRPM2, while TRPC4 silencing did not modify it.[11]

Endothelial TRPM2 activation may play a crucial role in oxidant-induced endothelial injury because excessive activation of these channels results in Ca^{2+} overloading and critical subsequent Ca^{2+}-dependent cellular responses.

2.3 Role of TRPM2 in Inflammation and Oxidant-Induced Vascular Hyperpermeability

At sites of inflammation, neutrophil and endothelial ROS that are produced in substantial amounts increase vascular permeability.[5–7] ROS-induced TRPM2 activation and subsequent increase in intracellular Ca^{2+} causes opening of interendothelial junctions, which is detectable as reduction in transendothelial electrical resistance (TER).[11] As illustrated in Fig. 10.2, we measured the changes in TER in control pulmonary endothelial cell monolayers, cells treated with a PARP inhibitor (DPQ or 3-AB),[11,13,17,20] and cells treated with TRPM2 small-interfering RNA (siRNA). H_2O_2

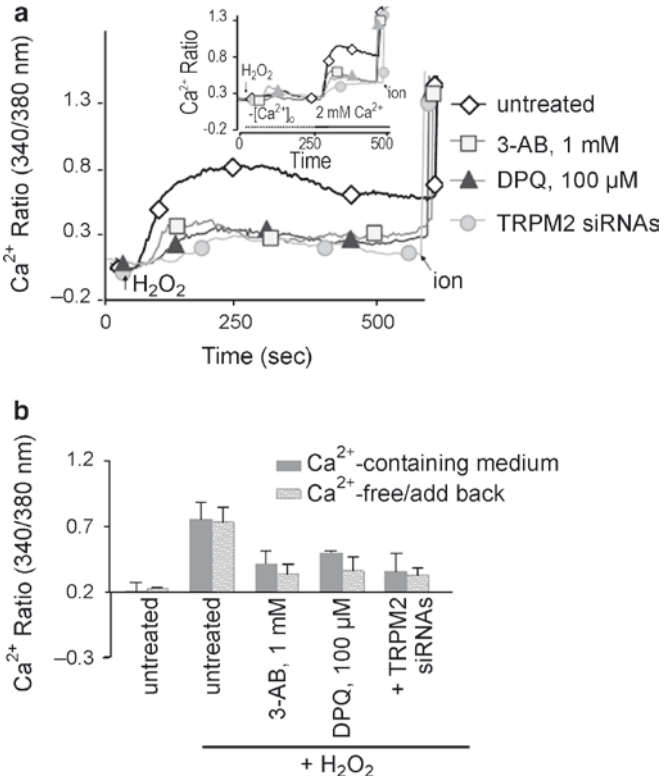

Fig. 10.1 H_2O_2-induced Ca^{2+} entry occurs entirely via TRPM2 channels in endothelial cells. Human lung endothelial cells untreated, transiently transfected with TRPM2 siRNAs, or pretreated with inhibitors of ADP-ribose polymerase (1 m*M* 3-AB or 100 µ*M* DPQ) for 45 min were loaded with fura-2AM. In response to H_2O_2 challenge, we measured intracellular Ca^{2+} in complete (**a**) versus Ca^{2+}-free Hank's Balanced Salt Solution (HBSS) (**a**, *inset*). (**a**) Original recordings of intracellular Ca^{2+} transients without or with pretreatment by specified inhibitors. H_2O_2 (100 µ*M*), $CaCl_2$ (2.0 m*M*), and Ca^{2+} ionophore ionomycin (ion) were added as indicated. The abscissa indicates time in seconds and the ordinate relative intracellular Ca^{2+} level. (**b**) Mean ratiometric values (±SEM) for steady-state intracellular $[Ca^{2+}]$ ($n = 4$). Addition of H_2O_2 to untreated cells elicited a marked cytosolic Ca^{2+} increase (**a**) or Ca^{2+} transient on Ca^{2+} repletion (**a**, *inset*) that was abolished by TRPM2 silencing or treatment with either ADP-ribose polymerase inhibitors; therefore, cytosolic Ca^{2+} increase reflected Ca^{2+} entry through TRPM2

(300 µ*M*) transiently decreased transmonolayer TER, indicating opening of interendothelial junctions. TRPM2 silencing attenuated the peak TER response to H_2O_2 by 44% relative to negative control (nonspecific siRNA transfection). Other approaches used to suppress TRPM2 expression or activity, including treatment of the cells with PARP inhibitor, TRPM2-blocking antibody, and overexpression of TRPM2-S (the nonconducting isoform), attenuated the peak TER response to H_2O_2 by 38–48% (summarized in Fig. 10.2b). Because the balance between short and long TRPM2

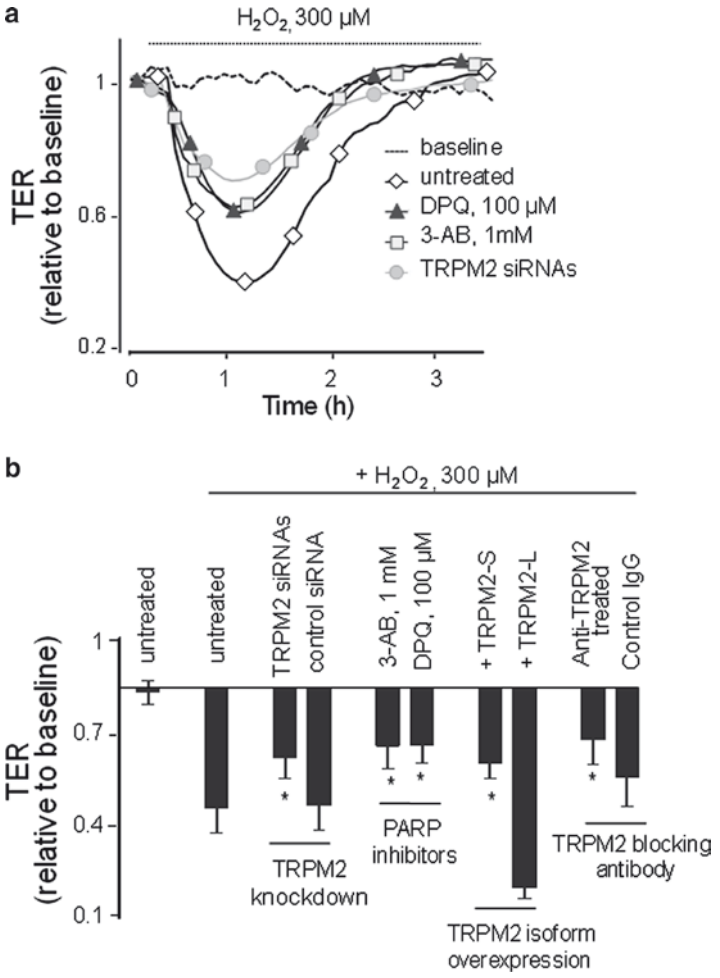

Fig. 10.2 Role of TRPM2 channels in H_2O_2-induced increase in endothelial barrier permeability. Confluent endothelial cell monolayers grown on gold microelectrodes were treated with H_2O_2 (300 μ*M*) or control buffer (for baseline). Changes in TER, reflecting the paracellular permeability of endothelial monolayers, were followed for 4 h. (**a**) Original TER recordings (each trace is the average of four responses). (**b**) Mean value (±SEM) of peak TER responses to H_2O_2. H_2O_2 (300 μ*M*) decreased transmonolayer TER, indicating opening of interendothelial junctions. TRPM2-silencing, treatment with a PARP inhibitor (3-AB or DPQ) or with a specific TRPM2-blocking antibody, and TRPM2-S overexpression attenuated the peak TER response to H_2O_2, while overexpression of TRPM2-L enhanced it. Thus, TRPM2 channels contribute to H_2O_2-induced endothelial barrier hyperpermeability

isoforms determines channel activity, overexpression of TRPM2-L isoform in pulmonary endothelial cells should enhance H_2O_2 effects. As anticipated, overexpression of TRPM2-L augmented H_2O_2-induced endothelial hyperpermeability. The effect was irreversible.[11] Therefore, TRPM2 channel activation plays a crucial role in mediating

increase in endothelial permeability caused by noncytolytic concentrations of H_2O_2. Noticeably, suppression of TRPM2 activity caused only a 50% reduction in the TER response of H_2O_2, suggesting that TRPM2 channels mediate about half of the permeability-increasing effect of H_2O_2. The residual effect of H_2O_2, which appears to be independent of Ca^{2+} entry, remains to be elucidated.

3 Role of TRPM2 Channel-Activated Ca^{2+} Entry in Mediating Neutrophil-Induced Lung Injury

In sepsis-induced acute lung injury, for instance, activated lung macrophages and infiltrated neutrophils generate inflammatory mediators that rapidly propagate to lung endothelial and epithelial cells. Among those, the generated oxygen metabolites and chemotactic cytokines, also called chemokines, increase endothelial adhesivity of neutrophils and vascular endothelial permeability, both critical factors governing tissue edema formation and neutrophil extravasation.[11,30–32] Although we have demonstrated the involvement of TRPM2 in mediating endothelial hyperpermeability in cultured endothelial cells, the in vivo functions of the TRPM2 channel in neutrophil activation-induced endothelial permeability elevation and neutrophil extravasation have not been assessed. Our current studies are showing that neutrophil activation-dependent increase in endothelial permeability requires TRPM2 in cultured endothelial cells.

3.1 Neutrophil-Induced Ca^{2+} Entry in Endothelial Cells Involves TRPM2 Channels

Lung endothelial cells in culture were loaded with fura-2, washed, and transferred to Ca^{2+}-free medium containing the chemoattractant *N*-formyl-Met-Leu-Phe (fMLP), another inflammatory mediator produced as a result of bacterial infection. Human neutrophils (5×10^5 cells/mL) were seeded onto the cells, and Ca^{2+} mobilization in endothelial followed. In response to fMLP, the pulmonary endothelium becomes activated and upregulates surface expression of adhesion molecules. This leads to neutrophil adhesion to endothelial cells and neutrophil activation to produce inflammatory mediators that include oxygen species such as H_2O_2. Ca^{2+} repletion elicited the Ca^{2+} transient, which reflects the Ca^{2+} entry stimulated by neutrophil oxidants. However, TRPM2 silencing and endothelial cell treatment with a PARP inhibitor, DPQ or 3-AB, prevented neutrophil-elicited Ca^{2+} entry observed in untreated cells (unpublished data). Thus, oxidants generated as a result of neutrophil stimulation activated theTRPM2 channel, which appears to be of major importance in the sepsis-induced increase in lung vascular permeability.

3.2 *Neutrophils Activate Endothelial TRPM2 as an Essential Step in Transendothelial Migration*

Because TRPM2 activation mediates endothelial hyperpermeability,[11] we investigated whether neutrophils through TRPM2 activation induce opening of interendothelial junctions, thereby allowing their infiltration into lung tissue. Pulmonary artery endothelial cells were grown to confluence on transwell filters (3-μm pore size). Culture medium containing the chemoattractant fMLP was added to the lower compartment. Neutrophils (5×10^5 cells/mL) were added to the top chambers and allowed to migrate to the lower wells for 2 h at 37°C. In untreated endothelial cells, the number of migrated neutrophils collected from the lower wells represented 61% of the initially seeded neutrophils. TRPM2 knockdown reduced neutrophil transendothelial migration to 32% (unpublished data), which therefore involves TRPM2 channel activation.

These observations suggest that increase in lung microvessel permeability and neutrophil sequestration depend on the activation of endothelial TRPM2 by the neutrophil oxidants. Moreover, Yamamoto[33] established the functional role of monocyte TRPM2 channels in mediating chemokine production and neutrophil-induced lung injury. Chemokine expression is inducible and is responsible for the recruitment of inflammatory cells to sites of infection or injury.[34,35] Thus, in site of sepsis, neutrophil oxidants evoke Ca^{2+} influx through TRPM2 not only in endothelial cells, but also in monocytes where TRPM2-elicited Ca^{2+} influx, via a Ca^{2+}-dependent tyrosine kinase Pyk2/Erk/Ras guanosine triphosphatase (GTPase) signaling pathway, elicits nuclear translocation of nuclear factor-κB (NF-κB) essential to increase the production of the chemokines. The increase in chemokine production in turn increases endothelial adhesivity of neutrophils and generation of ROS and thus aggravates endothelial inflammation and injury.[33] In chronic inflammation, the continued production of ROS by neutrophils causes extensive tissue damage.

In future studies, we will use the TRPM2-deficient mouse to address the crucial role of TRPM2 channel activation in mediating the neutrophil activation-induced increase in lung vascular permeability and edema formation.

4 PKCα Modulation of TRPM2 Channel Regulates H_2O_2-Induced Ca^{2+} Entry and Endothelial Permeability

4.1 *PKCα Modulation of TRPM2 Regulates H_2O_2–Ca^{2+} Entry in Endothelial Cells*

Because TRPM2 is implicated in endothelial dysfunction and many pathological states, elucidating the mechanisms of TRPM2 activation and regulation has gained significant interest. An interesting study by Zhang[36] showed rapid tyrosine phosphorylation of TRPM2-L after stimulation with H_2O_2 that is critical for its activation

and function, while TRPM2-L dephosphorylation by the widely expressed phosphotyrosine phosphatase L1 (PTPL1) resulted in channel inactivation. Although modulation of TRPM2 function by tyrosine dephosphorylation may be a mechanism through which PTPL1 protects the cells against oxidative damage,[36] our current studies are establishing the critical role of PKCα in the regulation of oxidant-induced TRPM2 activation in HPAE cells. PKCα regulates major endothelial cell functions important to maintenance of microvascular homeostasis, including angiogenesis, cell migration, and microvascular permeability.[4,5] Among the endothelial PKC isoforms α, β, δ, ε, and ζ, only PKCα and PKCε were found to change their intracellular distribution on H_2O_2 treatment.[37] Even though oxidants may react with the zinc thiolates in the PKCα regulatory domain to directly oxidize and stimulate PKCα,[37,38] it is still unclear whether ROS directly activate PKCα[38] in the endothelium during oxidative stress. PKCα is known to contribute to H_2O_2-induced increase in endothelial permeability[4,5]; nevertheless, the signaling mechanism is still yet to be defined. We observed that PKCα modulates Ca^{2+} entry through the TRPM2 channel. Using a Ca^{2+} add-back protocol, we have shown that H_2O_2-induced Ca^{2+} entry in endothelial cells occurs entirely via TRPM2 channels.[11] Treatment of the same cells with a pharmacological PKCα inhibitor (Gö6976) or PKCα siRNA partially inhibited Ca^{2+} entry through TRPM2 (unpublished data). Therefore, PKCα positively regulates oxidant-induced TRPM2 activation.

4.2 TRPM2-Activated Increase in Endothelial Permeability Involves a PKCα

Because the results implicate PKCα in the regulation of Ca^{2+} entry through TRPM2, we investigated the role of PKCα in the modulation of TRPM2-induced increase in endothelial barrier permeability. We measured the changes in TER in confluent control monolayers, cells treated with the PKCα inhibitor Gö6976, and cells treated with TRPM2 siRNA alone or with Gö6976 simultaneously. H_2O_2 (300 μ*M*) decreased transmonolayer TER, reflecting the paracellular permeability of endothelial monolayers. TRPM2 silencing reduced the peak TER response to H_2O_2 by 38% relative to untreated control, and PKCα silencing attenuated it by 36%. Interestingly, the peak TER response was decreased to a similar degree by PKCα and TRPM2 silencing (by 40%). Thus, PKCα may play a permissive role in the opening of TRPM2 channels responsible for a significant component of the oxidant-induced increase in TER.

4.3 H_2O_2 Induces PKCα Association with TRPM2-S

In pulmonary endothelial cells, both forms of TRPM2 (TRPM2-L and TRPM2-S) are expressed; therefore, the control of their physical interaction is an enticing potential regulatory mechanism of TRPM2 activity in these cells. In the endothelial

plasma membrane, TRPM2 is associated with its short isoform (TRPM2-S), which serves as a negative regulator of TRPM2 channel activity.[10,11,20] Because we identified a high-affinity binding site for PKCα in the N-terminal domain of TRPM2, we investigated the potential physical interaction of PKCα with a TRPM2 isoform in endothelial cells through a coimmunoprecipitation assay. As predicted, H_2O_2 induced a rapid colocalization of PKCα with TRPM2-S (the short-splice variant of TRPM2) that was not observed when TRPM2 was knocked down (unpublished data); it is therefore likely that PKCα regulates TRPM2-induced Ca^{2+} entry and endothelial injury by PKCα phosphorylation of TRPM2-S.

Further studies are needed to investigate the contribution of PKCα in the control of TRPM2 activity. Using a mutagenesis approach and PKCα-deficient mice, we will establish the mechanisms by which PKCα regulates Ca^{2+} entry through TRPM2 and how PKCα may affect neutrophil-induced lung vascular hyperpermeability and neutrophil infiltration in lung tissue.

5 Conclusions and Perspectives

Lung endothelial injury, particularly in the setting of sepsis, is the result of oxidant generation by endothelial cells themselves, neutrophils, and other inflammatory cells adherent to vessels.[36,39] The mechanism of oxidant-mediated disruption of endothelial barrier function, in part, is attributable to a rise in intracellular Ca^{2+} mediated by Ca^{2+} entry through oxidant-sensitive TRPM2 channels.[11] As represented in Fig. 10.3, our most recent observations imply the potentially crucial role of endothelial TRPM2 channel activation in mediating the neutrophil activation-induced endothelial permeability and neutrophil infiltration in lung tissue as well as the role of PKCα in the regulation of TRPM2 activation and TRPM2-induced increase in endothelial permeability. Because both isoforms of TRPM2 (TRPM2-L and TRPM2-S) are expressed in endothelial cells, the mechanisms that regulate their expression or control of their physical interaction could be targeted to reduce vascular endothelial injury due to oxidant production. PKCα may positively regulate TRPM2-induced Ca^{2+} entry and endothelial permeability by phosphorylation of TRPM2-S. Although these findings may provide possible strategies for modulating endothelial injury, further investigation is required to clarify the contribution of PKCα in the control of TRPM2 activity and the ensuing neutrophil activation-induced lung injury. In this regard, manipulating TRPM2 function in the endothelium represents a novel target to reduce vascular endothelial injury due to oxidant production and inflammation.

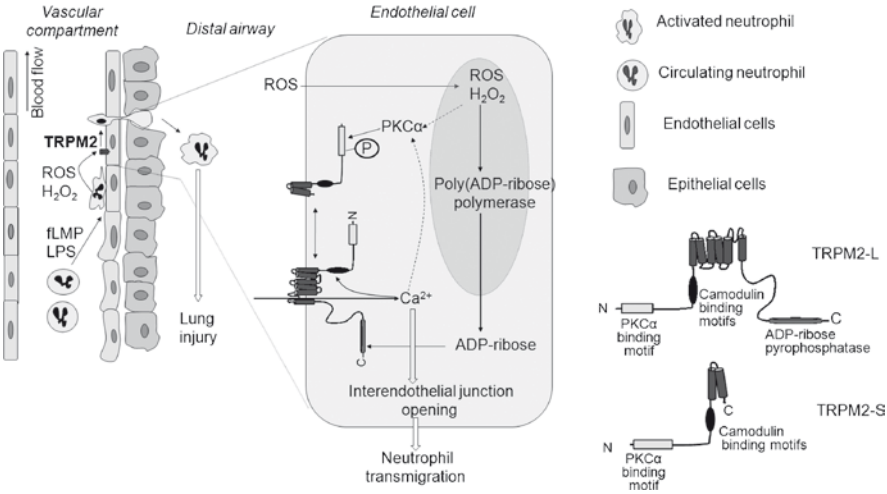

Fig. 10.3 Proposed mechanism for TRPM2 channel-activated Ca^{2+} entry in mediating increase in lung vascular permeability and neutrophil infiltration. In response to LPS and the chemoattractant fMLP, two inflammatory mediators produced as a result of bacterial infection, the pulmonary endothelium becomes activated and upregulates surface expression of adhesion molecules. This leads to neutrophil adhesion to endothelial cells and neutrophil activation to produce inflammatory mediators that include reactive oxygen species (ROS) such as H_2O_2. Neutrophils are also known to cause endothelial ROS production in substantial amounts. The generated ROS induce activation of nuclear poly(ADP-ribose) polymerases (PARPs) and mitochondrial NADases, resulting in the generation of the TRPM2 channel agonist ADP-ribose. It is still unclear whether the generated ROS oxidize and activate directly PKCα in the endothelium during oxidative stress. On ADP-ribose binding to its C terminus NUDTH-9 motif (ADP-ribose pyrophosphatase), TRPM2-evoked Ca^{2+} entry enhances interaction of calmodulin with TRPM2, providing a positive-feedback enhancement of TRPM2 activation.[40] Ca^{2+} entering through TRPM2 may also bind to the "Ca^{2+}-binding loops" of PKCα and trigger PKCα redistribution of this kinase to the membrane, where it interacts with TRPM2-S to regulate the channel activation. *Dotted lines* indicate minor pathways or relationships not generally accepted. Thus, ROS-induced TRPM2 activation mediates Ca^{2+} entry and Ca^{2+}-dependent opening of interendothelial junctions, causing activated neutrophil migration into pulmonary tissue, vascular injury, and edema formation

References

1. Johnson A, Phillips P, Hocking D, Tsan MF, Ferro T (1989) Protein kinase C inhibitor prevents pulmonary edema in response to H_2O_2. Am J Physiol Heart Circ Physiol 256:H1012–H1022
2. Stevens T, Garcia JG, Shasby DM, Bhattacharya J, Malik AB (2000) Mechanisms regulating endothelial cell barrier function. Am J Physiol Lung Cell Mol Physiol 279:L419–L422
3. Barnard ML, Matalon S (1992) Mechanisms of extracellular reactive oxygen species injury to the pulmonary microvasculature. J Appl Physiol 72:1724–1729
4. Lum H, Roebuck K (2001) Oxidant stress and endothelial cell dysfunction. Am J Physiol Cell Physiol 280:C719–C741
5. Siflinger-Birnboim A, Goligorsky MS, Delvecchio PJ, Malik AB (1992) Activation of protein kinase C pathway contributes to hydrogen peroxide-induced increase in endothelial permeability. Lab Invest 67:24–30

6. Dreher D, Junod AF (1995) Differential effects of superoxide, hydrogen peroxide, and hydroxyl radical on intracellular calcium in human endothelial cells. J Cell Physiol 162:147–153
7. Volk T, Hensel M, Kox WJ (1997) Transient Ca^{2+} changes in endothelial cells induced by low doses of reactive oxygen species: role of hydrogen peroxide. Mol Cell Biochem 171:11–21
8. Groschner K, Rosker C, Lukas M (2004) Role of TRP channels in oxidative stress. Novartis Found Symp 258:222–230
9. Kwan HY, Huang Y, Yao X (2007) TRP channels in endothelial function and dysfunction. Biochim Biophys Acta 1772:907–914
10. Dietrich A, Gudermann T (2008) Another TRP to endothelial dysfunction: TRPM2 and endothelial permeability. Circ Res 102:275–277
11. Hecquet CM, Ahmmed GU, Vogel SM, Malik AB (2008) Role of TRPM2 channel in mediating H_2O_2-induced Ca^{2+} entry and endothelial hyperpermeability. Circ Res 102:347–355
12. Sano Y, Inamura K, Miyake A et al (2001) Immunocyte Ca^{2+} influx system mediated by LTRPC2. Science 293:1327–1330
13. Fonfria E, Marshall IC, Benham CD et al (2004) TRPM2 channel opening in response to oxidative stress is dependent on activation of poly(ADP-ribose) polymerase. Br J Pharmacol 143:186–192
14. Perraud AL, Takanishi CL, Shen B et al (2005) Accumulation of free ADP-ribose from mitochondria mediates oxidative stress-induced gating of TRPM2 cation channels. J Biol Chem 280:6138–6148
15. Kraft R, Grimm C, Grosse K et al (2004) Hydrogen peroxide and ADP-ribose induce TRPM2-mediated calcium influx and cation currents in microglia. Am J Physiol 286:C129–C137
16. Heiner I, Eisfeld J, Luckhoff A (2003) Role and regulation of TRP channels in neutrophil granulocytes. Cell Calcium 33:533–540
17. Kolisek M, Beck A, Fleig A, Penner R (2005) Cyclic ADP-ribose and hydrogen peroxide synergize with ADP-ribose in the activation of TRPM2 channels. Mol Cell 18:61–69
18. Kühn FJ, Heiner I, Luckhoff A (2005) TRPM2: a calcium influx pathway regulated by oxidative stress and the novel second messenger ADP-ribose. Pflügers Arch 451:212–219
19. Maruyama Y, Ogura T, Mio K et al (2007) Three-dimensional reconstruction using transmission electron microscopy reveals a swollen, bell-shaped structure of transient receptor potential melastatin type 2 cation channel. J Biol Chem 282:36961–36970
20. Zhang W, Chu X, Tong Q, Cheung JY, Conrad K, Masker K, Miller BA (2003) A novel TRPM2 isoform inhibits calcium influx and susceptibility to cell death. J Biol Chem 278:16222–16229
21. Wysolmerski RB, Lagunoff D (1991) Regulation of permeabilized endothelial cell retraction by myosin phosphorylation. Am J Physiol 261:C32–C40
22. Tiruppathi C, Freichel M, Vogel SM et al (2002) Impairment of store-operated Ca^{2+} entry in $TRPC4^{-/-}$ mice interferes with increase in lung microvascular permeability. Circ Res 91:70–76
23. Paria BC, Vogel SM, Ahmmed GU et al (2004) Tumor necrosis factor-alpha-induced TRPC1 expression amplifies store-operated Ca^{2+} influx and endothelial permeability. Am J Physiol 287:L1303–L1313
24. Singh I, Knezevic N, Ahmmed GU, Kini V, Malik AB, Mehta D (2002) Gαq-TRPC6-mediated Ca^{2+} entry induces RhoA activation and resultant endothelial cell shape change in response to thrombin. J Biol Chem 282:7833–7843
25. Paria BC, Bair AM, Xue J, Yu Y, Malik AB, Tiruppathi C (2006) Ca^{2+} influx induced by protease-activated receptor-1 activates a feed-forward mechanism of TRPC1 expression via nuclear factor-κB activation in endothelial cells. J Biol Chem 281:20715–20727
26. Mehta D, Ahmmed GU, Paria BC et al (2003) RhoA interaction with inositol 1,4,5-trisphosphate receptor and transient receptor potential channel-1 regulates Ca^{2+} entry. Role in signaling increased endothelial permeability. J Biol Chem 278:33492–33500
27. Cioffi DL, Stevens T (2006) Regulation of endothelial cell barrier function by store-operated calcium entry. Microcirculation 13:709–723

28. Balzer M, Lintschinger B, Groschner K (1999) Evidence for a role of Trp proteins in the oxidative stress-induced membrane conductances of porcine aortic endothelial cells. Cardiovasc Res 42:543–549
29. Poteser M, Graziani A, Rosker C et al (2006) TRPC3 and TRPC4 associate to form a redox-sensitive cation channel. Evidence for expression of native $TRPC_3$–$TRPC_4$ heterometric channels in endothelial cells. J Biol Chem 281:13588–13595
30. Kaslovsky RA, Parker K, Siflinger-Birnboim A, Malik AB (1995) Increased endothelial permeability after neutrophil activation occurs by a diffusion-dependent mechanism. Microvasc Res 49:227–232
31. Wang Q, Doerschuk CM (2000) Neutrophil-induced changes in the biomechanical properties of endothelial cells: roles of ICAM-1 and reactive oxygen species. J Immunol 164:6487–6494
32. Siflinger-Birnboim A, Malik AB (1996) Regulation of endothelial permeability by second messengers. New Horiz 4:87–98
33. Yamamoto S, Shimizu S, Kiyonaka S et al (2008) TRPM2-mediated Ca^{2+} influx induces chemokine production in monocytes that aggravates inflammatory neutrophil infiltration. Nat Med 14:738–747
34. Han XB, Liu X, Hsueh W, De Plaen IG (2004) Macrophage inflammatory protein-2 mediates the bowel injury induced by platelet-activating factor. Am J Physiol Gastrointest Liver Physiol 287:G1220–G1226
35. Buanne P, Di Carlo E, Caputi L et al (2007) Crucial pathophysiological role of CXCR2 in experimental ulcerative colitis in mice. J Leukoc Biol 82:1239–1246
36. Zhang W, Tong Q, Conrad K, Wozney J, Cheung JY, Miller BA (2007) Regulation of TRP channel TRPM2 by the tyrosine phosphatase PTPL1. Am J Physiol Cell Physiol 292:C1746–C1758
37. Gopalakrishna R, Anderson WB (1989) Ca^{2+}- and phospholipid-independent activation of protein kinase C by selective oxidative modification of the regulatory domain. Proc Natl Acad Sci U S A 86:6758–6762
38. Gopalakrishna R, Jaken S (2000) Protein kinase C signaling and oxidative stress. Free Rad Biol Med 28:1349–1361
39. Nathan CF (1987) Neutrophil activation on biological surfaces. Massive secretion of hydrogen peroxide in response to products of macrophages and lymphocytes. J Clin Invest 80:1550–1560
40. Tong Q, Zhang W, Conrad K et al (2006) Regulation of the transient receptor potential channel TRPM2 by the Ca^{2+} sensor calmodulin. J Biol Chem 281:9076–9085

Part III
Pathogenic Role of Ion Channels in Pulmonary Vascular Disease

A Proposed Mitochondrial–Metabolic Mechanism for Initiation and Maintenance of Pulmonary Arterial Hypertension in Fawn-Hooded Rats: The Warburg Model of Pulmonary Arterial Hypertension

Jalees Rehman and Stephen L. Archer

Abstract Pulmonary arterial hypertension (PAH) is a disease of the pulmonary vasculature that is characterized by vascular obstruction and progressive right ventricular failure. One hallmark of clinical PAH is its very poor survival, with PAH mortality rates approximating those of many malignancies. The discovery that the fawn-hooded rat strain (FHR) spontaneously develops PAH has allowed for major insights into the pathophysiology of PAH. These findings have revealed that cancer and PAH not only share a similarly poor prognosis but also demonstrate similar resistance to apoptosis and activation of cell proliferation as a major pathophysiologic mechanism. One of the causes for the resistance to apoptosis and increased proliferation of pulmonary vascular smooth muscle cells in PAH is a cancer-like metabolic shift towards a glycolytic metabolism (Warburg effect) and down-regulation of mitochondrial glucose oxidation. This book chapter will review the role of such a metabolic shift in the pathophysiology of PAH and also highlight emerging anti-proliferative PAH therapies that correct the metabolic dysregulation in PAH.

Keywords pulmonary arterial hypertension (PAH) • vascular smooth muscle cell proliferation Warburg effect • cancer • epigenetic silencing • reactive oxygen species • mitochondrial electron transport chain • fawn-hooded rats (FHR) • pyruvate dehydrogenase kinase • glycolysis • hypoxia-inducible factor-1α

1 Introduction

Pulmonary arterial hypertension (PAH) is a disease of the pulmonary vasculature, which occurs in a rare idiopathic form (sporadic PAH-90%, familial PAH-10%) and, more commonly, as a syndrome associated with connective tissue diseases,

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congenital heart disease, anorexigen use, portopulmonary disease, or HIV. The reported prevalence of idiopathic PAH (iPAH) (1/1,000,000) is likely an underestimation, due to lack of data on sickle cell and schistosomiasis-associated PAH syndromes in Africa and Asia, and to insensitivity of the history and physical examination, as suggested by the high prevalence of moderate pulmonary hypertension in surveillance studies of high-risk cohorts with connective tissue diseases.[1] Despite important advances, such as the discovery of mutations in bone morphogenetic protein receptors (BMPR2) as a cause of familial PAH and the advent of effective oral therapies, such as phosphodiesterase 5 inhibitors and endothelin antagonists, mortality remains high (15% at 1 year).[2] Lack of a cure reflects ignorance of the cause of PAH and the related absence of optimally targeted therapies.

2 An Emerging Paradigm: The "Oncologic" View of Pulmonary Artery Hypertension

Recently several groups have concluded that PAH may be viewed, in part, as a disease of excess proliferation and impaired apoptosis of pulmonary artery smooth muscle cells (PASMC),[3–6] similar in some regards to neoplasia.[7,8] Similarities between PASMC in PAH and cancer cells include increased rates of cell proliferation, depressed rates of apoptosis, pathological activation of hypoxia-inducible factor 1α (HIF-1α) as well as metabolic shift towards glycolysis which is characterized by activation of pyruvate dehydrogenase kinase (PDK)[8] and downregulation of voltage-gated potassium channels (K_v)[9] (reviewed in Ref.[10]). Recently, we discovered that strategies which regress experimental PAH[8] such as PDK inhibition and K_v1.5 gene therapy also regress human cancers,[7] thus further highlighting that cancer and PAH may share some underlying pathophysiological pathways. While excessive vasoconstriction,[11–14] inflammation[15] and thrombosis[16] contribute to the pathogenesis of PAH, this new "oncologic view" of PAH, originally proposed by Tuder and Voelkel,[17] may constitute a paradigm-shift from the twentieth-century view of PAH as a primarily vasospastic disease.

3 Overview of Existing Mechanisms of PAH

Although the "oncologic" view of PAH emphasizes the critical role of PASMC hyperproliferation, it is important to recognize that PAH is a panvasculopathy. Abnormalities in each layer of the blood vessel contribute to this syndrome of obstructed, constricted small pulmonary arteries (PA), which ultimately results in right ventricular hypertrophy (RVH). Indicators of endothelial involvement in PAH include elevated plasma serotonin[18] and a decreased ratio of vasodilators/constrictors.[19–21] It is also hypothesized that widespread endothelial apoptosis in early PAH culminates in selection of apoptosis-resistant endothelial precursor cells that proliferate and eventually form plexiform lesions.[22] In the media of the

pulmonary artery, three groups, Rabinovitch's,[6,23] Yuan's[5,24,25] and ours[26–28] have independently shown that PASMC apoptosis is suppressed and proliferation is enhanced in experimental PAH, which is consistent with the findings in human PAH.[29] Many factors drive PASMC proliferation, including mutation[29] or downregulation[30] of BMPR-2, de novo expression of the anti-apoptotic protein survivin,[22,28] increased expression/activity of the serotonin transporter (SERT)[31,32] and increased expression/activity of platelet-derived growth factor (PDGF) receptor.[33] Another proliferative, anti-apoptotic PASMC abnormality is the selective decrease in expression of $K_v1.5$, a voltage-gated, O_2-sensitive potassium channel. The downregulation of $K_v1.5$, which is also the channel that is inhibited by hypoxia and initiates hypoxic pulmonary vasoconstriction,[34,35] is a hall-mark of PAH and occurs in human PAH,[36] experimental PAH models (induced by chronic hypoxia[26,37] or monocrotaline[28)] or genetic predisposition to PAH such as the fawn hooded rat[8] and transgenic mice with PAH due to overexpression of SERT[32] or a BMPR-2 dominant negative mutation.[38] In PAH, loss of $K_v1.5$, depolarizes the membrane and elevates cytosolic levels of K^+ and Ca^{2+}. The resulting calcium overload, which is later reinforced by activation of TRP channels,[39] leads to Ca^{2+}-calcineurin-dependent activation of the proliferative transcription factor, NFAT,[40] thus pointing to a causal role of $K_v1.5$. In the adventitia, metalloprotease activation causes architectural disruption, permitting cell migration and generating mitogenic peptides (tenascin).[23] Finally, infiltration of the lung with inflammatory cells, endothelial-precursor cells, and mesenchymal and bone-marrow-derived stem cells occurs in PAH,[41] and these cells may contribute to the syndrome.

With the discovery of BMPR-mutations in familial PAH,[42,43] the cause of PAH appeared to have been elucidated. These loss-of-function mutations favor PASMC proliferation and consistent with this model, a transgenic mouse with SMC-specific over-expression of a human dominant-negative BMPR2 transgene develops PAH.[44] Interestingly, PAH in BMPR-2 dominant-negative mice is initially not associated with vascular remodeling,[38] but manifests $K_v1.5$ deficiency and can be reversed with an L-type calcium channel blocker.[38] This suggests that disordered BMP signaling, leading to reduced $K_v1.5$ transcription, may be a link between an early vasospastic stage of familial PAH and the later fixed anatomic pathology. Eventually, impaired apoptosis and enhanced PASMC proliferation transforms PAH to a more fixed disease.[27] Indeed only 30–40% of PAH patients have significant vasodilator responses (>20% fall in PVR and PAP) to inhaled nitric oxide,[45,46] which points to vascular remodeling being a key component of the pathology in the majority of PAH patients. However, BMPR2 mutation does not appear to be a common cause of sporadic PAH. BMPR-2 mutations occur in only 10–20% of sporadic PAH patients and even in familial PAH, penetrance is low (~20%).[47] While modifier genes, such as CYP1B1, SERT and TGF-β may explain variable penetrance, aberrant BMPR2 function alone is neither a necessary nor sufficient precondition for most cases of PAH.[47] This literature highlights the multiplicity of putative "causes" for PAH and, in so doing, highlights the lack of a fundamental, initiating cause for nonfamilial PAH. While this partially reflects the fact that PAH is a syndrome, rather than a homogenous disease, it raises the question, "Does an additional unifying cause for PAH exist?" We believe the answer is yes and hypothesize that upstream mitochondrial abnor-

malities lead to metabolic dysregulation which in turn creates a downstream proliferative, anti-apoptotic phenotype.[8] This unifying model integrates the observed metabolic abnormalities in PAH with the critical increase in PASMC proliferation and vascular remodeling and is based on both experimental models of PAH in fawn hooded rats (FHR) and data from humans with PAH.

4 The Fawn-Hooded Rat as a Model for Idiopathic Pulmonary Artery Hypertension

The fawn hooded rats (FHR) are a mutant strain, named for their brown mantle of fur. FHR spontaneously develop PAH.[48] In studying PAH in FHR it is useful to compare FHR to consomic control rats. Consomic rats (FHR-BN1 control rats) were created by introgression of the hypoxia-resistant, Brown Norway rat's chromosome 1 into an isogenic FHR background using marker-assisted selection.[49] Other than chromosome 1 substitution, the consomics are identical to FHR. Importantly, the control FHR-BN1 strain does not develop PAH.[8]

The original FHR were an outbred strain created from "German brown," Lashley and Wistar albino, and Long Evans rats. Three major FHR strains are known: PAH prone FHH/EurMcwiCrl (which we use), a systemically hypertensive FHH strain (prone to glomerulonephritis) and a strain prone to depression/substance addiction. PAH in FHR is heritable and occurs in males and females.[50] In addition, FHR are hypoxia-sensitive, developing PAH and alveolar simplification when exposed to mild hypoxia, at levels that do not affect normal rodents.[50] FHR show exaggerated vasoconstrictor responses when raised at high altitudes.[12] The natural history of FHR likely varies based on concomitant hypoxic exposure. In Denver (elevation 5,200 ft) PAH develops within 1 month of birth.[50] In contrast, in Edmonton, Alberta, at roughly half the altitude, PAH develops at a similar prevalence, but later in life (between 20 and 40 weeks). PAH is ultimately lethal in FHR by ~60 weeks.[8] FHR PASMC share with human PAH PASMC, an exaggerated predilection to proliferate[8,29] and excessive rho kinase activity.[12] Additional similarities with human PAH include enhanced vasoconstriction to serotonin and a platelet storage-pool deficiency.[51] These characteristics of the FHR make it an excellent model to study the pathophysiology of PAH as well as develop novel treatment strategies.

5 A Metabolic Axis of Pulmonary Artery Hypertension

We recently discovered that FHR and humans with iPAH share an unexplained PASMC mitochondrial–metabolic phenotype that underlies their proliferation/apoptosis imbalance. PAH PASMC are characterized by: a fragmented, hyperpolarized

mitochondrial reticulum, decreased superoxide dismutase-2 (SOD2) expression/activity, a metabolic shift away from oxidative metabolism and normoxic activation of the transcription factor HIF-1α (hypoxia inducible factor-1) and the enzyme pyruvate dehydrogenase kinase (PDK). This dysregulation of the metabolic *SOD2-H_2O_2-HIF1-PDK* pathway increases proliferation and suppresses apoptosis in pulmonary artery smooth muscle cells. Since PAH patients demonstrate similar SOD2 downregulation and HIF-1α activation,[52,53] characterization of this metabolic-mitochondrial pathway has significant translational relevance that could impact the development of novel PAH therapies. This chapter reviews recent progress in identifying the mechanisms underlying these PASMC mitochondrial-metabolic abnormalities and in testing their potential as therapeutic targets. The discussion focuses on three key, related mitochondrial-metabolic abnormalities that not only contribute to PAH progression, but may also form a key underlying cause of idiopathic PAH: (1) Epigenetic silencing of SOD2, (2) Activation of the transcription factor HIF-1α and (3) Activation of PDK and glycolytic metabolism. These findings of metabolic dysregulation have paved the way for novel therapeutic strategies which restore SOD2 activity or inhibit HIF-1α and PDK, thus leading to a reduction in SMC proliferation and a regression of PAH.

5.1 Epigenetic Silencing of SOD2 and the Role of H_2O_2 in PAH

SOD2 is located in the mitochondria and is a major source of endogenous H_2O_2. At physiologic levels, H_2O_2 is a vasodilatory and antiproliferative redox-signaling molecule.[54–57] H_2O_2 is produced in mitochondria where SOD2 detoxifies the low basal amounts of superoxide generated by unpaired electron flux during normal activity of the electron transport chain (ETC). Several observations lead us to investigate the importance of SOD2, a mitochondrial protein that is encoded by a gene on rat chromosome 1. First, consomic control rats (FH-BN1), which are identical to FHR save for introgression of a normal chromosome 1 have normal SOD2 levels and do not develop PAH.[8] Second, serial DNA microarray analysis of resistance pulmonary arteries indicates that SOD2 mRNA is downregulated threefold in FHR, prior to onset of pulmonary hypertension. This suggests SOD2 as a candidate PAH gene. Normally, SOD2 expression is induced or repressed to match mitochondrial superoxide production (more superoxide = more SOD2). Consistent with this, oxidant stresses, such as ionizing radiation, induce SOD2 expression in normal animals/cells.[58] This avoids damage to the ETC and mitochondrial DNA. In FHR, decreased SOD2 expression/activity reduces H_2O_2 production.[8] Preliminary data show that restoring mitochondrial H_2O_2, by SOD2 replacement therapy, inhibits PASMC proliferation and enhances apoptosis (unpublished data-not shown). In this context it is critical to realize that H_2O_2 plays an important role in regulating cellular processes such as proliferation and that under-production of this signaling molecule can be harmful. Some groups have also found that in PAH induced by chronic hypoxia the levels of ROS can be increased,[59–61] which underscores the

importance of carefully distinguishing between the effects of specific types of ROS, methods to assess ROS, cellular sources of ROS as well as the dual role of ROS as cause and consequence of PAH during distinct phases of disease progression.

Our unpublished data also suggest that the SOD2 gene and promoter are normal in FHR. This, coupled with the heritable nature of FHR PAH, raises the possibility of epigenetic mechanisms for the SOD2 downregulation. Interestingly, epigenetic silencing of SOD2 is common in many cancers. The SOD2 gene is a putative tumor-suppressor gene (decreased expression is associated with proliferation of cancer cells).[62,63] Epigenetic silencing of SOD2, caused by hypermethylation of CpG dinucleotides within the SOD2 promoter, decreases SOD2 levels in multiple myeloma and pancreatic carcinoma.[64–66] SOD2 downregulation in breast cancer occurs by this mechanism plus a second epigenetic mechanism-histone hypoacetylation. Changes in chromatin acetylation create a repressive chromatin structure that impairs binding of SOD2 transcriptions factor, such as SP-1 and AP-1.[67] HDAC inhibitors, such as trichostatin A and sodium butyrate, restore SOD2 expression in cancer cells.[67] Thus, two epigenetic mechanisms of regulating SOD2 transcription collaborate to control SOD2 expression in cancer.[68,69] In breast cancer and other tumors restoration of SOD2 increases H_2O_2 and limits tumor growth.[65–67] In light of the similarities between cancer and PAH in regards to hyperproliferation of cells, it is likely that similar mechanisms of epigenetic SOD2 silencing may contribute to PASMC hyperproliferation, and our unpublished data suggest that epigenetic downregulation is indeed the cause of reduced SOD2 activity in PAH. This novel finding further points to pharmacological modulation of the epigenetic SOD2 suppression as a potential means for reducing PASMC proliferation and vascular remodeling in PAH.

5.2 HIF-1α Activation and the "Pseudohypoxic" State in PAH

Cancer cells are known to primarily use glycolysis as a source of energy and downregulate mitochondrial activity even in the presence of normal oxygen levels and thrive in this "pseudohypoxic" state. This seminal observation is named the "Warburg effect" because it was made in 1923 by the German scientist and Nobel Prize laureate Otto von Warburg, who also believed that this metabolic switch contributed to the progression of the malignant disease. Even though this observation was made nearly a century ago, the underlying mechanisms of this "pseudohypoxic" state have only recently been elucidated and point to abnormal mitochondrial oxygen-sensing and abnormal activation of the oxygen-sensitive transcription factor HIF-1 as mediators of the "pseudohypoxic" state. Interestingly, this "pseudohypoxic" activation is not only found in cancer cells but also in PAH, and appears to contribute to the pathological cell hyperproliferation in both diseases.

The mitochondrial ETC is the cell's major source of H_2O_2, most of which comes from superoxide anions, produced at complexes I and III, which are converted to H_2O_2 by SOD2.[70] H_2O_2, by virtue of its less toxic nature and moderate diffusion radius, serves as a physiological signaling molecule[71] communicating the "PO_2"

(sensed in the mitochondria) to the plasma membrane (K_v channels[35]) and to transcription factors, notably HIF-1α.[8] We (and others) have demonstrated a PO_2-sensitive ROS production in rodent PA (more oxygen = more ROS).[8,37,70,72–75] This ability to rapidly alter production of the relatively stable ROS H_2O_2 in direct proportion to PO_2 over a physiological PO_2 range (30–100 mmHg)[70,73] is unique to the PASMC mitochondria from small PAs (for example, ROS increase with hypoxia in renal artery SMC).[55] The fact that this superoxide production (and the associated H_2O_2 production by SOD2) is linked (through electron flux) to respiration[76] allows the mitochondria to serve as a cellular "O_2-sensor." Thus, "normoxia" is really a reflection of mitochondrial ROS production and does not always correlate with PO_2. This disconnect is evident for example in cancer or PAH, where low mitochondrial activity reduces superoxide levels and downstream hydrogen peroxide levels are further reduced by low SOD2 expression/activity, despite normal PO_2.

One of the targets of this "pseudohypoxic" state and reduction in H_2O_2 levels is activation of the transcription factor HIF-1α. HIF-1α is a heterodimeric transcription factor, consisting of HIF-1α and HIF-1β subunits.[77] Activation of HIF-1α switches metabolism to glycolysis by activating a panel of glycolytic genes while simultaneously suppressing the activity of the ETC by transactivating the PDK gene, thereby inhibiting the mitochondrial Krebs' (TCA) cycle.[78] Furthermore, HIF-1α activation decreases $K_v1.5$ expression, creating depolarized, calcium-overloaded FHR PASMCs with a proliferative, anti-apoptotic, phenotype[8] with increased activity of the proliferative transcription factor, nuclear factor activating T cells (NFAT).[40] Moreover, HIF-1α haploinsufficient mice are relatively resistant to chronic hypoxic pulmonary hypertension and do not develop downregulation of $K_v1.5$,[79] thus pointing to a causal role of the HIF-1α-$K_v1.5$. pathway in the pathogenesis of PAH. We speculate that the inappropriate normoxic activation of HIF-1α found in FHR and human PAH is triggered by a loss of endogenous H_2O_2. Other groups have also found, that H_2O_2 inactivates HIF-1α,[80,81] as do we.[8]

5.3 PDK Activation in PAH and Cancer

One of the key targets of HIF-1α activation is PDK. Kim et al. and our group have postulated that persistent activation of PDK and subsequent inhibition of pyruvate dehydrogenase (PDH) may be responsible for the "Warburg effect" in cancer cells.[7,78] PDH catalyses the irreversible oxidation of pyruvate, thus yielding acetyl CoA and CO_2, which then enter the TCA cycle and permit mitochondrial production of the electron donors NADH and FADH. PDH is thus a key enzyme controlling the rate of oxidative glycolysis. PDH is tightly controlled by the opposing effects of specific inhibitors (PDKs) and activators (PDH phosphatases). The PDH multi enzyme complex consists of multiple copies of three catalytic subunits, E1 (pyruvate decarboxylase), E2 (dihydrolipoamide acetyltransferase) and E3 (dihydrolipoamide dehydrogenase) in conjunction with the E3 binding protein. Phosphorylation of any of PDH's 3 regulatory serines in E1

by PDK completely inhibits PDH.[82] Four distinct but homologous PDK isoenzymes exist (PDK1–4); however, each isoenzyme displays distinct regulatory properties and tissue distributions. The tissue distribution of the PDK isoforms is similar in rats and humans.[83] PDK is a key regulator of mitochondrial activity since it phosphorylates and inhibits pyruvate dehydrogenase (PDH), thereby slowing the Krebs' cycle and restricting production of reducing equivalents (NADH, FADH) required to donate electrons to the ETC. This "inflow" obstruction may decrease respiration and reduce mitochondrial electron flux. This reduction in mitochondrial electron flux in turn decreases the associated the leak of superoxide which normally occurs in proportion to PO_2 as a result of the 3% of electron flux that is uncoupled.[7,8,84] The net effect of PDK activation is reduced oxidative metabolism and impairment of normoxic electron flux, which reduces mitochondrial ROS production.[37,70] In hypoxia, PDK's inhibition of the ETC is a beneficial, pro-survival mechanism, since ongoing electron transport without oxygen would not generate ATP but would instead cause detrimental mitochondrial superoxide formation by ETC autooxidation and overwhelm the superoxide detoxifying enzyme SOD2. However, even when O_2 is available, pathological activation of PDK can occur and suppress physiological O_2^- and H_2O_2 production in the mitochondria, thus creating a *pseudohypoxic state*, which allows cells to proliferate and prevents their removal by apoptosis. Kim et al. were able to show the central role of PDK as a cellular basis for the "Warburg effect" in P493–6 Burkitt's lymphoma cells, by demonstrating that PDK1 inhibition induces apoptosis in these malignant cells.[78] This is consistent with our demonstration that the PDK inhibitor, dichloroacetate, induces apoptosis and decreases proliferation in three human cancers, studied in a xenotransplantation model.[7] Using siRNA we have demonstrated that knocking down the PDK2 isoform of PDK in cancer cells depolarizes mitochondria and increases ROS production.[7]

PDK activation and impaired mitochondrial ROS production is also a common feature of FHR PAH.[8] Supporting the causal role of PDK activation in PAH is the observation that the PDK inhibitor dichloroacetate can regress all forms of experimental PAH tested to date (chronic hypoxic PHT, monocrotaline PAH and FHR PAH).[8] At effective doses (0.75 g/L of drinking water) dichloroacetate has no overt toxicity over 1–2 months of study.[8] While dichloroacetate inhibits all PDK isoforms, AZD7545 and the other new synthetic PDK inhibitors are selective for PDK2 (IC_{50} 6.4 ± 2.2 nM), with lesser effects on PDK1 (IC_{50} 36.8 ± 18 nM). In contrast, dichloroacetate's IC_{50} for PDK2 is only 200 μm.[83,85] Like dichloroacetate, new PDK2 selective inhibitors are very effective in activating PDH in vivo. However, there are tissue variations in PDH activation and little work has been done on the lung. Dichloroacetate not only restores mitochondrial ROS production, but also eliminates normoxic HIF-1α activation (as evidenced by loss of the nuclear localization of HIF-1α)[8]. This suggests that while HIF-1α can induce PDK expression, the converse is also true: PDK inhibition inactivates HIF-1α[8] and points to a feedback mechanism between HIF-1α and PDK, which based on our data is mediated by mitochondrial production of H_2O_2 (Fig. 11.1).

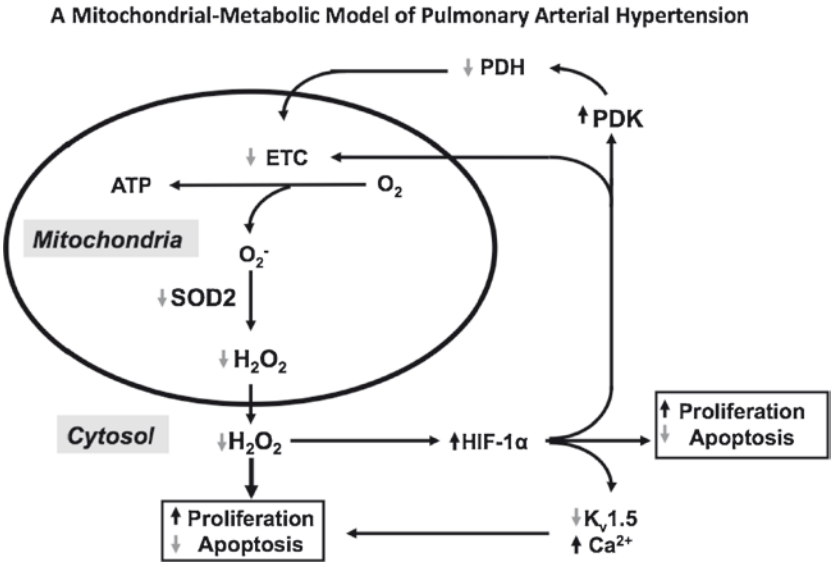

Fig. 11.1 Key aspects of the smooth muscle cell metabolic dysregulation in PAH. In aerobic metabolism, mitochondria are active producers of ATP via the electron transport chain (ETC), which allows electron donors (mitochondrial NADH and FADH) produced by the TCA (Krebs') cycle pass electrons down a redox-potential gradient in the ETC to molecular O_2. Side reactions of molecular O_2, accounting for ~3% of net electron flux, create the reactive oxygen species superoxide (O_2^-) in proportion to PO_2. SOD2 rapidly converts superoxide anion to H_2O_2, which serves as a signaling molecule, since it is less toxic than O_2^- and can diffuse out of the mitochondria due to its neutral charge. At physiological and supra-physiological levels H_2O_2 reduces cell proliferation and promotes apoptosis by either directly acting on the cell cycle or by inhibiting HIF-1α, which then inhibits PDK. This latter enzyme modulates PDH, which in turn enables pyruvate to enter the TCA cycle and aerobic metabolism to progress. In PAH, this balance is perturbed, since SOD2 activity and downstream H_2O_2 production are reduced. Decreased H_2O_2 production activates HIF-1α and reduces mitochondrial activity both by directly downregulating ETC enzymes as well as inhibiting PDH by activating PDK. Downregulation of mitochondrial activity further lowers mitochondrial O_2^- and H_2O_2 thus forming a feedback loop. This feedback loop and the abnormalities found in PAH are shown with arrows indicating the direction of change in activity of each element. The key pathology that contributes to vascular remodeling and PAH is the pro-proliferative and anti-apoptotic effect of this feedback loop. This is in part mediated by HIF-1α dependent and independent mechanisms, which can involve increases in $K_v1.5$ channels and higher cytosolic calcium levels

6 The Vicious Cycle of Metabolic Dysregulation in PAH

The mechanisms involved in the metabolic axis model of PAH that we have described offer numerous targets for the development of novel therapies in patients suffering from PAH. We propose that downregulation of the mitochondrial SOD2 in FHR decreases mitochondrial H_2O_2, production. Due to the neutral nature of the molecule, mitochondrial H_2O_2 can diffuse out of the mitochondria and therefore reduction of mitochondrial H_2O_2 also results in lower cytosolic levels of H_2O_2. This

not only has effects on possible direct biological targets of H_2O_2, but also activates the transcription factor HIF-1α. HIF-1α activation then enhances cell survival/proliferation and also increases PDK expression, which ultimately results in a vicious cycle as PDK inhibits oxidative metabolism and thus mitochondrial O_2^- and mitochondrial H_2O_2. While our unpublished data point to epigenetic silencing of SOD2 and reduction in mitochondrial H_2O_2 production as the initial trigger for this vicious cycle in PAH, the mitochondrial O_2^--SOD2-H_2O_2-HIF-1α-PDK-O_2^- feedback loop may also be initiated or exacerbated by additional triggers acting on elements of this loop (Fig. 11.1). Each of these abnormalities contributes to the abnormal *mitochondria-SOD2-ROS-HIF-PDK* pathway that we have described in human PAH and FHR PAH[8] as well as human cancer.[7]

7 Integrating Other Theories of PAH with the Mitochondrial–Metabolic Model

We acknowledge that many theories and models exist for the etiology of PAH and our focus in this chapter on *mitochondrial–metabolic* pathway does not imply that it is the only or the most important cause of PAH. The proliferation/apoptosis imbalance in PAH likely results from several, intersecting and reinforcing abnormalities that, including BMPR2 mutations,[29] de novo expression of the anti-apoptotic protein survivin[22,28] increased expression/activity of the SERT[31,32] and K_v1.5 downregulation.[8,26,36,37] Likewise, while we focus on a disorder that disturbs proliferation, we acknowledge the importance of endothelial dysfunction due to excess levels of endothelin and insufficient nitric oxide[19] or excessive rho-kinase mediated vasoconstriction.[12] In light of the high morbidity and mortality of PAH and our relatively limited armamentarium to help patients with this terminal disease it is critical that all avenues pointing to novel therapies be pursued. The mitochondrial-metabolic model offers both an explanation of the disease etiology based on numerous published studies as well as novel therapeutic avenues that can be used clinically in a very timely fashion, thus making this a very attractive approach.

8 Summary

Recognition of the central and interrelated roles of SOD2 downregulation, HIF-1α and PDK activation offers many promising therapeutic targets in PAH, including SOD2 replacement therapy, PDK inhibition and HIF-1α inhibition. The translational potential of this hypothesis is strengthened by the availability of drugs that are in clinical use to treat other human diseases, such as the PDK inhibitor dichloroacetate, or pharmacological agents used to act on epigenetic processes such as the DNA methyltransferase inhibitor 5-azacytidine. Dichloroacetate is used to treat

lactic acidosis related to mitochondrial diseases. Other PDK inhibitors are being tested in type II diabetes.[83] 5-Azacytidine (Decitabine®) is used to treat myeloproliferative disorders.[86] The current clinical use of such agents, which act on various components of the described metabolic dysregulation, in diseases such as diabetes or cancer facilitates testing them in clinical PAH trials.

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References

1. Wigley FM, Lima JA, Mayes M, McLain D, Chapin JL, Ward-Able C (2005) The prevalence of undiagnosed pulmonary arterial hypertension in subjects with connective tissue disease at the secondary health care level of community-based rheumatologists (the UNCOVER study). Arthritis Rheum 52:2125–2132
2. Thenappan T, Shah SJ, Rich S, Gomberg-Maitland M (2007) A USA-based registry for pulmonary arterial hypertension: 1982–2006. Eur Respir J 30:1103–1110
3. Platoshyn O, Zhang S, McDaniel SS, Yuan JX-J (2002) Cytochrome c activates K^+ channels before inducing apoptosis. Am J Physiol Cell Physiol 283:C1298–C1305
4. Pozeg ZI, Michelakis ED, McMurtry MS et al (2003) In vivo gene transfer of the O_2-sensitive potassium channel Kv1.5 reduces pulmonary hypertension and restores hypoxic pulmonary vasoconstriction in chronically hypoxic rats. Circulation 107:2037–2044
5. Remillard CV, Yuan JX-J (2004) Activation of K^+ channels: an essential pathway in programmed cell death. Am J Physiol Lung Cell Mol Physiol 286:L49–L67
6. Merklinger SL, Jones PL, Martinez EC, Rabinovitch M (2005) Epidermal growth factor receptor blockade mediates smooth muscle cell apoptosis and improves survival in rats with pulmonary hypertension. Circulation 112:423–431
7. Bonnet S, Archer SL, Allalunis-Turner J et al (2007) A mitochondria-K^+ channel axis is suppressed in cancer and its normalization promotes apoptosis and inhibits cancer growth. Cancer Cell 11:37–51
8. Bonnet S, Michelakis ED, Porter CJ et al (2006) An abnormal mitochondrial-HIF-1-Kv channel pathway disrupts oxygen-sensing and triggers pulmonary arterial hypertension (PAH) in fawn-hooded rats: similarities to human PAH. Circulation 113:2630–2641
9. Yuan JX-J, Aldinger AM, Juhaszova M et al (1998) Dysfunctional voltage-gated K^+ channels in pulmonary artery smooth muscle cells of patients with primary pulmonary hypertension. Circulation 98:1400–1406
10. Archer SL, Gomberg-Maitland M, Maitland ML, Rich S, Garcia JG, Weir EK (2008) Mitochondrial metabolism, redox signaling, and fusion: a mitochondria-ROS-HIF-1(-Kv1.5 O_2-sensing pathway at the intersection of pulmonary hypertension and cancer. Am J Physiol Heart Circ Physiol 294:H570–H578
11. Ishikura K, Yamada N, Ito M et al (2006) Beneficial acute effects of rho-kinase inhibitor in patients with pulmonary arterial hypertension. Circ J 70:174–178
12. Nagaoka T, Gebb SA, Karoor V et al (2006) Involvement of RhoA/Rho kinase signaling in pulmonary hypertension of the fawn-hooded rat. J Appl Physiol 100:996–1002
13. Parker TA, Roe G, Grover TR, Abman SH (2006) Rho kinase activation maintains high pulmonary vascular resistance in the ovine fetal lung. Am J Physiol Lung Cell Mol Physiol 291:L976–L982
14. Xing XQ, Gan Y, Wu SJ, Chen P, Zhou R, Xiang XD (2006) Rho-kinase as a potential therapeutic target for the treatment of pulmonary hypertension. Drug News Perspect 19:517–522

15. Dorfmuller P, Perros F, Balabanian K, Humbert M (2003) Inflammation in pulmonary arterial hypertension. Eur Respir J 22:358–363
16. Otterdal K, Andreassen AK, Yndestad A et al (2008) Raised LIGHT levels in pulmonary arterial hypertension: potential role in thrombus formation. Am J Respir Crit Care Med 177:202–207
17. Voelkel NF, Cool C, Lee SD, Wright L, Geraci MW, Tuder RM (1998) Primary pulmonary hypertension between inflammation and cancer. Chest 114:225S–230S
18. Herve P, Launay JM, Scrobohaci ML et al (1995) Increased plasma serotonin in primary pulmonary hypertension. Am J Med 99:249–254
19. Christman BW, McPherson CD, Newman JH et al (1992) An imbalance between the excretion of thromboxane and prostacyclin metabolites in pulmonary hypertension. N Engl J Med 327:70–75
20. Steudel W, Ichinose F, Huang PL et al (1997) Pulmonary vasoconstriction and hypertension in mice with targeted disruption of the endothelial nitric oxide synthase (NOS 3) gene. Circ Res 81:34–41
21. Stewart DJ, Levy RD, Cernacek P, Langleben D (1991) Increased plasma endothelin-1 in pulmonary hypertension: marker or mediator of disease? Ann Intern Med 114:464–469
22. Sakao S, Taraseviciene-Stewart L, Lee JD, Wood K, Cool CD, Voelkel NF (2005) Initial apoptosis is followed by increased proliferation of apoptosis-resistant endothelial cells. FASEB J 19(9):1178–1180
23. Cowan KN, Jones PL, Rabinovitch M (2000) Elastase and matrix metalloproteinase inhibitors induce regression, and tenascin-C antisense prevents progression, of vascular disease. J Clin Invest 105:21–34
24. Krick S, Platoshyn O, Sweeney M, Kim H, Yuan JX-J (2001) Activation of K^+ channels induces apoptosis in vascular smooth muscle cells. Am J Physiol Cell Physiol 280:C970–C979
25. Ekhterae D, Platoshyn O, Krick S, Yu Y, McDaniel SS, Yuan JX-J (2001) Bcl-2 decreases voltage-gated K^+ channel activity and enhances survival in vascular smooth muscle cells. Am J Physiol Cell Physiol 281:C157–C165
26. Michelakis ED, McMurtry MS, Wu X-C et al (2002) Dichloroacetate, a metabolic modulator, prevents and reverses chronic hypoxic pulmonary hypertension in rats: role of increased expression and activity of voltage-gated potassium channels. Circulation 105:244–250
27. McMurtry MS, Bonnet S, Wu X et al (2004) Dichloroacetate prevents and reverses pulmonary hypertension by inducing pulmonary artery smooth muscle cell apoptosis. Circ Res 95:830–840
28. McMurtry MS, Archer SL, Altieri DC et al (2005) Gene therapy targeting survivin selectively induces pulmonary vascular apoptosis and reverses pulmonary arterial hypertension. J Clin Invest 115:1479–1491
29. Morrell NW, Yang X, Upton PD et al (2001) Altered growth responses of pulmonary artery smooth muscle cells from patients with primary pulmonary hypertension to transforming growth factor-β_1 and bone morphogenetic proteins. Circulation 104:790–795
30. McMurtry MS, Moudgil R, Hashimoto K, Bonnet S, Michelakis ED, Archer SL (2007) Overexpression of human bone morphogenetic protein receptor 2 does not ameliorate monocrotaline pulmonary arterial hypertension. Am J Physiol Lung Cell Mol Physiol 292:L872–L878
31. Eddahibi S, Raffestin B, Hamon M, Adnot S (2002) Is the serotonin transporter involved in the pathogenesis of pulmonary hypertension? J Lab Clin Med 139:194–201
32. Guignabert C, Izikki M, Tu LI et al (2006) Transgenic mice overexpressing the 5-hydroxytryptamine transporter gene in smooth muscle develop pulmonary hypertension. Circ Res 98:1323–1330
33. Schermuly RT, Dony E, Ghofrani HA et al (2005) Reversal of experimental pulmonary hypertension by PDGF inhibition. J Clin Invest 115:2811–2821
34. Archer SL, Souil E, Dinh-Xuan AT et al (1998) Molecular identification of the role of voltage-gated K^+ channels, Kv1.5 and Kv2.1, in hypoxic pulmonary vasoconstriction and control of resting membrane potential in rat pulmonary artery myocytes. J Clin Invest 101:2319–2330

35. Archer SL, Wu X-C, Thébaud B et al (2004) Preferential expression and function of voltage-gated, O_2-sensitive K^+ channels in resistance pulmonary arteries explains regional heterogeneity in hypoxic pulmonary vasoconstriction. ionic diversity in smooth muscle cells. Circ Res 95:308–318
36. Yuan XJ, Wang J, Juhaszova M, Gaine SP, Rubin LJ (1998) Attenuated K^+ channel gene transcription in primary pulmonary hypertension. Lancet 351:726–727
37. Reeve HL, Michelakis E, Nelson DP, Weir EK, Archer SL (2001) Alterations in a redox oxygen sensing mechanism in chronic hypoxia. J Appl Physiol 90:2249–2256
38. Young KA, Ivester C, West J, Carr M, Rodman DM (2006) BMP signaling controls PASMC K_V channel expression in vitro and in vivo. Am J Physiol Lung Cell Mol Physiol 290:L841–L848
39. Landsberg JW, Yuan JX-J (2004) Calcium and TRP channels in pulmonary vascular smooth muscle cell proliferation. News Physiol Sci 19:44–50
40. Bonnet S, Rochefort G, Sutendra G et al (2007) The nuclear factor of activated T cells in pulmonary arterial hypertension can be therapeutically targeted. Proc Natl Acad Sci USA 104:11418–11423
41. Davie NJ, Crossno JT, Jr., Frid MG et al (2004) Hypoxia-induced pulmonary artery adventitial remodeling and neovascularization: contribution of progenitor cells. Am J Physiol Lung Cell Mol Physiol 286:L668–L678
42. Deng Z, Morse JH, Slager SL et al (2000) Familial primary pulmonary hypertension (gene PPH1) is caused by mutations in the bone morphogenetic protein receptor-II gene. Am J Hum Genet 67:737–744
43. Thomson JR, Machado RD, Pauciulo MW et al (2000) Sporadic primary pulmonary hypertension is associated with germline mutations of the gene encoding BMPR-II, a receptor member of the TGF-β family. J Med Genet 37:741–745
44. West J, Fagan K, Steudel W et al (2004) Pulmonary hypertension in transgenic mice expressing a dominant-negative BMPRII gene in smooth muscle. Circ Res 94:1109–1114
45. Ricciardi MJ, Knight BP, Martinez FJ, Rubenfire M (1998) Inhaled nitric oxide in primary pulmonary hypertension: a safe and effective agent for predicting response to nifedipine. J Am Coll Cardiol 32:1068–1073
46. Sitbon O, Humbert M, Jagot JL et al (1998) Inhaled nitric oxide as a screening agent for safely identifying responders to oral calcium-channel blockers in primary pulmonary hypertension. Eur Respir J 12:265–270
47. West J, Cogan J, Geraci M et al (2008) Gene expression in BMPR2 mutation carriers with and without evidence of pulmonary arterial hypertension suggests pathways relevant to disease penetrance. BMC Med Genomics 1:45
48. Kentera D, Susic D, Veljkovic V, Tucakovic G, Koko V (1988) Pulmonary artery pressure in rats with hereditary platelet function defect. Respiration 54:110–114
49. Cowley AW, Jr., Liang M, Roman RJ, Greene AS, Jacob HJ (2004) Consomic rat model systems for physiological genomics. Acta Physiol Scand 181:585–592
50. Sato K, Webb S, Tucker A et al (1992) Factors influencing the idiopathic development of pulmonary hypertension in the fawn hooded rat. Am Rev Respir Dis 145:793–797
51. Ashmore RC, Rodman DM, Sato K et al (1991) Paradoxical constriction to platelets by arteries from rats with pulmonary hypertension. Am J Physiol 260:H1929–H1934
52. Bowers R, Cool C, Murphy RC et al (2004) Oxidative stress in severe pulmonary hypertension. Am J Respir Crit Care Med 169:764–769
53. Tuder RM, Chacon M, Alger L et al (2001) Expression of angiogenesis-related molecules in plexiform lesions in severe pulmonary hypertension: evidence for a process of disordered angiogenesis. J Pathol 195:367–374
54. Burke-Wolin T, Wolin MS (1989) H2O2 and cGMP may function as an O_2 sensor in the pulmonary artery. J Appl Physiol 66:167–170
55. Michelakis ED, Hampl V, Nsair A et al (2002) Diversity in mitochondrial function explains differences in vascular oxygen sensing. Circ Res 90:1307–1315

56. Wolin MS, Burke TM (1987) Hydrogen peroxide elicits activation of bovine pulmonary arterial soluble guanylate cyclase by a mechanism associated with its metabolism by catalase. Biochem Biophys Res Commun 143:20–25
57. Michelakis ED, Rebeyka I, Wu X et al (2002) O_2 sensing in the human ductus arteriosus: regulation of voltage-gated K^+ channels in smooth muscle cells by a mitochondrial redox sensor. Circ Res 91:478–486
58. Guo G, Yan-Sanders Y, Lyn-Cook BD et al (2003) Manganese superoxide dismutase-mediated gene expression in radiation-induced adaptive responses. Mol Cell Biol 23:2362–2378
59. Redout EM, Wagner MJ, Zuidwijk MJ et al (2007) Right-ventricular failure is associated with increased mitochondrial complex II activity and production of reactive oxygen species. Cardiovasc Res 75:770–781
60. Liu JQ, Zelko IN, Erbynn EM, Sham JSK, Folz RJ (2006) Hypoxic pulmonary hypertension: role of superoxide and NADPH oxidase (gp91phox). Am J Physiol Lung Cell Mol Physiol 290:L2–L10
61. Nozik-Grayck E, Suliman HB, Majka S et al (2008) Lung EC-SOD overexpression attenuates hypoxic induction of Egr-1 and chronic hypoxic pulmonary vascular remodeling. Am J Physiol Lung Cell Mol Physiol 295:L422–L430
62. Li N, Oberley TD, Oberley LW, Zhong W (1998) Overexpression of manganese superoxide dismutase in DU145 human prostate carcinoma cells has multiple effects on cell phenotype. Prostate 35:221–233
63. Bravard A, Sabatier L, Hoffschir F, Ricoul M, Luccioni C, Dutrillaux B (1992) SOD2: a new type of tumor-suppressor gene? Int J Cancer 51:476–480
64. Hodge DR, Xiao W, Peng B, Cherry JC, Munroe DJ, Farrar WL (2005) Enforced expression of superoxide dismutase 2/manganese superoxide dismutase disrupts autocrine interleukin-6 stimulation in human multiple myeloma cells and enhances dexamethasone-induced apoptosis. Cancer Res 65:6255–6263
65. Hurt EM, Thomas SB, Peng B, Farrar WL (2007) Molecular consequences of SOD2 expression in epigenetically silenced pancreatic carcinoma cell lines. Br J Cancer 97:1116–1123
66. Hurt EM, Thomas SB, Peng B, Farrar WL (2007) Integrated molecular profiling of SOD2 expression in multiple myeloma. Blood 109:3953–3962
67. Hitchler MJ, Oberley LW, Domann FE (2008) Epigenetic silencing of SOD2 by histone modifications in human breast cancer cells. Free Radic Biol Med 45(11):1573–1580
68. Fuks F, Hurd PJ, Deplus R, Kouzarides T (2003) The DNA methyltransferases associate with HP1 and the SUV39H1 histone methyltransferase. Nucleic Acids Res 31:2305–2312
69. Fuks F, Hurd PJ, Wolf D, Nan X, Bird AP, Kouzarides T (2003) The methyl-CpG-binding protein MeCP2 links DNA methylation to histone methylation. J Biol Chem 278:4035–4040
70. Archer SL, Huang J, Henry T, Peterson D, Weir EK (1993) A redox-based O_2 sensor in rat pulmonary vasculature. Circ Res 73:1100–1112
71. Schroder E, Eaton P (2008) Hydrogen peroxide as an endogenous mediator and exogenous tool in cardiovascular research: issues and considerations. Curr Opin Pharmacol 8:153–159
72. Archer SL, Will JA, Weir EK (1986) Redox status in the control of pulmonary vascular tone. Herz 11:127–141
73. Archer SL, Nelson DP, Weir EK (1989) Simultaneous measurement of O_2 radicals and pulmonary vascular reactivity in rat lung. J Appl Physiol 67:1903–1911
74. Archer SL, Weir EK, Reeve HL, Michelakis E (2000) Molecular identification of O_2 sensors and O_2-sensitive potassium channels in the pulmonary circulation. Adv Exp Med Biol 475:219–240
75. Archer SL, Michelakis ED, Thebaudt B et al (2006) A central role for oxygen-sensitive K^+ channels and mitochondria in the specialized oxygen-sensing system. Novartis Found Symp 272:157–171; discussion 71–75, 214–217
76. Cadenas E, Davies KJ (2000) Mitochondrial free radical generation, oxidative stress, and aging. Free Radic Biol Med 29:222–230

77. Semenza GL (2004) O_2-regulated gene expression: transcriptional control of cardiorespiratory physiology by HIF-1. J Appl Physiol 96:1173–1177; discussion 0–2
78. Kim JW, Tchernyshyov I, Semenza GL, Dang CV (2006) HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. Cell Metab 3:177–185
79. Shimoda LA, Manalo DJ, Sham JSK, Semenza GL, Sylvester JT (2001) Partial HIF-1α deficiency impairs pulmonary arterial myocyte electrophysiological responses to hypoxia. Am J Physiol Lung Cell Mol Physiol 281:L202–L208
80. Huang LE, Arany Z, Livingston DM, Bunn HF (1996) Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit. J Biol Chem 271:32253–32259
81. Wang GL, Jiang BH, Semenza GL (1995) Effect of altered redox states on expression and DNA-binding activity of hypoxia-inducible factor 1. Biochem Biophys Res Commun 212:550–556
82. Roche TE, Baker JC, Yan X et al (2001) Distinct regulatory properties of pyruvate dehydrogenase kinase and phosphatase isoforms. Prog Nucleic Acid Res Mol Biol 70:33–75
83. Mayers RM, Leighton B, Kilgour E (2005) PDH kinase inhibitors: a novel therapy for Type II diabetes? Biochem Soc Trans 33:367–370
84. Freeman BA, Crapo JD (1981) Hyperoxia increases oxygen radical production in rat lungs and lung mitochondria. J Biol Chem 256:10986–10992
85. Morrell JA, Orme J, Butlin RJ, Roche TE, Mayers RM, Kilgour E (2003) AZD7545 is a selective inhibitor of pyruvate dehydrogenase kinase 2. Biochem Soc Trans 31:1168–1170
86. Silverman LR, Mufti GJ (2005) Methylation inhibitor therapy in the treatment of myelodysplastic syndrome. Nat Clin Pract Oncol 2(Suppl 1):S12–S23

The Role of Classical Transient Receptor Potential Channels in the Regulation of Hypoxic Pulmonary Vasoconstriction

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Abstract Hypoxic pulmonary vasoconstriction (HPV) is an essential mechanism of the lung matching blood perfusion to ventilation during local alveolar hypoxia. HPV thus optimizes pulmonary gas exchange. In contrast chronic and generalized hypoxia leads to pulmonary vascular remodeling with subsequent pulmonary hypertension and right heart hypertrophy. Among other non-selective cation channels, the family of classical transient receptor potential channels (TRPC) has been shown to be expressed in pulmonary arterial smooth muscle cells. Among this family, TRPC6 is essential for the regulation of acute HPV in mice. Against this background, in this chapter we give an overview about the TRPC family and their role in HPV.

Keywords Hypoxia • lung • TRPC • pulmonary arterial smooth muscle cells

1 Introduction

Hypoxic pulmonary vasoconstriction (HPV) is a physiological lung mechanism that directs blood perfusion from poorly ventilated to well-ventilated lung areas to optimize gas exchange.[1] In contrast to the systemic circulation, in which hypoxia leads to vasodilation, HPV is unique to the pulmonary vasculature[2] (Fig. 12.1a) and is triggered by mild hypoxia (alveolar $pO_2 < 100$ mmHg).[1] A disturbance of this mechanism may result in life-threatening hypoxemia[3] (Fig. 12.1b). Furthermore, when hypoxia is generalized and chronic, as in many lung diseases (e.g., chronic obstructive pulmonary disease [COPD], pneumonia, fibrosis) or in residents at high

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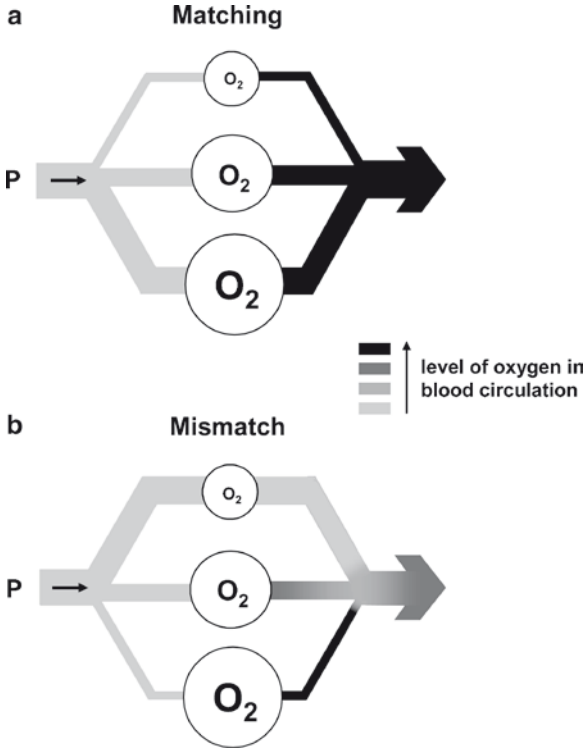

Fig. 12.1 Ventilation-perfusion matching by hypoxic pulmonary vasoconstriction (HPV) in the lung under (patho)physiological conditions (P = blood flow). (**a**) Under physiological conditions, perfusion is matched to the alveolar oxygen partial pressure (pO_2) by HPV. Reproduced with permission.[9] (**b**) Under disturbed HPV, a mismatch of blood flow and alveolar ventilation may result in hypoxemia. The blood perfusion color indicates the oxygen concentration of the blood in a graded manner: the darker the color, the higher the oxygen concentration. *Large circles* high alveolar pO_2, *small circles* low alveolar pO_2

altitude, the subsequent pulmonary vasoconstriction leads to chronic pulmonary hypertension, vascular remodeling, and right heart failure.[4,5]

Although the physiological function of HPV was originally described in 1946 by Euler and Liljestrand, the underlying oxygen-sensing and signal transduction processes remain unclear.[6] Several hypothetical mechanisms for the O_2-sensing in pulmonary artery smooth muscle cells (PASMC) are currently proposed, for the most part related to reactive oxygen species (ROS).[7] Nicotinamide adenine dinucleotide phosphate-oxidase (NAD(P)H) oxidases, similar to that found in neutrophils, as well as mitochondria are strongly suggested to affect superoxide generation during hypoxia. However, it is unclear whether ROS generation is increased or decreased under hypoxic conditions. Moreover, there is also evidence for a hypoxia-induced increase of cyclic adenosine diphosphate-ribose (cADPR), resulting in a rise of intracellular Ca^{2+} ($[Ca^{2+}]_i$) as well as a role for cytochrome P450-dependent processes in HPV.[1]

The smooth muscle layer of the precapillary vessels has been identified as the effector cell type. Moreover, isolated PASMCs respond to acute hypoxia by an increase of the $[Ca^{2+}]_i$ concentration and subsequent contraction, indicating that these cells function as sensor as well as effector cells.[8]

In addition to L-type voltage-operated Ca^{2+} channels (VOCC), nonselective cation channels have been identified as important players in the regulation of vascular tone by their role in mediating the entry of cations like Ca^{2+} and Na^{+}.[9] Among other nonselective cation channels, the family of classical transient receptor potential (TRPC) channels has been shown to be expressed in pulmonary arterial smooth muscle[10–12] and to play a major role in the regulation of HPV under acute hypoxia.[5,13,14]

2 The Classical Transient Receptor Potential Family of Nonselective Cation Channels

2.1 Introduction

Transient receptor potential (TRP) channels were first discovered in the fruit fly *Drosophila melanogaster* and constitute a superfamily of cation channels. In contrast to vertebrates, the transduction of visual stimuli in *Drosophila melanogaster* is a phospholipase C-dependent process that leads to an activation of membrane channels with subsequent membrane depolarization.[15,16] Interestingly, *Drosophila melanogaster* with a mutation in the *trp* locus exhibited a transient instead of a sustained response to light due to a defect in Ca^{2+} influx following the initial Na^{+} influx. The channels responsible for this light-induced Ca^{2+} influx were named TRP channels.[17,18]

2.2 Identification and Structural Properties of Mammalian TRP Channels

The first mammalian TRP channel closely related to *Drosophila melanogaster* TRP channels was identified in 1995 and founded a new TRP family called the classical or canonical TRP (TRPC) channels.[19] In the ensuing years, six other TRPC family members and other TRP families, such as TRPM (for melastatin), TRPV (for vanilloid receptor), TRPP (for polycystic kidney disease (PKD) proteins), TRPML (for mucolipidins), and TRPA (for ankyrin-rich proteins) were identified. Figure 12.2a shows the phylogenetic tree of the TRP superfamily.[15] The classification of the mammalian TRP proteins in these six groups results from their structure (≥90% amino acid similarity within each group), whereas the characteristic features of TRP proteins in general are the six transmembrane domains (S1–S6), cytoplasmic N- and C-termini, and a hydrophobic loop between S5 and S6, as well as cation permeability[15] (Fig. 12.2b). Thus, the channel pore is thought to be formed

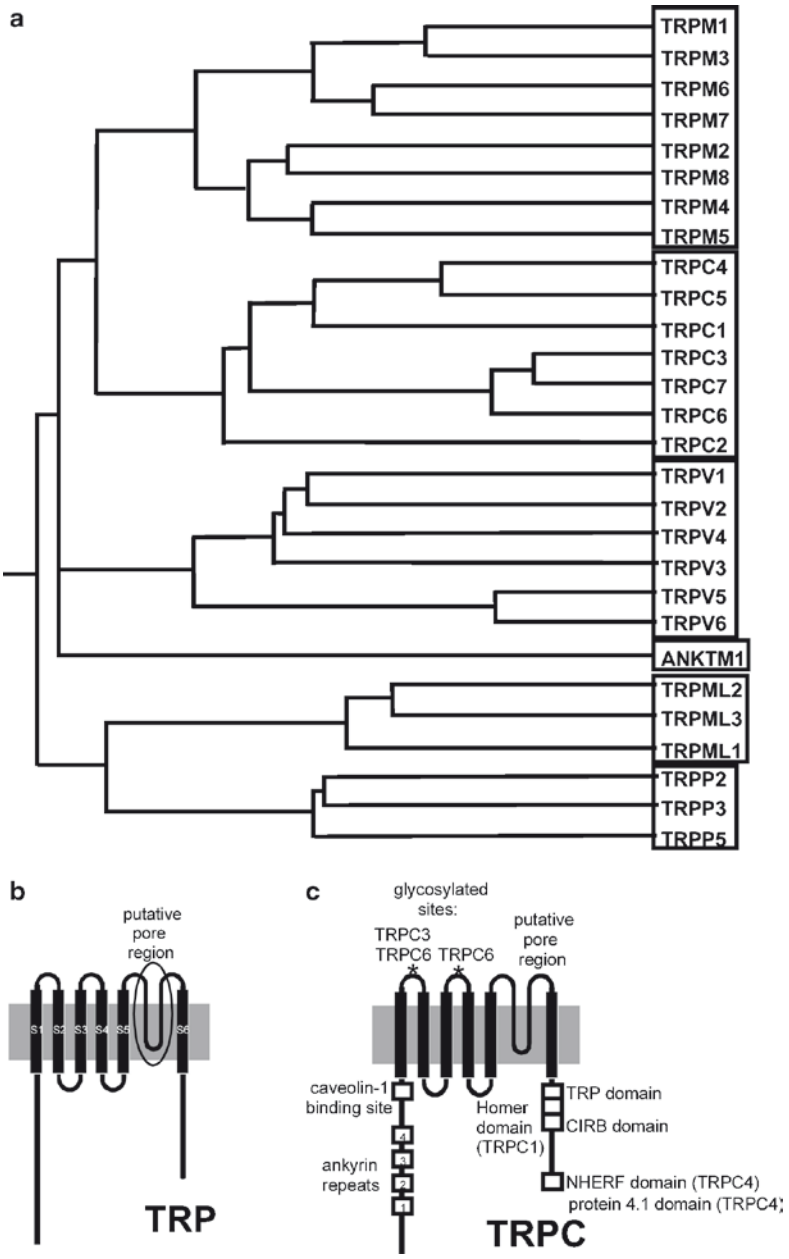

Fig. 12.2 Phylogenetic tree and plasma membrane topology of the TRP proteins. (**a**) Phylogenetic tree of the TRP superfamily subdivided into six families according to their amino acid homology (≥90% within each group).[15] (**b**) Plasma membrane topology of TRP proteins: six transmembrane domains (S1–S6) with a hydrophobic putative pore region between S5 and S6 and the cytoplasmic N- and C-termini.[15] (**c**) Plasma membrane topology and functional domains of TRPC channels. TRPC channels have typically four ankyrin repeats, a caveolin-1-binding site, and a TRPC domain as well as a calmodulin IP_3-receptor-binding domain (CIRB). In addition, there is one glycosylation site in TRPC3, two glycosylation sites in TRPC6, a protein 4.1-binding domain, and an Asparagin-Histidin-Glutamat-Arginin-Phenylalanin-binding domain in TRPC4, as well as a homer domain in TRPC1. Reproduced with permission[9]

by the loop between S5 and S6. TRP channels are homo- or heterotetramers with members of the same subfamily[15] and are permeable to Na^+, K^+, Cs^+, Li^+, Ca^{2+}, Mn^{2+}, and Mg^{2+} after voltage-independent activation.[20]

2.3 Characteristics of the TRPC Channel Subfamily

The seven members of the TRPC family share common structural features in addition to the six transmembrane-spanning domains (S1–S6) and the putative pore region between S5 and S6 as the typical TRP structure: four N-terminal ankyrin repeats as well as a TRP box (amino acid sequence EWKFAR) and a calmodulin inositol 1,4,5-trisphosphate (IP_3)-receptor-binding (CIRB) site in the C-terminal tail. Other protein-binding domains vary between the different TRPC proteins (Fig. 12.2c). The TRPC family can be divided into three subfamilies on the basis of their amino acid homology: TRPC1, TRPC4/5, and TRPC3/6/7. TRPC2 is a pseudogene in humans but plays an important role in the sexual recognition of mice.[9,21]

Whereas TRPC4 and TRPC5 share approximately 65% of the amino acid sequence, the members of the TRPC3/6/7 subfamily form a structural and functional subfamily with 70–80% homology of the amino acid sequence and direct activation by diacylglycerol (DAG).[9] DAG production results from activation of G protein-coupled receptors or receptor tyrosine kinases and subsequent activation of phospholipase C isoforms (PLCβ or PLCγ, respectively), leading to hydrolysis of phosphatidylinositol 4,5-bisphosphate.[22] While DAG mediates cation entry through receptor-operated channels (ROCs), IP_3, the second product of this signal cascade, induces Ca^{2+} depletion of the endoplasmatic reticulum (ER), which subsequently activates Ca^{2+} influx from the extracellular space through store-operated channels (SOCs), also called capacitative Ca^{2+} entry (CCE). SOCs are discussed as mechanisms for TRPC1, 4, and 5 as well as for TRPC3 and 7.[9,23] The varying function of TRPC3 and TRPC7 as ROCs or SOCs is probably caused by the formation of heterotetrameric channels as a consequence of the interaction with other TRPC isoforms.[20] Thus, TRPC channels can be gated by different stimuli, leading to multisided channel activation.[24] In general, the properties of heteromultimer proteins depend on their protein composition. Furthermore, the function of TRPC channels may also depend on many additional modulators, making the mechanisms more complex.[21]

2.4 Expression Pattern and Function of TRPC Channels

After identification of the TRPC channel subfamily, the expression levels of TRPC family members in different organs and cell types, as well as their functional roles, have been intensely investigated.

TRPC1 exhibits a widespread, but not ubiquitous, expression in different cell types, whereas an important role for vascular smooth muscle has been suggested that is related to the contractile and proliferative functions of muscle. However, there is evidence that

TRPC1 only contributes to a heterotetrameric ion channel complexed with other TRPC isoforms. Heterologous expression studies and biochemical investigations indicated a possible association of TRPC1 with TRPC4, TRPC5, TRPC3, and TRPP2.[15,24] This interaction with other TRPC isoforms seems to be required for the translocation of TRPC1 to the plasma membrane, as shown in TRPC1–TRPC4 coexpression studies.[20]

TRPC3 is highly expressed in brain as well as cardiac and smooth muscle cells (SMCs) and has been detected in the slow oxidative myofibers in skeletal muscle after neuromuscular activity.[15] Numerous functions of TRPC3 have been suggested in agonist-induced contraction in SMCs.[24] Interestingly, TRPC3 has high basal activity, which is decreased after the addition of a second glycosylated site at the extracellular portion of the channel, as found in TRPC6.[15] Moreover, basal and agonist-induced cation influx into SMCs as well as smooth muscle contractility were reportedly increased after the replacement of TRPC6 by TRPC3 in TRPC6-deficient ($TRPC6^{-/-}$) mice.[25] Thus, protein glycosylation seems to play an important role for channel activity. TRPC3 also forms functional heteromultimeric channels with TRPC6.[24]

TRPC4 is particularly expressed in the endothelium, where it regulates microvascular permeability, endothelium-dependent vasorelaxation of SMCs, and gene transcription. In SMCs, TRPC4 regulates cell proliferation and contraction.[20] TRPC4 as well as TRPC5 are reportedly thought to be assisted in their function as nonselective cation channels by TRPC1.[15]

TRPC5 was first found to be primarily expressed in the brain, but there are conflicting reports on the expression of TRPC5 in the pulmonary vasculature. Since TRPC5 is only poorly characterized, the role of this channel is unclear.[15,20]

TRPC6 is expressed in many tissues rich in SMCs but is most prominently expressed in lung tissue. Many studies suggest an important role of TRPC6 in vascular and pulmonary SMCs.[15] Investigations in $TRPC6^{-/-}$ mice in comparison to wild-type (WT) mice have also revealed a unique role for TRPC6 in the regulation of airway and vascular smooth muscle contractility.[25,26] Most interestingly, small precapillary pulmonary arteries, in contrast to large pulmonary arteries, do not express TRPC3.[9] Besides a role for TRPC6 for SMC contraction, there is evidence for a role of TRPC6 in cell proliferation.[24]

TRPC7 was first identified in mouse brain, but expression is also found in SMCs from the aortic and renal arteries as well as in endothelial cells from the cerebral and coronary arteries.[20] TRPC7 expression is also reported in the eye, spleen, and testis.[15]

3 Role of the TRPC Channels in Acute Hypoxic Pulmonary Vasoconstriction

3.1 Role of Ca^{2+}Channels in Acute HPV

Under physiological conditions, acute alveolar hypoxia leads to vasoconstriction of the precapillary arteries in the lung. Therefore, a rise of $[Ca^{2+}]_i$ in PASMCs is a key event in this process, inducing Ca^{2+}/calmodulin-dependent activation of

myosin light chain kinase, phosphorylation of the myosin light chains, actin-myosin interactions, and contraction.[13,27] However, the regulation of $[Ca^{2+}]_i$ has not yet been resolved. In general, $[Ca^{2+}]_i$ can be increased by Ca^{2+} influx from the extracellular space or by release from intracellular Ca^{2+} stores. At least three classes of Ca^{2+}-permeable channels in the plasma membrane are known: the L-type VOCCs, which are regulated by the resting membrane potential; the ROCs, which are activated by agonists; and SOCs, which are opened by depletion of Ca^{2+} from the sarcoplasmic reticulum (SR).[4,28] Concerning the Ca^{2+} release from intracellular Ca^{2+} stores, IP_3 receptor-mediated Ca^{2+} release from IP_3-sensitive SR as well as ryanodine receptor-mediated Ca^{2+} release from ryanodine-sensitive SR are known.[29]

One well-documented concept for the regulation of HPV proposes that a hypoxia-induced inhibition of voltage-gated K^+ (K_v) channels leads to membrane depolarization and Ca^{2+} entry through VOCCs.[30] However, since antagonists of K_v channels did not block HPV and VOCC antagonists exhibited no or only partial prevention of the hypoxic response, the mechanism of HPV seems to be more complex.[12,13] Growing evidence in the literature indicates a role for hypoxia-induced Ca^{2+} release from intracellular stores activating SOCs and CCE, possibly in addition to VOCCs.[13,14,30] Thus, depletion of intracellular Ca^{2+} stores, and thereby activation of SOCs by cyclopiazonic acid (CPA) and simultaneous application of nifedipine to prevent Ca^{2+} influx through VOCCs, was shown to cause an increase of $[Ca^{2+}]_i$ that was markedly enhanced under hypoxic compared to normoxic conditions.[13,14,30] Moreover, the pharmacological agents SKF-96365, Ni^{2+}, and La^{3+}, which block influx through nonselective cation channels in PASMCs, were potent inhibitors of HPV at concentrations that did not affect VOCCs. This is the first direct evidence that nonselective cation channels may play an important role in HPV.[14] Nevertheless, the VOCC inhibitor nifedipine was quite effective in preventing and reversing HPV, suggesting a hypoxia-induced influx through both SOCCs and VOCCs.[14]

Nonselective cation channels, which are associated with both SOCs and ROCs, are reported to be likely formed of homo- or heteromultimers of TRP proteins.[31,32] Among the TRP channels, especially TRPC proteins are expressed in SMCs of distal pulmonary arteries, which are suggested to be O_2 sensor and effector cells, at least of acute HPV.[11] However, less is known about the functional role of TRPC channels in the pulmonary circulation.

3.2 Importance of TRPC6 Channels in Acute HPV

TRPC6 is highly expressed in lung tissue as well as pulmonary and vascular SMCs.[23] Since there is a lack of specific TRPC channel blockers, a $TRPC6^{-/-}$ mouse model was developed by gene inactivation in embryonic stem cells to investigate

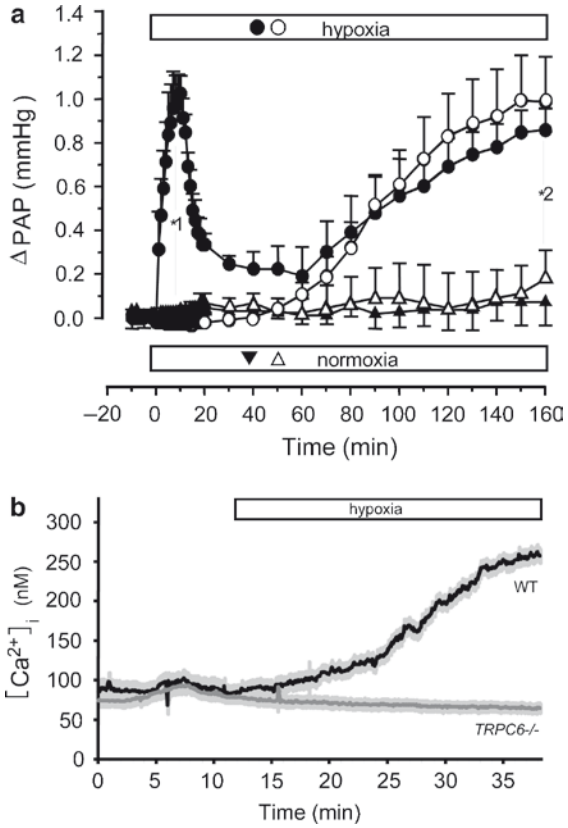

Fig. 12.3 Involvement of TRPC6 in acute hypoxic pulmonary vasoconstriction. (**a**) Time course of the increase in pulmonary arterial pressure (ΔPAP) in isolated, buffer-perfused, and ventilated mouse lungs (*filled circles* WT; *open circles* TRPC6$^{-/-}$) during 160 min of hypoxic ventilation (1% O_2). Control lungs were ventilated normoxically (*filled triangles* WT; *open triangles* TRPC6$^{-/-}$). $_*1$ indicates a significant difference ($p < 0.05$) between WT and TRPC6$^{-/-}$ mice after applying acute hypoxia; $_*2$ indicates significant differences ($p < 0.05$) between normoxic (WT and TRPC6$^{-/-}$) and hypoxic (WT and TRPC6$^{-/-}$) mice. (**b**) Time course of $[Ca^{2+}]_i$ in primary cultured PASMCs from WT and TRPC6$^{-/-}$ mice on exposure to hypoxia. Cells were loaded with fura-2 and analyzed by single-cell fluorescence imaging. Horizontal bars indicate endothelin priming (4 n*M* ET-1) and hypoxic perfusion (hypoxia) of PASMCs. Reproduced with permission[5]

functional parameters in comparison to WT mice.[22,25] Interestingly, TRPC6$^{-/-}$ mice showed increased vascular smooth muscle contractility, suggesting a critical role for TRPC6 in regulating vascular smooth muscle tone.[25]

To assess the role of TRPC6 in HPV, we analyzed the pressor response in isolated ventilated and perfused lungs from WT and TRPC6$^{-/-}$ mice during acute (<20 min) and sustained (60 – 160 min) hypoxia.[5] As shown in Fig. 12.3a, ventilation of lungs from WT mice with 1% O_2 provoked a biphasic profile of pulmonary arterial

pressure (PAP), with a first transient increase followed by a second progressive increase of PAP. Interestingly, the acute phase of HPV was completely absent in TRPC6$^{-/-}$ mice, while the sustained phase was not significantly different compared to WT mice. Thus, the general muscular contractility was not affected, the vasoconstriction induced by the thromboxane mimetic U46619 being unchanged.[5] Under normoxic conditions, the PAP did not differ between WT and TRPC6$^{-/-}$.[5] These results clearly show the indispensable role of TRPC6 in acute HPV as well as the differential regulation of the acute and sustained phase of HPV. Moreover, since partial occlusion of alveolar ventilation provoked severe hypoxemia in TRPC6$^{-/-}$ mice but not in WT mice, the profound physiological relevance of TRPC6 in acute HPV was confirmed.[5]

3.3 Role of TRPC6 Channels in the Increase of Intracellular Ca^{2+} Concentration in Acute HPV

To investigate the cellular mechanism of the TRPC6 dependency of HPV, alterations of $[Ca^{2+}]_i$ were investigated in hypoxia-incubated PASMCs from TRPC6$^{-/-}$ and WT mice, using an established method based on fluorescence imaging of single cells loaded with the fluorescent dye fura-2.[33,34] The expression level of the TRPC subtypes was unchanged in the PASMCs from TRPC6$^{-/-}$ mice except for TRPC6, as expected.[5] In contrast to WT PASMCs, the hypoxia-induced rise of $[Ca^{2+}]_i$ after priming with endothelin 1 (ET-1) was completely absent in PASMCs from TRPC6$^{-/-}$ (Fig. 12.3b).[5] The "priming" with a low dose of vasoactive agents such as angiotensin II, ET-1, or prostaglandin F2α turned out to be a prerequisite for HPV.[35–37]

Interestingly, the observed increase of $[Ca^{2+}]_i$ in WT PASMCs was completely dependent on extracellular Ca^{2+}, although ET-1 caused a rise of $[Ca^{2+}]_i$ in both cell types in the absence of extracellular Ca^{2+}.[5] This finding challenges the suggested contribution of SOCs and CCE to the regulation of HPV[12–14] and may be related to the fact that PASMCs of the precapillary resistance vessels were investigated in this study.

However, since the potent blocker of VOCC nicardipine almost completely inhibited Ca^{2+} entry in WT PASMCs and acute HPV in isolated lungs, the increase of $[Ca^{2+}]_i$ appears to be mostly attributable to VOCCs.[5] Then, what role do TRPC6 channels play? As described in the literature, TRPC6 channels are predominantly permeable to Na^+, and only a small percentage of the whole-cell current is caused by Ca^{2+} in the presence of extracellular Na^+.[38] In contrast to VOCCs, TRPC6 channels are permeable for Mn^{2+}. Thus, the hypoxia-induced influx of non-Ca^{2+} ions through TRPC6 channels was analyzed by a method called Mn^{2+} quenching. These experiments showed that hypoxia induced an increase in the Mn^{2+} quenching rate as a result of increased Mn^{2+} influx in WT but not in TRPC6$^{-/-}$ PASMCs.[5] This result is in line with the concept that Na^+ influx through TRPC6 channels leads to membrane depolarization and activation of VOCCs.[38–40] Moreover, an increase of

intracellular Na^+ has been shown to block the K_v channels[41] known to activate VOCCs. The important role of K_v channels was demonstrated by the impairment of HPV in mice lacking the K_v channel.[4,42]

3.4 Activation of TRPC6 Channels in Acute HPV

The receptor-operated, store-independent TRPC6 channel was the first ion channel shown to be DAG activated in a membrane-delimited fashion, independently of protein kinase C. However, the exact location of the DAG-binding site in the TRPC6 protein is still unresolved. In addition, sensitivity to the arachidonic acid metabolite 20-hydroxyeicosatetraenoic acid (HETE) as well as an activating effect by Ca^{2+}/calmodulin or protein phosphorylation have been reported.[23]

Since the recombinant TRPC6 channels heterologously expressed in human embryonic kidney (HEK) 293 cells were not activated by hypoxia, a direct activation of TRPC6 channels by hypoxia could be excluded.[5] Interestingly, a fluorescent DAG sensor expressed in PASMCs revealed the localization of DAG in the cytosol under normoxic conditions. Under hypoxia, DAG was translocated to the plasma membrane, suggesting gating of TRPC6 via DAG. In addition, a DAG kinase inhibitor activated the TRPC6 channels, supporting the hypothesis of hypoxia-induced DAG accumulation mediated by inhibition of DAG kinase or phospholipases.[5] Speculatively, the activation of TRPC6 in HPV may occur via ROS as it has been proposed that the O_2-sensing mechanism underlying HPV involves ROS.[5] NAD(P)H oxidase isoforms, different from those found in neutrophils, as well as mitochondria have been shown to be involved in the regulation of HPV in this regard. However, it is still unclear whether ROS generation is increased or decreased under hypoxic conditions.[1] Moreover, a role for a decreased adenosine monophosphate/adenosine triphosphate ratio as well as a role for cytochrome P450-dependent processes have been suggested for HPV.[1,43]

3.5 Hypothesis of the Mechanism of Acute HPV

A hypothetic model of the signal transduction pathway in PASMCs underlying acute HPV is described in Fig. 12.4. Initial priming of ET-1 activates PLC, producing a basal DAG concentration without activation of TRPC6 itself, but which is required for hypoxia-induced TRPC6 activation. Hypoxia-induced DAG accumulation results from the activation of PLCs[1] or phospholipase D (PLD)[2] or inhibition of DAG-degrading DAG kinases,[3] probably induced by changes in ROS production, and ultimately leads to Na^+ influx through TRPC6 channels.

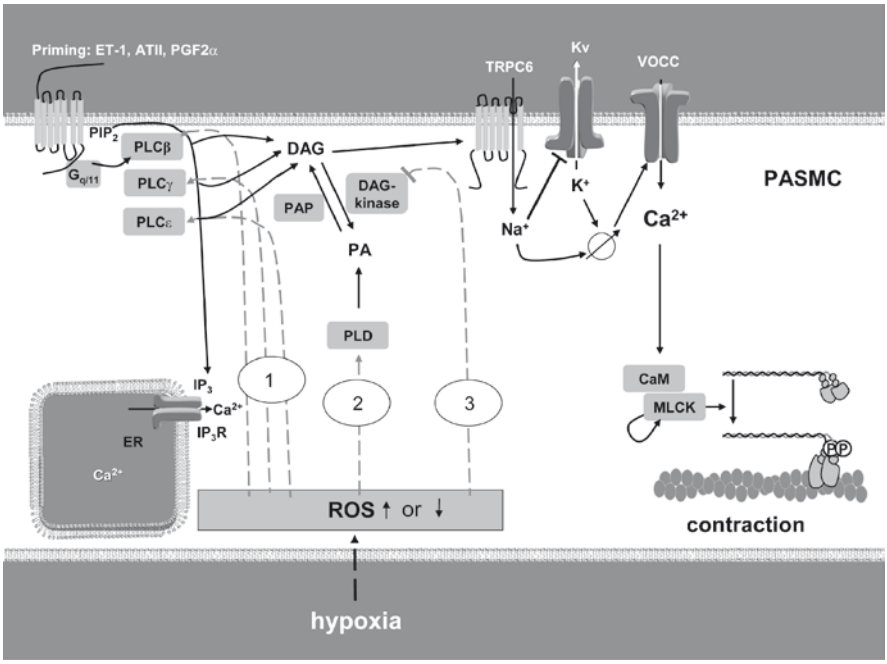

Fig. 12.4 Hypothetical model of the signal transduction pathway underlying acute hypoxic pulmonary vasoconstriction in PASMCs. According to this model, hypoxia-induced diacylglycerol (DAG) accumulation causes a cation influx through TRPC6. The DAG increase may be caused by activation of either phospholipase C (1), phospholipase D (2) or by inhibition of DAG-degrading DAG kinases (3). *ET-1* endothelin-1; *ATII* angiotensin II; *PGF2α* prostaglandin F2α; *Gq/11* G protein type q and 11; *PLC* phospholipase C; PIP_2 phosphatidylinositol 4,5-bisphosphate; IP_3 inositol-1,4,5 trisphosphate; *VOCC* voltage gated calcium channel; *ER* endoplasmatic reticulum; IP_3-*R* IP_3-receptor; *PLD* phospholipase D; *ROS* reactive oxygen species; *PA* phosphatidic acid; *PAP* phosphatidic acid phosphatase; *CaM* calmodulin; *MLCK* myosin-light chain kinase. Reproduced with permission[9]

A subsequent membrane depolarization, probably also caused by Na^+-induced inhibition of K_v channels, activates Ca^{2+} influx through VOCCs, resulting in contraction of the PASMCs.

4 Conclusion

HPV is an important mechanism in the lung that has been under investigation for more than 60 years. The nonselective cation channel TRPC6 has been identified as playing an essential role in this mechanism, as demonstrated by a complete absence of the acute response to hypoxia in mice lacking this ion channel.

Therefore, the TRPC6 channels offer a promising therapeutic target for pharmacological intervention in the control of pulmonary hemodynamics and gas exchange.

References

1. Weissmann N, Sommer N, Schermuly RT, Ghofrani HA, Seeger W, Grimminger F (2006) Oxygen sensors in hypoxic pulmonary vasoconstriction. Cardiovasc Res 71:620–629
2. Ward JPT, Aaronson PI (1999) Mechanisms of hypoxic pulmonary vasoconstriction: can anyone be right? Respir Physiol 115:261–271
3. Naeije R, Brimioulle S (2001) Physiology in medicine: importance of hypoxic pulmonary vasoconstriction in maintaining arterial oxygenation during acute respiratory failure. Crit Care 5:67–71
4. Weir EK, Olschewski A (2006) Role of ion channels in acute and chronic responses of the pulmonary vasculature to hypoxia. Cardiovasc Res 71:630–641
5. Weissmann N, Dietrich A, Fuchs B et al (2006) Classical transient receptor potential channel 6 (TRPC6) is essential for hypoxic pulmonary vasoconstriction and alveolar gas exchange. Proc Natl Acad Sci U S A 103:19093–19098
6. Weissmann N, Grimminger F, Walmrath D, Seeger W (1995) Hypoxic vasoconstriction in buffer-perfused rabbit lungs. Respir Physiol 100:159–169
7. Sham JSK (2002) Hypoxic pulmonary vasoconstriction: ups and downs of reactive oxygen species. Circ Res 91:649–651
8. Weissmann N, Grimminger F, Olschewski A, Seeger W (2001) Hypoxic pulmonary vasoconstriction: a multifactorial response? Am J Physiol Lung Cell Mol Physiol 281:L314–L317
9. Dietrich A, Kalwa H, Fuchs B, Grimminger F, Weissmann N (2007) Gudermann T. In vivo TRPC functions in the cardiopulmonary vasculature. Cell Calcium 42:233–244
10. McDaniel SS, Platoshyn O, Wang J et al (2001) Capacitative Ca^{2+} entry in agonist-induced pulmonary vasoconstriction. Am J Physiol Lung Cell Mol Physiol 280:L870–L880
11. Wang J, Shimoda LA, Sylvester JT (2004) Capacitative calcium entry and TRPC channel proteins are expressed in rat distal pulmonary arterial smooth muscle. Am J Physiol Lung Cell Mol Physiol 286:L848–L858
12. Ward JPT, Robertson TP, Aaronson PI (2005) Capacitative calcium entry: a central role in hypoxic pulmonary vasoconstriction? Am J Physiol Lung Cell Mol Physiol 289:L2–L4
13. Wang J, Shimoda LA, Weigand L, Wang W, Sun D, Sylvester JT (2005) Acute hypoxia increases intracellular [Ca^{2+}] in pulmonary arterial smooth muscle by enhancing capacitative Ca^{2+} entry. Am J Physiol Lung Cell Mol Physiol 288:L1059–L1069
14. Weigand L, Foxson J, Wang J, Shimoda LA, Sylvester JT (2005) Inhibition of hypoxic pulmonary vasoconstriction by antagonists of store-operated Ca^{2+} and nonselective cation channels. Am J Physiol Lung Cell Mol Physiol 289:L5–L13
15. Dietrich A, Chubanov V, Kalwa H, Rost BR, Gudermann T (2006) Cation channels of the transient receptor potential superfamily: their role in physiological and pathophysiological processes of smooth muscle cells. Pharmacol Ther 112:744–760
16. Hardie RC, Minke B (1993) Novel Ca^{2+} channels underlying transduction in Drosophila photoreceptors: implications for phosphoinositide-mediated Ca^{2+} mobilization. Trends Neurosci 16:371–376
17. Montell C, Rubin GM (1989) Molecular characterization of the Drosophila trp locus: a putative integral membrane protein required for phototransduction. Neuron 2:1313–1323
18. Minke B, Selinger Z (1996) The roles of trp and calcium in regulating photoreceptor function in Drosophila. Curr Opin Neurobiol 6:459–466

19. Zhu X, Chu PB, Peyton M, Birnbaumer L (1995) Molecular cloning of a widely expressed human homologue for the Drosophila trp gene. FEBS Lett 373:193–198
20. Firth AL, Remillard CV, Yuan JX-J (2007) TRP channels in hypertension. Biochim Biophys Acta 1772:895–906
21. Targos B, Ska J, Pomorski P (2005) Store-operated calcium entry in physiology and pathology of mammalian cells. Acta Biochim Pol 52:379–409
22. Freichel M, Vennekens R, Olausson J et al (2004) Functional role of TRPC proteins in vivo: lessons from TRPC-deficient mouse models. Biochem Biophys Res Commun 322:1352–1358
23. Dietrich A, Schnitzler M, Kalwa H, Storch U, Gudermann T (2005) Functional characterization and physiological relevance of the TRPC3/6/7 subfamily of cation channels. Naunyn Schmiedebergs Arch Pharmacol 371:257–265
24. Beech DJ (2005) Emerging functions of 10 types of TRP cationic channel in vascular smooth muscle. Clin Exp Pharmacol Physiol 32:597–603
25. Dietrich A, Mederos YS, Gollasch M et al (2005) Increased vascular smooth muscle contractility in TRPC6$^{-/-}$ mice. Mol Cell Biol 25:6980–6989
26. Sel S, Rost BR, Yildirim AO et al (2008) Loss of classical transient receptor potential 6 channel reduces allergic airway response. Clin Exp Allergy 38:1548–1558
27. Ward JPT, Snetkov VA, Aaronson PI (2004) Calcium, mitochondria and oxygen sensing in the pulmonary circulation. Cell Calcium 36:209–220
28. Sweeney M, Yuan JX-J (2000) Hypoxic pulmonary vasoconstriction: role of voltage-gated potassium channels. Respir Res 1:40–48
29. Landsberg JW, Yuan JX-J (2004) Calcium and TRP channels in pulmonary vascular smooth muscle cell proliferation. News Physiol Sci 1944–1950
30. Aaronson PI, Robertson TP, Knock GA et al (2006) Hypoxic pulmonary vasoconstriction: mechanisms and controversies. J Physiol 570:53–58
31. Beech DJ, Muraki K, Flemming R (2004) Non-selective cationic channels of smooth muscle and the mammalian homologues of Drosophila TRP. J Physiol 559:685–706
32. Birnbaumer L, Zhu X, Jiang M et al (1996) On the molecular basis and regulation of cellular capacitative calcium entry: roles for Trp proteins. Proc Natl Acad Sci U S A 93:15195–15202
33. Marshall C, Mamary AJ, Verhoeven AJ, Marshall BE (1996) Pulmonary artery NADPH-oxidase is activated in hypoxic pulmonary vasoconstriction. Am J Respir Cell Mol Biol 15:633–644
34. Waypa GB, Chandel NS, Schumacker PT (2001) Model for hypoxic pulmonary vasoconstriction involving mitochondrial oxygen sensing. Circ Res 88:1259–1266
35. Waypa GB, Marks JD, Mack MM, Boriboun C, Mungai PT, Schumacker PT (2002) Mitochondrial reactive oxygen species trigger calcium increases during hypoxia in pulmonary arterial myocytes. Circ Res 91:719–726
36. Sham JSK, Crenshaw BR Jr, Deng LH, Shimoda LA, Sylvester JT (2000) Effects of hypoxia in porcine pulmonary arterial myocytes: roles of K_V channel and endothelin-1. Am J Physiol Lung Cell Mol Physiol 279:L262–L272
37. Turner JL, Kozlowski RZ (1997) Relationship between membrane potential, delayed rectifier K^+ currents and hypoxia in rat pulmonary arterial myocytes. Exp Physiol 82:629–645
38. Estacion M, Sinkins WG, Jones SW, Applegate MA, Schilling WP (2006) Human TRPC6 expressed in HEK 293 cells forms non-selective cation channels with limited Ca^{2+} permeability. J Physiol 572:359–377
39. Gudermann T, Mederos y Schnitzler M, Dietrich A (2004) Receptor-operated cation entry – more than esoteric terminology? Sci STKE 2004:e35
40. Soboloff J, Spassova M, Xu W, He LP, Cuesta N, Gill DL (2005) Role of endogenous TRPC6 channels in Ca^{2+} signal generation in A7r5 smooth muscle cells. J Biol Chem 280:39786–39794

41. French RJ, Wells JB (1977) Sodium ions as blocking agents and charge carriers in the potassium channel of the squid giant axon. J Gen Physiol 70:707–724
42. Archer SL, London B, Hampl V et al (2001) Impairment of hypoxic pulmonary vasoconstriction in mice lacking the voltage-gated potassium channel Kv1.5. FASEB J 15:1801–1803
43. Keserü B, Barbosa-Sicard E, Popp R et al (2008) Epoxyeicosatrienoic acids and the soluble epoxide hydrolase are determinants of pulmonary artery pressure and the acute hypoxic pulmonary vasoconstrictor response. FASEB J 22:4306–4315

Developmental Regulation of Oxygen Sensing and Ion Channels in the Pulmonary Vasculature

David N. Cornfield

Abstract The increase in oxygen tension occurring at birth causes sustained and progressive pulmonary vasodilation. The oxygen-induced perinatal pulmonary vasodilation depends on the production of nitric oxide (NO) from the pulmonary endothelium and activation of various K^+ channels in pulmonary artery smooth muscle cells. This chapter reviews a) the oxygen-sensing mechanism that stimulates endothelial NO production; b) how K^+ channels sense changes in oxygen tension; c) whether hypoxia-inducible factor-1α (HIF-1α), a well defined hypoxia-sensitive transcription factor in adult, contributes to the regulation of NO production and K^+ channel activation; and d) whether and how dysfunctional K^+ channels contribute to the development of pulmonary hypertension in the newborns.

Keywords Oxygen-sensing • Calcium-sensitive potassium channels • Smooth muscle cells

1 Introduction

In 1953, Dawes and coworkers published a seminal article demonstrating that ventilation and establishment of an air–liquid interface caused an immediate increase in pulmonary blood flow and a decrease in pulmonary arterial (PA) blood pressure.[1] Evidence for an integral role for O_2 in the postnatal adaptation of the pulmonary circulation came first with the finding that while ventilation with nitrogen caused pulmonary vasodilation, ventilation with O_2 caused even greater pulmonary vasodilation.[2] The demonstration that fetal blood flow increased more than threefold when pregnant ewes with chronically instrumented fetal lambs were placed in a hyperbaric chamber provided clear evidence that an increase in fetal oxygen tension, absent any other physiologic stimulus, could cause fetal pulmonary vasodilation.[3]

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The increase in prostaglandin production immediately after birth demonstrated a role for the elaboration of vasoactive mediators from the pulmonary endothelium in the transition of the pulmonary circulation.[4] Blockade of prostaglandin production did not prevent either postnatal adaptation of the pulmonary circulation[5] or fetal pulmonary vasodilation caused by an increase in fetal O_2 tension alone.[6] The observation that pharmacologic blockade of endothelium-derived relaxing factor (EDRF), later identified as nitric oxide (NO),[7–9] prevented the postnatal adaptation of the pulmonary circulation in lambs ventilated with 100% O_2,[10] demonstrated the critical importance of the pulmonary endothelium in the postnatal adaptation of the pulmonary circulation.

The observation that pharmacologic blockade of NO production attenuated the decrease in pulmonary vascular resistance with both ventilation alone and ventilation with 100% O_2 provided direct evidence that NO production played a key role in O_2-induced fetal pulmonary vasodilation.[11] Two separate studies found that O_2-induced pulmonary vasodilation was either attenuated or prevented by pharmacologic blockade of NO in the chronically instrumented fetal lamb.[12,13] These findings, together with the observation that O_2 tension is capable of modulating NO production in fetal PA endothelial cells,[14] implied that the increase in O_2 tension that occurs at birth may contribute to sustained and progressive pulmonary vasodilation by providing a stimulus for augmented NO production by the pulmonary endothelium.

Concomitant with the emergence of data demonstrating a critically important role for NO in the transition of the perinatal pulmonary circulation were studies indicating a link between the vasodilation caused by NO and K^+ channel activation in vascular smooth muscle cells (SMCs). Robertson et al. demonstrated that in cerebral artery SMCs cyclic guanosine 3′,5′-monophosphate (cGMP)-dependent protein kinase (PK) acts to phosphorylate the large conductance K_{Ca} channel.[15] In pulmonary artery smooth muscle cells (PASMCs), NO-induced increases in intracellular levels of cGMP cause activation of a cGMP-sensitive kinase; this in turn activates a K_{Ca} channel, resulting in vasodilation.[16] NO has also been shown to directly activate K_{Ca} channels.[17] Taken together, these data indicated a putative role for K^+ channel activation in mediating perinatal pulmonary vasodilation.

2 K^+ Channels in the Pulmonary Circulation

The first data that demonstrated a role for K^+ channels in the developing pulmonary circulation came from three separate and not completely consistent studies.[18–20] Each group of investigators demonstrated that adenosine triphosphate (ATP)-sensitive potassium (K_{ATP}) channels were present in the pulmonary circulation, and activation resulted in fetal pulmonary vasodilation. Activation of the channels caused profound and sustained fetal pulmonary vasodilation.[20] However, whether a component of the vasodilation caused by K_{ATP} activation results from NO release remains controversial. Two groups of investigators demonstrated that activation of the K_{ATP} channel causes NO-independent vasodilation,[19,20] while another group demonstrated that pharmacologic inhibition of NO prevented K_{ATP} channel activation

from causing fetal pulmonary vasodilation.[18] Subsequent work has demonstrated that in the pulmonary circulation, K_{ATP} channel activity increases with maturation, especially in resistance vessels.[21]

3 Role of the Calcium-Sensitive K^+ Channel in the Perinatal Lung

The first definitive evidence that K_{Ca} channel activation mediates O_2-induced fetal pulmonary vasodilation came from studies in acutely instrumented late-gestation ovine fetuses.[22] O_2-induced fetal pulmonary vasodilation was blocked by either tetraethylammonium (TEA), a K^+ channel blocker,[23,24] or iberiotoxin, a specific K_{Ca} channel antagonist,[25] but unaffected by glibenclamide (GLI), a blocker of the K_{ATP} channel[26,27] (Fig. 13.1). The data suggested that O_2 causes fetal pulmonary vasodilation through K_{Ca} channel activation. Inhibitors of either guanylate cyclase or cyclic nucleotide dependent kinases also attenuated O_2-dependent fetal pulmonary vasodilation, implying that elevated fetal O_2 acts to increase guanylate cyclase activity, cGMP concentration, and activate cyclic nucleotide dependent kinases, causing K_{Ca} channel activation and vasodilation.[22]

Evidence that K^+ channel activity mediates ventilation induced pulmonary vasodilation derives from studies wherein the effect of K^+ channel inhibition on (1) mechanical ventilation with low inspired oxygen concentrations and (2) mechanical ventilation with high concentrations of inspired oxygen was studies. Acutely instrumented fetal lambs were treated with TEA (a K^+ channel blocker), GLI (a blocker of

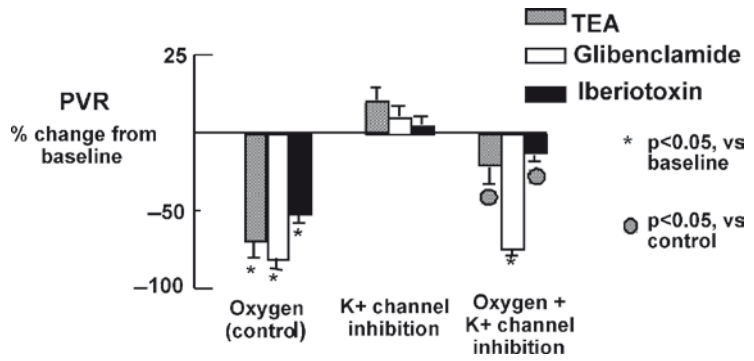


Fig. 13.1 The effect of K^+ channel inhibition on oxygen-induced fetal pulmonary vasodilation. In instrumented fetal lambs, delivery of supplemental oxygen to the maternal ewe increased fetal oxygen tension and in the control periods caused a marked decrease in pulmonary vascular resistance (PVR) ($p < 0.05$) compared to baseline. K^+ channel inhibition had no effect on basal PVR. Following administration of tetraethylammonium (TEA) ($p < 0.05$) and iberiotoxin, a specific K_{Ca} channel blocker, to the pulmonary circulation, oxygen-induced fetal pulmonary vasodilation was blocked. Glibenclamide, an ATP-sensitive K^+ channel antagonist, had no effect on attenuated oxygen-induced fetal pulmonary vasodilation ($p < 0.05$)

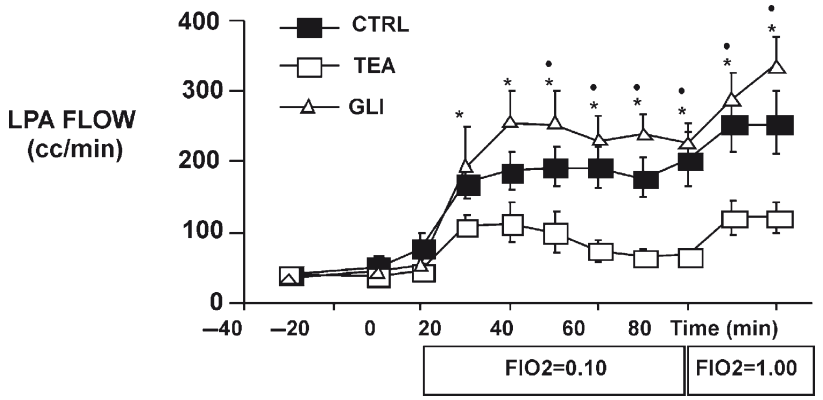


Fig. 13.2 Effect of K^+ channel inhibition with either tetraethylammonium (TEA) or glibenclamide (GLI) on perinatal pulmonary vasodilation. The figure shows left pulmonary artery (LPA) blood flow in response to sequential ventilation with low and high O_2. LPA flow in the TEA group was attenuated compared to control (CTRL) in response to both low and high O_2. There was no difference in LPA flow between CTRL and GLI groups. *$p < 0.01$ all groups compared to baseline value. •$p < 0.01$ CTRL versus TEA

ATP-sensitive K^+ channels), or saline. TEA attenuated and GLI had no effect on the increase in left pulmonary artery (LPA) blood flow and the decrease in pulmonary vascular resistance in response to mechanical ventilation with 0.10 and 1.0 FIO_2 (fraction of inspired air) (Fig. 13.2). These results provided clear evidence that K^+ channel activation is required for the progressive and sustained perinatal pulmonary vasodilation that characterizes normal postnatal adaptation of the pulmonary circulation.[28] Interestingly, ventilation and oxygen-induced pulmonary vasodilation are similarly attenuated in the presence of endothelium-derived nitric oxide (EDNO) inhibition.[11]

4 Ontogeny of the Pulmonary Vascular K^+ Channel and O_2 Sensing

While the K_{Ca} channel is crucially important in the perinatal pulmonary circulation, the importance of the K_{Ca} channel seems to decrease with maturation. The observation that the K^+ channel setting resting membrane potential (RMP) in the pulmonary circulation changes following birth, going from a K_{Ca} to a K_v (voltage-gated K^+) channel, suggests developmental regulation of K^+ channels in the pulmonary circulation.[29] In coordination with a maturation-related decrease in K_{Ca} channel expression and activation, the K_v channel increases with maturation. There is relatively more $K_v2.1$ channel protein and message in the adult than in the fetal and neonatal pulmonary circulation.[30] The increase in $K_v2.1$ channel parallels the increasing capacity of PASMCs to sense and respond to acute decreases in O_2 tension, as in response to acute hypoxia, intracellular Ca^{2+} ($[Ca^{2+}]_i$) increases more rapidly and to a greater degree in adult, as compared to fetal, PASMCs.[30] The relatively greater abundance of

the $K_v2.1$ channel in the adult may represent an adaptation that allows the pulmonary circulation to respond to a specific physiologic signal that is relevant to a particular developmental stage. Reports that the O_2 sensor in the adult PASMCs is a K_v channel are consistent with this construct[31,32] since the K_v channel is inactivated by acute hypoxia, causing PASMC depolarization, opening of voltage-operated Ca^{2+} channels (VOCCs), increase in $[Ca^{2+}]_i$, and vasoconstriction. Thus, the maturation-related increase in hypoxic pulmonary vasoconstriction that has been previously reported[33,34] might derive from the parallel increases in $K_v2.1$ channel activity, protein, and message with maturation. Protein data are consistent with messenger RNA (mRNA) data as large-conductance Ca^{2+}-activated K^+ channel (BK_{Ca}) protein is more abundantly expressed in the fetal as compared to the adult pulmonary circulation[35] (Fig. 13.3), suggesting that BK_{Ca} activity might be modulated at the transcriptional level.

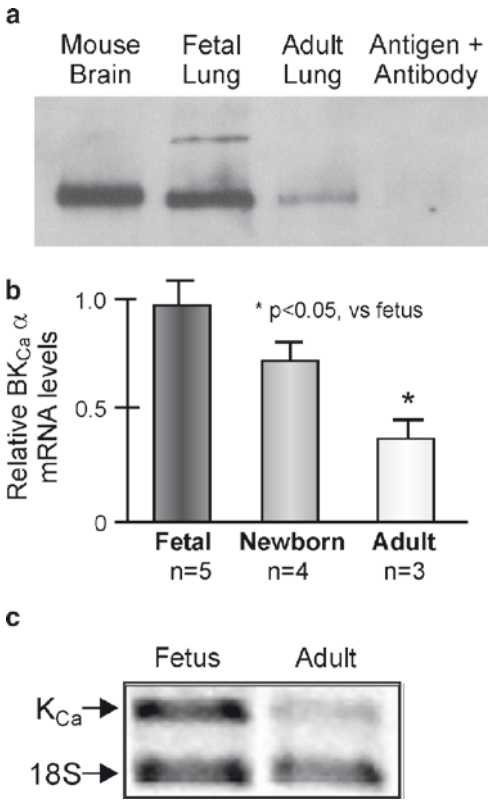


Fig. 13.3 (**a**) Western blot of protein extract from the fetal and adult pulmonary vasculature. More BK_{Ca} channel protein is present in the fetus compared to the adult. Mouse brain served as the positive control. Coincubation of the antigen and antibody did not produce a band, indicating specificity of the antibody.[35] (**b**) BK_{Ca} channel α subunit mRNA levels in the distal pulmonary vasculature decreased with maturation. BK_{Ca} channel band was normalized to 18S band intensity. mRNA was isolated from fetal, newborn, and adult animal pulmonary arteries. [35] (**c**) Representative gel of a reverse-transcription polymerase chain reaction (RT-PCR) analysis of BK_{Ca} channel mRNA expression during development. The BK_{Ca} band was compared with the control 18S band in fetal and adult samples. BK_{Ca} channel band intensity was determined by densitometry and normalized to that of the 18S band[35]

The physiologic response of the PASMCs to an increase in oxygen tension is consistent with molecular data. Microfluorimetry studies demonstrated that only fetal PASMCs respond to an acute increase in O_2 tension with a decrease in $[Ca^{2+}]_i$. Thus, maturational-related changes in the ability of the pulmonary vasculature to respond to an acute increase in O_2 tension are intrinsic to the PASMCs. The O_2-induced decrease in fetal PASMC $[Ca^{2+}]_i$ was blocked by iberiotoxin, a specific blocker of the BK_{Ca} channel. Further support for a developmental role for the BK_{Ca} channel comes from the observation that fetal, but not adult, PASMCs respond to iberiotoxin with an increase in basal $[Ca^{2+}]_i$, suggesting that the BK_{Ca} channel determines basal $[Ca^{2+}]_i$ in fetal PASMCs.[35,36]

Further evidence of the consistency between the physiology and molecular biology of the pulmonary vascular SMCs derives from electrophysiology studies. In fetal PASMCs, the RMP is determined by the BK_{Ca} channel, while in adult PASMCs K_v channel activity determines RMP.[29] In fetal PASMCs, an acute increase in oxygen tension results in membrane hyperpolarization and a decrease in PASMC $[Ca^{2+}]_i$. In adult PASMCs, an acute increase in oxygen tension has no effect on membrane potential or $[Ca^{2+}]_i$ (Fig. 13.4).[29,35] Taken together, these observations indicate that the developmental regulation of BK_{Ca} channel expression allows for the fetal PASMCs to be uniquely well adapted to respond to an acute increase in oxygen tension with a decrease in $[Ca^{2+}]_i$ and vasorelaxation. The molecular mechanisms by which a maturation-related decrease in BK_{Ca} and increase in K_v channel activity occurs remain unknown.

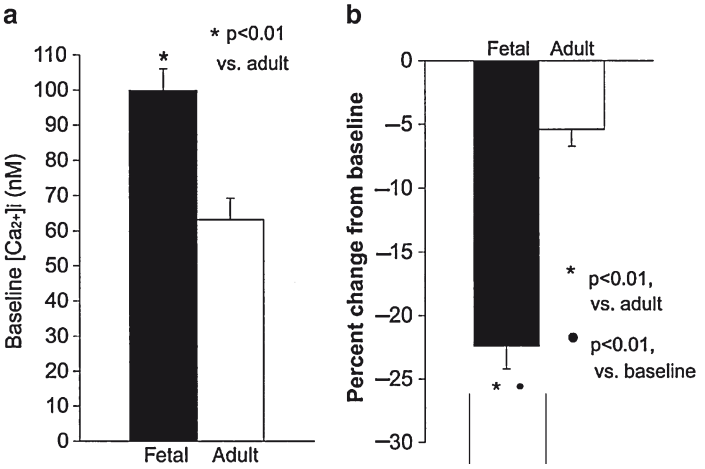


Fig. 13.4 (**a**) Comparison of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in fetal ($n = 113$ cells; six animals) and adult ($n = 99$ cells; four animals) pulmonary artery smooth muscle cells (PASMCs) maintained in hypoxia (25 torr). Under hypoxic conditions, basal $[Ca^{2+}]_i$ was significantly higher in fetal compared with adult PASMCs. (**b**) Effect of an acute increase in O_2 tension (125 torr) on $[Ca^{2+}]_i$.[35]

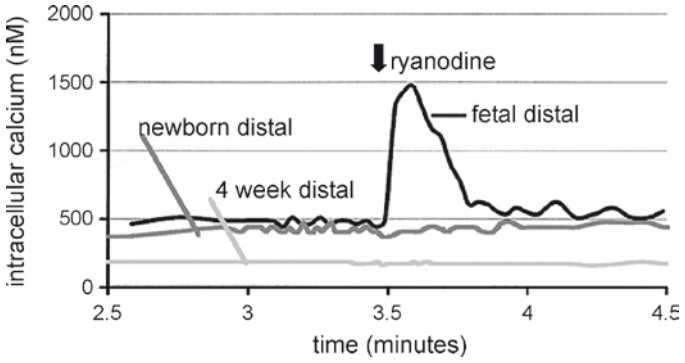


Fig. 13.5 Ryanodine increases $[Ca^{2+}]_i$ in PASMCs from fetal, but not newborn or 4-week-old, rabbits[37]

5 Developmental Regulation of Oxygen-Induced Vasodilation

To determine the subcellular mechanism by which the developmentally regulated response to an acute increase in oxygen occurs, the hypothesis that sensitivity to ryanodine is specific to fetal and neonatal PASMCs was tested. PASMCs were freshly dispersed from the proximal and distal PAs of fetal (0.9 term), neonatal (<24 h old), and juvenile rabbits. In this model, ryanodine caused a substantial increase in $[Ca^{2+}]_i$ in PASMCs isolated from the fetal, but not neonatal or juvenile, distal PAs[37] (Fig. 13.5).

To determine the mechanism by which ryanodine causes an increase in $[Ca^{2+}]_i$, experiments was probed by removal of external calcium or application of diltiazem, a blocker of the voltage-operated calcium channels (VOCC or Ca_V). Both suppressed the ryanodine-induced increase in $[Ca^{2+}]_i$, providing evidence that ryanodine causes an increase in $[Ca^{2+}]_i$ through an influx of extracellular calcium via Ca_V.[37]

The similar pattern of developmental regulation between the BK_{Ca} channel and the ryanodine-sensitive stores in PASMCs suggested that quantal release of calcium from ryanodine-sensitive stores, termed *calcium sparks*, and BK_{Ca} channels work in a coordinated fashion to control PASMC $[Ca^{2+}]_i$. To demonstrate a causal link, confocal microscopy demonstrated the presence of calcium sparks in fetal PASMCs[38] but not in PASMCs from neonatal or adult animals. While calcium sparks had been demonstrated in other vascular structures,[39–42] our report represents the first evidence of calcium sparks in the fetal pulmonary circulation. The observations that pretreatment with iberiotoxin, a specific blocker of the BK_{Ca} channel, blocked the ryanodine-induced increase in $[Ca^{2+}]_i$ while pretreatment with ryanodine blocked the increase in $[Ca^{2+}]_i$ caused by iberiotoxin demonstrated a link between release of calcium from ryanodine-sensitive stores and BK_{Ca} channel activity.[37,38] These data suggests that quantal

release of calcium from ryanodine-sensitive stores exerts a tonic stimulus on BK_{Ca} channels, thereby limiting tone. With the stimulus of oxygen, the frequency of calcium sparks increases, leading to activation of the BK_{Ca} channels, membrane hyperpolarization, closure of Ca_V, a decrease in PASMC $[Ca^{2+}]_i$, and ultimately vasodilation.

6 Oxygen-Induced Fetal Pulmonary Vasodilation Is Ryanodine Sensitive

Further detail surrounding the cellular mechanism by which oxygen causes fetal pulmonary vasodilation derives from in vivo studies in acutely instrumented fetal sheep. In these studies, the pregnant ewe was administered 100% O_2 for 30 min, with a resulting increase in fetal oxygen tension from 18 ± 2 to 28 ± 3 torr. LPA blood flow increased from 27.9 ± 8.9 to 241.5 ± 71 cc/min. After a 1-h recovery period, ryanodine (100 μg) was administered to the fetus via the LPA over 10 min. Despite a similar increase in fetal arterial oxygen tension, the increase in LPA flow was attenuated compared to the control period ($n = 5$; $p < 0.05$ versus control period).[38]

In vitro studies were performed in freshly dispersed fetal ovine PASMCs maintained in primary culture. Ryanodine prevented the O_2-induced decrease in PASMCs $[Ca^{2+}]_i$.[38] Electrophysiology experiments with freshly dispersed PASMCs demonstrated spontaneous transient outward currents (STOCs), previously identified as a critical link between calcium sparks and membrane hyperpolarization. Calcium sparks have been shown to cause activation of the BK_{Ca} channel, leading to a transient, but robust, increase in K^+ efflux (STOC). While we initially demonstrated that fetal PASMCs possess STOCs,[29] the evidence that an acute increase in oxygen tension increased both calcium sparks and STOCs provided a link between calcium sparks and membrane hyperpolarization. These observations led to the conclusion that Ca^{2+} release from the ryanodine-sensitive store mediates the O_2-induced decrease in PASMC $[Ca^{2+}]_i$ by increasing STOC activity, resulting in membrane hyperpolarization, closure of Ca_V, and a decrease in $[Ca^{2+}]_i$. Figure 13.6 is a cartoon that represents the putative subcellular pathways by which O_2 decreases the contractile tone of the PASMCs.

Both oxygen and NO play a central role in the transition of the pulmonary circulation at the onset of air-breathing life. While both NO and oxygen stimulate perinatal pulmonary vasodilation via a guanylate cyclase-sensitive pathway,[11,13] an NO-induced increase in cytosolic cGMP leads to perinatal pulmonary vasodilation through activation of the BK_{Ca} channel.

Data from our laboratory demonstrated that NO activated BK_{Ca} channels, increasing I_K by 253 ± 28% (at +40 mV).[22] To determine whether NO causes perinatal pulmonary vasodilation through BK_{Ca} channel activation via Ca^{2+} release from ryanodine-sensitive stores, inhaled NO was administered to acutely instrumented, late-gestation fetal lambs in the presence and absence of pharmacologic blockade of the BK_{Ca} channel, ryanodine receptor, or cGMP kinase. TEA (in millimolar concentrations) and ryanodine, but not 4-aminopyridine, a blocker of the voltage-

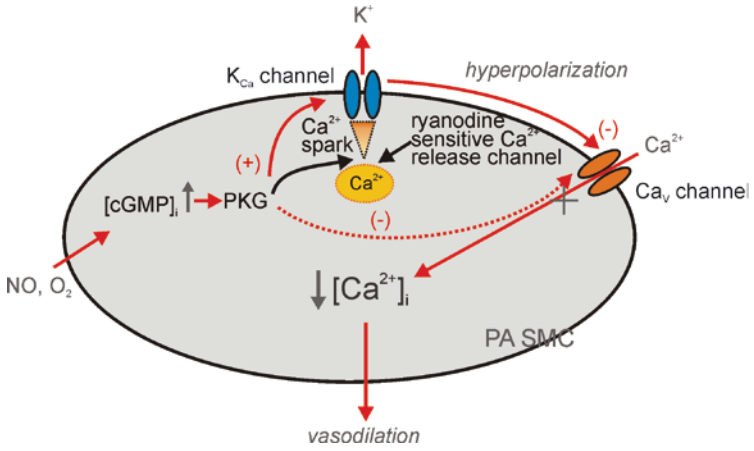


Fig. 13.6 O_2-induced decrease in pulmonary artery smooth muscle cell cytosolic Ca^{2+}. Local release of Ca^{2+}, a calcium spark, from an internal, ryanodine-sensitive store causes elevation of Ca^{2+} in the region of the BK_{Ca} channel. Activation of the BK_{Ca} channel causes K^+ efflux, membrane hyperpolarization, and closure of the voltage-operated Ca^{2+} channel, a decrease in cytosolic Ca^{2+}, and vasodilation. Vasodilatory stimuli, such as nitric oxide (NO) or oxygen (O_2), act through cyclic nucleotide-dependent kinases like protein kinase G (PKG) to affect ryanodine receptor function and calcium pumps on internal stores. PKG may also directly affect both K^+ and Ca^{2+} channel activity

sensitive K^+ (K_v) channel, attenuates the perinatal pulmonary vasodilation caused by NO. The interpretation of the data is that BK_{Ca}, but not K_v, channel activation plays a key role in NO-induced perinatal pulmonary vasodilation and requires release of Ca^{2+} from ryanodine-sensitive stores.[43]

Considered in concert, these data support the notion that the pulmonary vascular SMC BK_{Ca} channel plays an essential role in mediating pulmonary vasodilation at a biologically critical point in development. Fetal, but not adult, PASMCs respond directly to an increase in oxygen tension through activation of the BK_{Ca} channel. Transient activation of the PASMC BK_{Ca} channel results from the localized calcium release in the form of calcium sparks from developmentally regulated intracellular ryanodine-sensitive stores. The effect of opening multiple channels is membrane hyperpolarization, a decrease in PASMC $[Ca^{2+}]_i$, and vasodilation. These data suggest that the BK_{Ca} channel is a potential molecular target for modulating pulmonary vascular tone in general and, specifically, the abnormal perinatal pulmonary vasoreactivity that characterizes persistent pulmonary hypertension of the newborn (PPHN). Thus, in the perinatal pulmonary circulation, when pulmonary vasodilation is biologically imperative, BK_{Ca} channel activation mediates the response to birth-related physiologic stimuli. The remainder of this chapter outlines how the environment of the fetus with normally low oxygen tension contributes to perinatal pulmonary vascular BK_{Ca} channel expression and molecular mechanisms that determine fetal pulmonary vascular BK_{Ca} channel expression.

7 Oxygen Tension Modulates the Expression of Pulmonary Vascular BK_{Ca} Channel

Recent data underscored the notion that hypoxia increases BK_{Ca} channel subunit expression, and that hypoxia has a greater effect on BK_{Ca} channel α and β1 subunit expression than on β2. The observation implies that the environment of low oxygen tension of the normal fetus plays a role in preparing the pulmonary vasculature to respond to perinatal vasodilator stimuli by enhancing calcium-sensitive K^+ channel expression. Data demonstrating that BK_{Ca} channel expression increases with hypoxia in both in vivo and in vitro systems as well as in both adult and fetal pulmonary vasculature indicate that expression of the BK_{Ca} is intrinsically oxygen sensitive.

Moreover, the effect of oxygen on K_{Ca} channel subunit expression is not specific to a given developmental stage as hypobaric hypoxia caused an increase BK_{Ca} channel subunit expression in the lungs of adult Sprague-Dawley rats as well fetal PASMCs in primary culture. That hypoxia increases β1 subunit expression is, potentially, highly significant as the β1 subunit renders the BK_{Ca} channel more sensitive to an increase in calcium concentration,[44,45] thereby increasing the "open probability" of the channel in response to an elevation of calcium in the region of the channel.

8 Hypoxia-Inducible Factor 1 Modulates BK_{Ca} Expression

Deferoxamine mesylate (DFX), a known hypoxia mimic, is an iron chelator capable of inducing hypoxia-inducible factor 1α (HIF-1α) protein stability and hypoxia-dependent gene expression, probably by inhibiting the action of the prolyl hydroxylase (PHD) enzyme responsible for HIF-1α degradation. Normoxic fetal PASMCs supplemented with 200 m*M* DFX demonstrated increased expression of the BK_{Ca} channel. DFX-treated cells showed an increased K_{Ca} α subunit mRNA expression, at levels comparable to those observed under hypoxia (Fig. 13.7). As deferoxamine prevents HIF-1α degradation, expression of the BK_{Ca} channel is augmented, implicating a role for HIF-1 in the regulation of the BK_{Ca} channel.

Consistent with the notion that HIF-1 regulates BK_{Ca} expression is the presence putative hypoxia response elements on the 5′ genomic sequences of the human K_{Ca} channel gene β1 subunit.[46] Two elements that closely matched the consensus sequence for the HIF-1-binding site (HBS), 5′-RCGTG-3′.[47] One of the sequences perfectly matches the "extended" consensus (5′-GCACGTA-3′), as found in many hypoxia-related genes.[48–52] The position of these sites proximal to the start of transcription and their precise match to elements previously shown to contain HBS strongly suggest the presence of hormone responsive elements (HREs) on the BK_{Ca} channel.

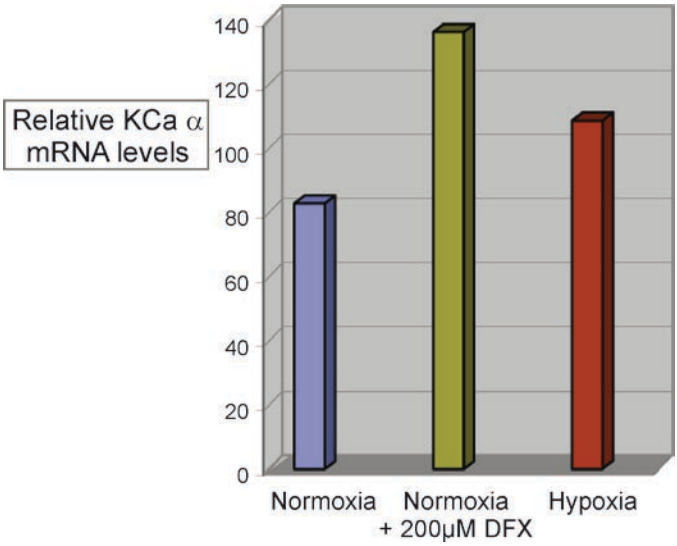
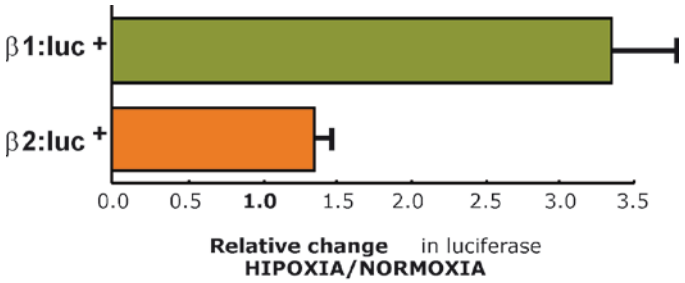


Fig. 13.7 Deferoxamine mesylate (DFX) mimics the effect of hypoxia on the calcium-sensitive K^+ channel α-subunit gene expression, suggesting a possible involvement of HIF-1. This figure shows the aggregate RT-PCR data ($n = 3$) of the relative expression of K_{Ca} α in normal and DFX-treated cells. Hypoxic cells were used as controls. The β-actin band was used to normalize the results for total mRNA content

Definitive evidence for the sensitivity of BK_{Ca} to hypoxia derives from the fetal PASMCS transfected with 0.5–1 μg of *KCa:luc*$^+$ plasmids and a plasmid control (to assess transfection efficiency) for 1–2 h. Cell cultures were placed under normoxic (pO_2 = 120 torr) or hypoxic (pO_2 = 30 torr) conditions. Interestingly, while the β1 subunit of the K_{Ca} channel showed a significant threefold induction under hypoxic conditions, β2 showed only a 38% increase in luciferase activity (Fig. 13.8).

HIF-1α, a key transcription factor that is central to oxygen homeostasis, enables the cell to respond to changes in oxygen (O_2) availability, an essential response in many developmental, physiological, and pathological processes. Semenza and others have shown that HIF-1 regulates the transcription of many genes involved in the cellular and systemic response to oxygen availability, including genes involved in angiogenesis (i.e., vascular endothelial growth factor [VEGF]), oxygen transport (i.e., erythropoietin), and energy metabolism (i.e., glycolytic enzymes).[53] HIF-1 is also a key player in pathophysiological processes such as cancer because hypoxic microenvironments in a tumor trigger expression of angiogenic genes that promote the growth of the newly vascularized tumor.[54,55]

From a teleologic perspective, the transition from the intrauterine environment with relatively low O_2 tension to air-breathing life entails an unprecedented and absolutely required response to an increase in oxygen availability. The physiologically low-O_2 environment of the fetus might be essential for fetal vascular growth and lung morphogenesis[56] as well as other early embryo developmental programs.[57] As a master regulator of the oxygen response, it follows that the molecular mechanisms responsible for fetal life and its transition to a newborn might involve HIF-1. Under normoxic



	Relative Luciferase activity Hypoxia/Normoxia
β1:luc +	3.35 ± 0.48
β2:luc +	1.38 ± 0.18

p<0.01 n=12

Fig. 13.8 Ovine PASMCs in primary cell cultures were transfected with 0.5–1 μg of *KCa:luc*$^+$ plasmids and a plasmid control for 1–2 h. Cell cultures were placed under normoxic (pO_2 = 120 torr) or hypoxic (pO_2 = 30 torr) conditions. After 24–36 h in culture, cells were washed and lysed. Protein extracts were assayed for luciferase activity and normalized for transfection efficiency and protein concentration. While the β1 subunit of the K_{Ca} channel showed a significant threefold induction under hypoxic conditions, β2 showed only a 38% increase in luciferase activity

conditions, the α chain of HIF-1 is eliminated by degradation, rendering HIF-1 inactive. In low-O_2 environments, HIF-1 forms a dimer, is stabilized, and can translocate to the cell nucleus, thereby promoting transcription of hypoxia-sensitive genes.[58,59]

The molecular expression and activity of the HIF-1 protein are tightly regulated by at least two complementary mechanisms, one involving the degradation of HIF-1 under normal oxygen levels via the ubiquitination–degradation pathway mediated by hydroxylases and the other involving the blocking of HIF-1 transcriptional activity by either hydroxylation or domain competition.[60] Hypoxia inactivates these pathways to limit HIF-1 activity. Ratcliffe and others demonstrated that ubiquitination and proteasomal degradation render HIF-1α unstable under normal oxygen conditions[61,62] through O_2-dependent hydroxylation of two proline residues in its α chain (Pro^{564} and Pro^{402}), thereby targeting HIF-1 for proteasomal degradation by the von Hippel–Lindau (pVHL) ubiquitin E3 ligase complex.[61,62] Enzymes that catalyze proline hydroxylation, termed *prolyl hydroxylases* or (PHDs) (1, 2, and 3), use molecular O_2 as a substrate. As O_2 availability determines PHD activity, these enzymes may be the primary oxygen sensors, linking O_2 concentration and HIF-1 protein levels.[63] While the three PHDs are capable of regulating HIF-1, their activity and cellular distribution vary.[64,65] PHD2 likely has the dominant role[66] since "silencing" of PHD2 with short-interfering RNAs stabilizes and activates HIF-1 in normoxia, while "silencing" of PHD1 and PHD3 has no effect on the stability of HIF-1α. PHD2

and PHD3 expression are upregulated by hypoxia via HIF-1, providing an autoregulatory mechanism of HIF-1 stability driven by oxygen tension.[67] Paradoxically, the expression of these hydroxylases is induced in hypoxic cell culture despite the fact that the reduced availability of the O_2 cosubstrate renders them inactive, suggesting that the PHD function is to rapidly terminate the HIF-1 response on reoxygenation.[67]

An additional layer of O_2-dependent regulation of the HIF-1 response centers on its transactivating function. The ability of HIF-1α to interact with cotranscription factors such as CREB binding protein (CPB/p300) is also regulated by a hydroxylation-dependent switch. Asparagine hydroxylation of the C-terminal transactivation domain (CTAD) of HIF-1 prevents its interaction with CBP/p300, a necessary cofactor for HIF-1-mediated transcription. As with the case of PHDs, asparagyl hydroxylation is blocked by hypoxia.[68] The enzyme responsible for this was originally called factor-inhibiting HIF-1 (FIH-1), a CTAD-interacting factor later found to be an asparagyl hydroxylase. As with the PHDs, the fact that O_2 is a principal substrate of FIH-1 makes this enzyme a legitimate oxygen sensor that represents an added level of regulation of the hypoxic response.

CBP/p300 interacting transactivator with ED-rich tail (CITED2) is similar to FIH-1 in its ability to block the HIF-1 transactivation domain from interacting with the CBP/p300 cofactor but differs in its mechanism of action. CITED2 is a ubiquitously expressed protein that competes with HIF-1α for binding to the CBP/p300 domain.[69] CITED2 is activated by hypoxia via HIF-1 itself, providing an additional layer of HIF-1 autoregulation by controlling HIF-1α access to CBP/p300 and therefore limiting HIF-1-dependent hypoxic response. Inactivation of HIF-1 target genes in CITED2 knockout mice seems to support the role of CITED2 as an HIF-1 competitor.[70] Similar to PHD2 and FIH, CITED2 activation by hypoxia might indicate a role for this molecule in the rapid inactivation of the hypoxic response on reoxygenation. CITED2 may play a role in heart morphogenesis and the establishment of left–right asymmetry in the early embryo.[70,71]

Since changes in oxygen tension at birth might signal an important transcriptional adaptation of the fetus to the air-breathing life of the newborn infant, we investigated the role of HIF-1 in that transition. We found that expression of HIF-1α is developmentally regulated, as is the expression of the PHDs and asparagyl hydroxylases and other competing factors that control HIF-1α expression and activity. The well-coordinated developmental regulation of the components of the HIF-1 pathway in the fetus suggests that the fetus represents a particular transcriptional paradigm that, in response to differential oxygen availability, produces a specific physiological and developmental response.

9 Developmentally Regulated Expression of HIF-1

HIF-1 protein levels are oxygen insensitive in fetal PASMCs, while relatively subtle levels of hypoxia (PaO_2 = 25 torr) increased HIF-1 protein in adult PASMCs. Figure 13.8 demonstrates the results of Western blotting for HIF-1 protein normalized to β actin. Further evidence of the oxygen insensitivity of HIF-1 in fetal as compared

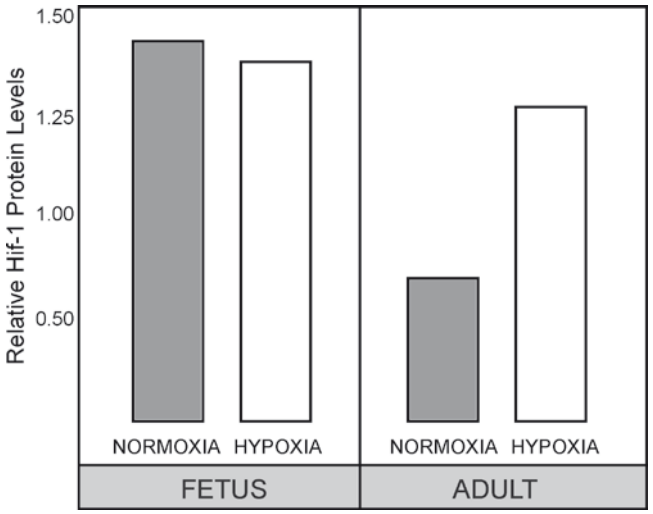


Fig. 13.9 Relative levels of HIF-1 protein expression, as determined by Western blotting, between fetal (n = 4 animals; eight experiments) and adult (n = 4 animals; eight experiments) PASMCs under conditions of either normoxia or hypoxia. Hypoxia decreased HIF-1 protein expression in adult, but not fetal, PASMCs. Protein was normalized to β-actin concentration. *$p < 0.01$ versus fetus; **$p < 0.01$

to adult PASMCs derive from protein measurements using enzyme-linked immunosorbent assay (ELISA) on the nuclear extract. Hypoxia increased HIF-1 protein levels in adult PASMCs (normoxia = 0.58 ± 0.13 arbitrary units [a.u.], hypoxia = 1.32 ± 0.15 a.u.; $p < 0.001$), but had no effect on fetal PASMCs (normoxia = 1.42 ± 0.13 a.u., hypoxia = 1.37 ± 0.20 a.u.). Surprisingly, in a separate set of experiments, even anoxia had no effect on fetal HIF-1 protein levels (normoxia = 1.0 ± 0.12 a.u., anoxia = 0.85 ± 0.20 a.u.) in fetal PASMCs.[72]

In contrast to the effect of hypoxia on protein expression, hypoxia had no effect on HIF-1 gene expression in adult PASMC HIF-1 (Fig. 13.9) but caused a marked increase in fetal PASMC HIF-1 α mRNA expression (26.7 ± 4.4%; $p < 0.01$).[72] These observations suggest that in the fetus, HIF-1 protein expression is O_2 insensitive, while mRNA expression is sensitive to hypoxia. To elucidate the mechanisms by which fetal HIF-1 is rendered O_2 insensitive, molecules involved in the regulation of HIF-1α stability were interrogated. In specific, we sought to determine whether differential regulation in the fetus prevents degradation of HIF-1.

10 Prolyl Hydroxylases PHD2 and PHD3 are Developmentally Regulated

The PHDs are O_2 sensitive.[63] PHD2 levels are increased by hypoxia.[66] Consistent with these results, we found that hypoxia significantly increased PHD2 protein expression in adult, but not fetal, PASMCs. Neither PHD2 protein nor mRNA expression

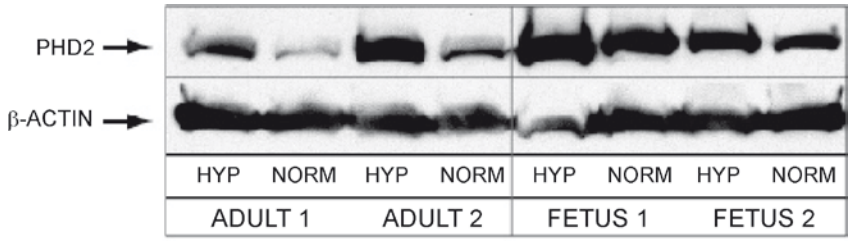


Fig. 13.10 Protein expression of prolyl hydoxylase 2 in pulmonary artery smooth muscle cells from the late-gestation ovine fetus and adult sheep. PHD2 expression is increased in fetal and adult PASMCs. Expression of PHD2 protein is greater in fetal compared to adult pulmonary artery smooth muscle cells

changed with hypoxia in fetal PASMCs. Consistent with HIF-1, the oxygen sensitivity of PHD2 protein expression is developmentally regulated. PHD2 protein and gene expression was significantly greater in fetal compared to adult PASMCs.

The teleologic reason for the differential expression of PHD2 between fetus and adult is unclear but may relate to the critical role of the PHDs on reoxygenation[60,67] to ensure rapid cessation of the hypoxic response. Similarly, PHD2 mRNA and protein levels might be relatively high in the term mammalian fetus to ready the fetus to rapidly silence the HIF-1-dependent transcription immediately on initiation of air-breathing life. The critical changes that quickly occur at birth, like pulmonary vascular relaxation and vascular remodeling (such as closure of the ductus arteriosus), might require an immediate reprogramming of the cellular transcriptional machinery. A buildup of the enzymes that destroy and inactivate HIF-1 prior to birth may represent a necessary step that enables the swift termination of the HIF-1 pathway. At the same time, the low oxygen availability in the fetus likely prevents the accumulated PHD2 from promoting HIF-1α degradation in the fetus, where the HIF-1α expression is required for fetal development.

Consistent with the notion of PHD2 "buildup" before birth, gene expression of PHD2 IS also increased in the late-gestation fetus compared to an adult[72] (Fig. 13.10). mRNA expression of PHD2 is fourfold greater in the fetus compared to the adult. The difference in expression might serve to ensure that PHD2 protein can be produced in sufficient quantities at birth to enable an almost immediate halt in HIF-1α protein production on introduction to air-breathing life. The relative differences in PHD2 protein and mRNA expression could be due to differential hypoxia-induced inhibition of transcription.[73]

Similarly, PHD3 mRNA levels are increased in the fetus compared to the adult, albeit to a lesser degree than PHD2, as PHD3 mRNA expression is 25% higher ($p < 0.03$) in the fetus compared to the adult. These results demonstrate that both mRNA and protein expression of PHD expression (mainly of PHD2) are increased in the term fetus. Despite the elevation of PHD expression, HIF-1α protein expression is O_2 insensitive.[71] We speculate that the role of PHD may be to "turn off" the HIF-1α machinery immediately after birth. While the O_2 insensitivity of HIF-1 is unprecedented, it suggests that the molecule is absolutely required for normal fetal

growth and development. This interpretation begs the question of the control of HIF-1 expression and activity in the normal fetus.

11 Asparagyl Hydroxylase FIH-1 and CITED2 are Developmentally Regulated

While the level of protein expression of the HIF-1 transactivator blocker FIH is slight in fetal cells, compared to the adult, its gene expression is greatly increased relative to the adult cells. The teleologic reason for the relative increases in both the PHDs and asparagyl hydroxylases in the fetus compared to the adult may be informed by divergent O_2 affinities of the molecules. While the fetal environment is relatively low in oxygen, it is not an oxygen-free system. The accumulation of PHD in the preterm fetus might be "safe," from an HIF-1 standpoint, as the fetal environment might be insufficiently hypoxic to elicit the hydroxylation reaction that targets HIF-1 for degradation. Alternatively, FIH hydroxylase activity is likely to be comparatively active in that same environment[74] in such a way that its expression at high levels could hinder HIF-1-dependent transcription in the fetus. In support of this hypothesis, the FIH K_m for O_2 in vitro (90 μM) is clearly lower than the K_m of the HIF PHDs (230–250 μM).[75] The difference in the K_m for O_2 in FIH and PHDs suggests that a minor decrease in O_2 concentration is likely to effect HIF PHD activity,[63] while a larger decrease in O_2 concentration is needed for a significant decrease in the activity of FIH.[74] The observation that CITED2 mRNA expression is developmentally regulated, with genetic expression fourfold greater in the fetus compared to the adult is consistent with the notion that HIF-1 expression and activity are essential in the fetus and must be amenable to rapid inactivation, via multiple yet complementary mechanisms, on introduction to the postnatal oxygen-rich environment.

12 Conclusions

In conclusion, we report that in the fetal pulmonary vasculature, HIF-1 protein is oxygen insensitive while HIF-1 gene expression is sensitive to a change in oxygen tension. These results stand in sharp contrast to the interaction between either HIF-1 protein or mRNA in the pulmonary vasculature of the adult, in which protein is highly sensitive to a change in oxygen tension, while genetic expression does not vary with oxygen tension. Moreover, the expression of several molecules critical for HIF-1 activity differs with respect to both oxygen sensitivity and developmental stage. Evidence that the present findings have functional implications includes the observations that molecular expression of both, vascular endothelial growth factor (VEGF) and protein kinase G-1 (PKG-1) decrease with hypoxia in fetal, but not adult, PASMCs. These findings have important implications for lung development in both normal newborns and infants born prematurely.

References

1. Dawes GS, Mott JC, Widdicombe JG, Wyatt DG (1953) Changes in the lungs of the newborn lamb. J Physiol 121:141–162
2. Cassin S, Dawes GS, Ross BB (1964) Pulmonary blood flow and vascular resistance in immature fetal lambs. J Physiol 171:80–89
3. Assali NS, Kirchbaum TH, Dilts PV (1968) Effects of hyperbaric oxygen on utero placental and fetal circulation. Circ Res 22:573–588
4. Leffler CW, Hessler JR, Green RS (1984) The onset of breathing at birth stimulates pulmonary vascular prostacyclin synthesis. Pediatr Res 18:938–942
5. Leffler CW, Tyler TL, Cassin S (1978) Effect of indomethacin on pulmonary vascular response to ventilation of fetal goats. Am J Physiol 235:H346–H351
6. Morin F, Eagan E, Ferguson W, Lundgren CEG (1988) Development of pulmonary vascular response to oxygen. Am J Physiol 254:H542–H546
7. Furchgott RF (1998) Studies on relaxation of rabbit aorta by sodium nitrite: the basis for the proposal that the acid-activatable inhibitory factor from bovine retractor penis is inorganic nitrite and the endothelium-derived relaxing factor is nitric oxide. In: Vanhoutte PM (ed) Vasodilatation, Raven, New York, NY, pp 410–414
8. Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G (1987) Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc Natl Acad Sci U S A 84:9265–9269
9. Amezcua JL, Palmer RMJ, de Souza BM, Moncada S (1989) Nitric oxide synthesized from L-arginine regulates vascular tone in the coronary circulation of the rabbit. Br J Pharmacol 97:1019–1024
10. Abman SH, Chatfield BA, Hall SL, McMurtry IF (1990) Role of endothelium-derived relaxing factor during transition of pulmonary circulation at birth. Am J Physiol 259:H1921–H1927
11. Cornfield D, Chatfield B, McQueston J, McMurtry I, Abman S (1992) Effects of birth-related stimuli on L-arginine-dependent pulmonary vasodilation in ovine fetus. Am J Physiol 262:H1474–H1481
12. McQueston JA, Cornfield DN, McMurtry IF, Abman SH (1993) Effects of oxygen and exogenous L-arginine on EDRF activity in fetal pulmonary circulation. Am J Physol 264: H865–H871
13. Tiktinsky MH, Morin FC 3rd (1993) Increasing oxygen tension dilates fetal pulmonary circulation via endothelium-derived relaxing factor. Am J Physiol 265:H376–H380
14. Shaul PW, Wells LB (1994) Oxygen modulates nitric oxide production selectively in fetal pulmonary endothelial cells. Am J Respir Cell Mol Biol 11:432–438
15. Robertson BE, Schubert R, Hescheler J, Nelson M (1993) cGMP-dependent protein kinase activates Ca-activated K channels in cerebral artery smooth muscle cells. Am J Physiol 265:C299–C303
16. Archer SL, Huang JM-C, Hampl V, Nelson DP, Shultz PJ, Weir EK (1994) Nitric oxide and cGMP cause vasorelaxation by activation of a charybdotoxin-sensitive K channel by cGMP-dependent protein kinase. Proc Natl Acad Sci U S A 91:7583–7587
17. Bolotina VM, Najibi S, Palacino JJ, Pagano PJ, Cohen RA (1994) Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. Nature 368:850–853
18. Chang J-K, Moore P, Fineman JR, Soifer SJ, Heymann MA (1992) K^+ channel pulmonary vasodilation in fetal lambs: role of endothelium-derived nitric oxide. J Appl Physiol 73:188–194
19. Pinheiro JM, Malik AB (1992) K^+_{ATP}-channel activation causes marked vasodilation in the hypertensive neonatal pig lung. Am J Physiol 263:H1532–H1536
20. Cornfield DN, McQueston JA, McMurtry IF, Rodman DM, Abman SH (1992) Role of ATP-sensitive potassium channels in ovine fetal pulmonary vascular tone. Am J Physiol 263:H1363–H1368

21. Boels PJ, Gao B, Deutsch J, Haworth SG (1997) ATP-dependent K^+ channel activation in isolated normal and hypertensive newborn and adult porcine pulmonary vessels. Pediatr Res 42:317–326
22. Cornfield DN, Reeve HL, Tolarova S, Weir EK, Archer SL (1996) Oxygen causes fetal pulmonary vasodilation through activation of a calcium-dependent potassium channel. Proc Natl Acad Sci U S A 93:8089–8094
23. Langton P, Nelson MT, Huang Y, Standen N (1991) Block of calcium-activated potassium channels in mammalian arterial myocytes by tetraethylammonium ions. Am J Physiol 260: H927–H935
24. Nelson MT, Quayle JM (1995) Physiological roles and properties of potassium channels in arterial smooth muscle. Am J Physiol 268:C799–C822
25. Wallner M, Meera P, Toro L (1999) Molecular basis of fast inactivation in voltage and Ca^{2+}-activated K^+ channels: a transmembrane β-subunit homolog. Proc Natl Acad Sci U S A 96: 4137–4142
26. Cook N (1988) The pharmacology of potassium channels and their therapeutic potential. Trends Pharmacol Sci 9:21–28
27. Cook NS (1990) Potassium channels: structure, classification, function, and therapeutic potential. Ellis Horwood Limited, Chichester, England
28. Tristani-Firouzi M, Martin EB, Tolarova S, Weir EK, Archer SL, Cornfield DN (1996) Ventilation-induced pulmonary vasodilation at birth is modulated by potassium channel activity. Am J Physiol 271:H2353–H2359
29. Reeve HL, Archer SL, Weir EK, Cornfield DN (1998) Maturational changes in K^+ channel activity and oxygen sensing in the ovine pulmonary vasculature. Am J Physiol 275: L1019–L1025
30. Cornfield DN, Saqueton CB, Porter VA et al (2000) Voltage-gated K^+ channel activity in the ovine pulmonary vasculature is developmentally regulated. Am J Physiol 278:L1297–L1304
31. Archer SL, Souil E, Dinh-Xuan AT et al (1998) Molecular identification of the role of voltage-gated K^+ channels, Kv1.5 and Kv2.1, in hypoxic pulmonary vasoconstriction and control of resting membrane potential in rat pulmonary artery myocytes. J Clin Invest 101:2319–2330
32. Yuan XJ (1995) Voltage-gated K^+ currents regulate resting membrane potential and $[Ca^{2+}]_i$ in pulmonary arterial myocytes. Circ Res 77:370–378
33. Gordon JB, Hartup J, Hakim A (1989) Developmental effects of hypoxia and indomethacin on distribution of vascular responses in lamb lungs. Pediatr Res 26:325–329
34. Rendas A, Reid L (1982) Response of the pulmonary circulation to acute hypoxia in the growing pig. J Appl Physiol 52:811–814
35. Rhodes MT, Porter VA, Saqueton CB, Herron JM, Resnik ER, Cornfield DN (2001) Pulmonary vascular response to normoxia and K_{Ca} channel activity is developmentally regulated. Am J Physiol 280:L1250–L1257
36. Cornfield DN, Stevens T, McMurtry IF, Abman SH, Rodman DM (1994) Acute hypoxia causes membrane depolarization and calcium influx in fetal pulmonary artery smooth muscle cells. Am J Physiol 266:L469–L475
37. Porter VA, Reeve HL, Cornfield DN (2000) Fetal rabbit pulmonary artery smooth muscle cell response to ryanodine is developmentally regulated. Am J Physiol Lung Cell Mol Physiol 279:L751–L757
38. Porter VA, Rhodes MT, Reeve HL, Cornfield DN (2001) Oxygen-induced perinatal pulmonary vasodilation is mediated by ryanodine-sensitive activation of a calcium-sensitive K^+ channel. Am J Physiol Lung Cell Mol Physiol 281:L1379–L1385
39. Gomez AM, Valdivia HH, Cheng H et al (1997) Defective excitation-contraction coupling in experimental cardiac hypertrophy and heart failure. Science 276:800–806
40. Porter VA, Bonev AD, Knot HJ et al (1998) Frequency modulation of Ca^{2+} sparks is involved in regulation of arterial diameter by cyclic nucleotides. Am J Physiol Cell Physiol 274:C1346–C1355

41. Jaggar JH, Wellman GC, Heppner TJ et al (1998) Ca^{2+} channels, ryanodine receptors and Ca^{2+}-activated K^+ channels: a functional unit for regulating arterial tone. Acta Physiol Scand 164:577–587
42. Nelson MT, Cheng H, Rubart M et al (1995) Relaxation of arterial smooth muscle by arterial sparks. Science 270:633–637
43. Saqueton CB, Miller RM, Porter VA, Millla CM, Cornfield DN (1999) Nitric oxide causes perinatal pulmonary vasodilation through K^+ channel activation and requires intracellular calcium release. Am J Physiol 276:L925–L932
44. Tanaka Y, Meera P, Song M, Knaus HG, Toro L (1997) Molecular constituents of maxi K_{Ca} channels in human coronary smooth muscle: predominant $\alpha + \beta$ subunit complexes. J Physiol 502:545–557
45. Brenner R, Peréz GJ, Bonev AD et al (2000) Vasoregulation by the β1 subunit of the calcium-activated potassium channel. Nature 407:870–876
46. Jiang Z, Wallner M, Meera P, Toro L (1999) Human and rodent maxiK channel β-subunit genes: cloning and characterization. Genomics 55:57–67
47. Semenza G, Jiang B-H, Leung S et al (1996) Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. J Biol Chem 271:32529–32537
48. Beck I, Ramirez S, Weinmann R, Caro J (1991) Enhancer element at the 3′-flanking region controls transcriptional response to hypoxia in the human erythropoietin gene. J Biol Chem 266:15563–15566
49. Semenza GL, Roth PH, Fang HM, Wang GL (1994) Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. J Biol Chem 269:23757–23763
50. Melillo G, Musso T, Sica A, Taylor LS, Cox GW, Varesio LA (1995) Hypoxia-responsive element mediates a novel pathway of activation of the inducible nitric oxide synthase promoter. J Exp Med 182:1683–1693
51. Lok CN, Ponka P (1999) Identification of a hypoxia response element in the transferrin receptor gene. J Biol Chem 274:24147–24152
52. Rolfs A, Kvietikova I, Gassmann M, Wenger RH (1997) Oxygen-regulated transferrin expression is mediated by hypoxia-inducible factor-1. J Biol Chem 272:20055–20062
53. Bruick RK, McKnight SL (2001) A conserved family of prolyl-4-hydroxylases that modify HIF. Science 294:1337–1340
54. Maxwell PH, Weisener MS, Chang GW et al (1999) The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen dependent proteolysis. Nature 399:271–275
55. Semenza GL (2008) Vasculogenesis, angiogenesis, and arteriogenesis: mechanisms of blood vessel formation and remodeling. J Cell Biochem 102:840–847
56. Semenza GL (1999) Regulation of mammalian O_2 homeostasis by hypoxia-inducible factor 1. Annu Rev Cell Dev Biol 15:551–578
57. Sainson RC, Harris AL (2008) Regulation of angiogenesis by homotypic and heterotypic notch signalling in endothelial cells and pericytes: from basic research to potential therapies. Angiogenesis 11:41–51
58. Huang LE, Gu J, Schau M, Bunn HF (1998) Regulation of hypoxia-inducible factor 1α is mediated by an O_2-dependent degradation domain via the ubiquitin-proteasome pathway. Proc Natl Acad Sci U S A 95:7987–7992
59. Salceda S, Caro J (1997) Hypoxia-inducible factor 1α (HIF-1α) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. J Biol Chem 272:22642–22647
60. Kaelin WJ (2005) The von Hippel-Lindau protein, HIF hydroxylation, and oxygen sensing. Biochem Biophys Res Commun 338:627–638
61. Ivan M, Kondo K, Yang H et al (2001) HIF-1α targeted for VHL-mediated destruction by proline hydroxylation: implications for O_2 sensing. Science 292:464–468
62. Jaakola P, Mole DR, Tian YM et al (2001) Targeting of HIF-1α to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. Science 292:468–472

63. Hirsilä M, Koivunen P, Gunzler V, Kivirikko KI, Myllyharju J (2003) Characterization of the human prolyl 4-hydroxylases that modify the hypoxia-inducible factor. J Biol Chem 278:30772–30780
64. Cioffi CL, Liu XQ, Kosinski PA, Garay M, Bowen BR (2003) Differential regulation of HIF-1α prolyl-4-hydroxylase genes by hypoxia in human cardiovascular cells. Biochem Biophys Res Commun 303:947–953
65. Lieb ME, Menzies K, Moschella MC, Ni R, Taubman MB (2002) Mammalian EGLN genes have distinct patterns of mRNA expression and regulation. Biochem Cell Biol 80:421–426
66. Berra E, Benizr iE, Ginouvès A, Volmat V, Roux D, Pouysségur J (2003) HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1α in normoxia. EMBO J 22:4082–4090
67. D'Angelo G, Duplan E, Boyer N, Vigne P, Frelin C (2003) Hypoxia up-regulates prolyl hydroxylase activity: a feedback mechanism that limits HIF-1 responses during reoxygenation. J Biol Chem 278:38183–38187
68. Lando D, Peet D, Whelan D, Gorman J, Whitelaw M (2002) Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch. Science 295:858–861
69. Bhattacharya S, Ratcliffe PJ (2003) ExCITED about HIF. Nat Struct Biol 10:501–503
70. Yin Z, Haynie J, Yang X et al (2002) The essential role of Cited2, a negative regulator for HIF-1α, in heart development and neurulation. Proc Natl Acad Sci U S A 99:10488–10493
71. Weninger WJ, Lopes Floro K, Bennett MB et al (2005) Cited2 is required both for heart morphogenesis and establishment of the left-right axis in mouse development. Development 132:1337–1348
72. Resnik ER, Herron JM, Lyu SC, Cornfield DN (2007) Developmental regulation of hypoxia-inducible factor 1 and prolyl-hydroxylases in pulmonary vascular smooth muscle cells. Proc Natl Acad Sci U S A 104:18789–18794
73. Koritzinsky M, Wouters BG (2007) Hypoxia and regulation of messenger RNA translation. Methods Enzymol 435:247–273
74. Stolze IP, Tian YM, Appelhoff RJ et al (2004) Genetic analysis of the role of the asparaginyl hydroxylase factor inhibiting hypoxia-inducible factor (HIF) in regulating HIF transcriptional target genes. J Biol Chem 279:42719–42725
75. Koivunen P, Hirsilä M, Günzler V, Kivirikko KI, Myllyharju J (2004) Catalytic properties of the asparaginyl hydroxylase (FIH) in the oxygen sensing pathway are distinct from those of its prolyl 4-hydroxylases. J Biol Chem 279:9899–9904

Hypoxic Regulation of Ion Channels and Transporters in Pulmonary Vascular Smooth Muscle

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Abstract Exposure to prolonged alveolar hypoxia, as occurs with many chronic lung diseases or residence at high altitude, results in the development of pulmonary hypertension, significantly worsening patient prognosis. While the structural and functional changes that occur in the pulmonary vasculature in response to chronic hypoxia have been well characterized, less is known regarding the cellular mechanisms underlying this process. The use of animals models of hypoxic pulmonary hypertension have provided important insights into the changes that occur in the pulmonary vascular smooth muscle cells and some of the mediators involved. In this chapter, the effect of chronic hypoxia on various pulmonary arterial smooth muscle cell ion channels and transporters, and the role of the transcription factor, hypoxia-inducible factor 1, in regulating these changes, will be discussed.

Keywords Hypoxia-inducible factor 1 • Na+/H+ exchanger • Intracellular calcium • Intracellular pH • Chronic hypoxia • Pulmonary vascular smooth muscle • Hypoxic pulmonary hypertension

1 Introduction

All organisms require the ability to sense and respond to changes in oxygen levels for survival. Over time, oxygen homeostasis has evolved into a complex system to regulate oxygen delivery and use. Immediate responses to changes in oxygen concentration usually involve modifications of preexisting proteins, such as changes in phosphorylation, secretion, cleavage, or redox state. In contrast, long-term adaptation to hypoxia or hyperoxia typically results in altered gene expression and changes in the levels of various proteins.

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Many chronic lung diseases, including emphysema, chronic bronchitis, chronic asthma, and cystic fibrosis, are associated with reduced levels of alveolar oxygen, resulting in the development of pulmonary arterial hypertension. In an effort to better understand the effects of long-term hypoxic exposure on the pulmonary circulation, many investigators turned to animal models. A widely used, well-established method for inducing hypoxic pulmonary hypertension is to expose rats or mice to normobaric or hypobaric hypoxia (10% O_2) for 14–28 days in an environmental chamber. Studies using chronically hypoxic animals have demonstrated that the elevation in pulmonary arterial pressure observed under these conditions is due to both structural remodeling of the pulmonary vasculature, characterized by smooth muscle cell proliferation, intimal thickening, and extension of smooth muscle into previously nonmuscular arterioles,[1,2] and active contraction of vascular smooth muscle, evidenced by acute reduction in pulmonary arterial pressure in response to vasodilatory agents.[3,4] The development of pulmonary arterial hypertension has a devastating impact on patient mortality and morbidity due to eventual right heart failure, with current treatment modalities limited primarily to supplemental oxygen, mechanical ventilation, and lung transplant. The key to development of new therapeutic approaches to prevent or reverse pulmonary hypertension lies in understanding the processes involved in pulmonary vascular responses to prolonged hypoxia. Unfortunately, despite advances in knowledge with respect to the structural and functional changes that occur in the pulmonary vasculature in response to chronic hypoxia (CH), the cellular mechanisms underlying hypoxic pulmonary vasoconstriction (HPV), pulmonary vascular smooth muscle cell migration, hypertrophy and hyperplasia, and the subsequent development of pulmonary hypertension remain poorly understood.

Emerging evidence suggests that both the sustained vasoconstriction and vascular remodeling associated with CH may be related to abnormalities in the pulmonary arterial smooth muscle cells (PASMCs). In particular, a number of studies have now suggested that changes in PASMC function may be related to changes in membrane transporter/channel expression and intracellular ion concentrations. This chapter describes CH-induced changes in transporter/channel activity in PASMCs and evidence for alterations in channel/transporter expression. The role of the transcription factor hypoxia-inducible factor 1 (HIF-1) in mediating these responses is also discussed.

2 Changes in K^+ channels with Chronic Hypoxia

In PASMCs, K^+ channels are the major regulators of resting membrane potential (E_m),[5,6] which in turn modulates intracellular calcium concentration ($[Ca^{2+}]_i$) due to the voltage dependence of Ca^{2+} influx through sarcolemmal Ca^{2+} channels. Changes in intracellular Ca^{2+} homeostasis are important for PASMC function as a rise in $[Ca^{2+}]_i$ is required for both HPV[7–9] and smooth muscle growth and proliferation.[10–12] Of the various K^+ channel families present in PASMCs, under normal conditions voltage-gated K^+ (K_v) channels are the main subtype responsible for control of basal E_m in PASMCs, with inhibition of these channels causing membrane depolarization,

activation of voltage-dependent Ca^{2+} channels (VDCCs) and increased $[Ca^{2+}]_i$.[5,6] Early experiments reported that depolarization[13] and reduced K_v channel activity[14] were observed in PASMCs from rats exposed to CH, results confirmed in later studies (Fig. 17.1a, b). These data suggested that hypoxia caused alterations in K^+ channel regulation or expression. Since these effects were observed for several hours after return to normoxic conditions and most adaptations to CH are accomplished by changes in gene expression, it is likely that the decrease in K_v channel activity observed in these studies was mediated by transcriptional regulation. Indeed, this hypothesis was first tested in vitro; PASMCs cultured under hypoxic conditions for 72 h exhibited decreased expression of messenger RNAs (mRNAs) encoding several K_v channel α (pore-forming) subunits, including $K_v1.1$, $K_v1.5$, and $K_v2.1$,[15] suggesting that hypoxia could repress K^+ channel expression.

However, since the effect of short-term, in vitro exposure to hypoxia in cultured cells may not accurately reflect the effects of CH on K^+ channel expression in the intact animal, where the duration of exposure, level of hypoxia, or changes in pulmonary arterial pressure and circulating factors may alter the response, several laboratories then explored the effect of CH on K_v channel expression in vivo. In intact animals, the protein expression of $K_v2.1$ and $K_v1.5$ was shown to decrease in pulmonary arteries isolated from rats exposed to 2–3 weeks of hypoxia.[16,17] The decrease in $K_v1.5$ protein levels was associated with a reduction in mRNA expression, although the effect on $K_v2.1$ gene expression was not examined.

Subsequent experiments from our laboratory examined the effect of CH on the expression of the *Shaker* family K_v channels, $K_v1.1$–1.6, the Shab family channel $K_v2.1$, the *Shal* family member $K_v4.3$, the subunit that assembles with *Shab* family members to form functional heteromeric channels ($K_v9.3$), and the regulatory β subunits $K_vβ1.1$–1.3 in distal pulmonary arteries from rats.[18] Exposure to CH reduced the expression of several subtypes of K_v channel α subunits in pulmonary arteries, including $K_v1.1$, $K_v1.2$, $K_v1.5$, $K_v1.6$, $K_v2.1$, $K_v4.3$, and $K_v9.3$. These results suggest that the reduction in K_v current observed by our lab and others in PASMCs from chronically hypoxic rats could be due to a reduction in K_v channel density. Moreover, K_v β-subunit expression was unaffected by in vitro[15] or in vivo[18] hypoxia. Under these conditions, a decrease in K_v channel number with a concomitant increase in association with inhibitory β subunits could result in reduced K_v current density. In contrast to the effects of CH on K_v channel expression in pulmonary arteries, K_v channel expression was unaltered by CH in aorta from the same animals,[18] indicating that the CH-induced reduction in K_v channel expression and activity is a pulmonary-specific response, although the reason for this difference is still under investigation.

3 Effect of Chronic Hypoxia on Ca^{2+} Channels

Both acute hypoxic vasoconstriction and in vitro smooth muscle proliferation are associated with alterations in Ca^{2+} homeostasis[7–11] and can be prevented by administration of voltage-gated Ca^{2+} channel antagonists.[7,12,19] Since PASMCs from

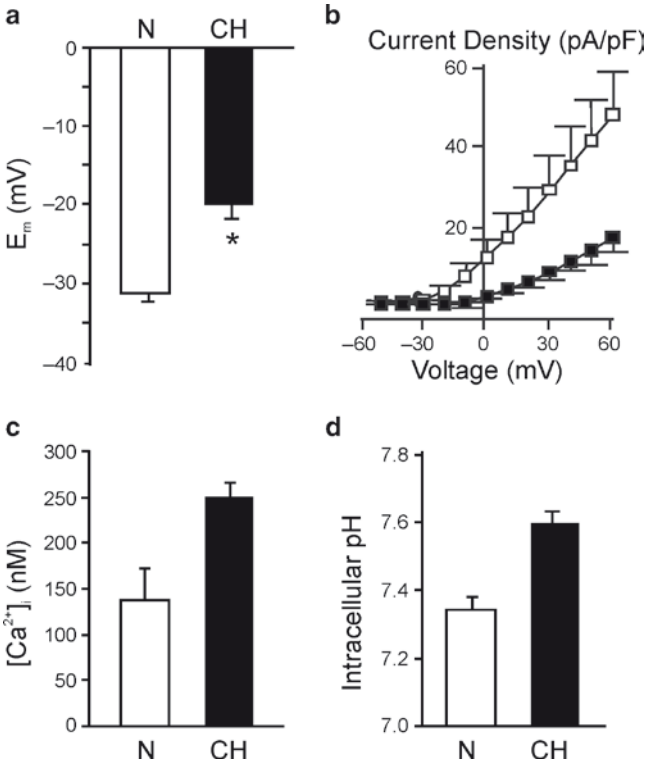

Fig. 14.1 Effect of chronic hypoxia on pulmonary arterial smooth muscle cell: (**a**) membrane potential E_m, (**b**) voltage-gated K^+ channel current density, (**c**) intracellular calcium concentration ($[Ca^{2+}]_i$), (**d**) intracellular pH. All measurements were performed on cells isolated from normoxic (N) or chronically hypoxic (CH) rats (**a**–**c**) or mice (**d**)

chronically hypoxic animals are depolarized, presumably secondary to decreased voltage-gated K^+ channel activity, it was initially hypothesized that an increase in $[Ca^{2+}]_i$ due to activation of VDCCs was the mechanism underlying hypoxic pulmonary hypertension.[13,14] This speculation was contradicted, however, by data indicating that voltage-gated Ca^{2+} channel antagonists did not prevent development of hypertension secondary to CH.[20] Furthermore, acute administration of vasodilators,[21] but not Ca^{2+} channel antagonists,[20] reduced pulmonary artery pressure in patients with hypoxic pulmonary hypertension due to chronic obstructive pulmonary disease. To answer whether activation of VDCCs was an important component underlying the development of hypoxic pulmonary hypertension, we initially demonstrated that basal $[Ca^{2+}]_i$ was increased in PASMCs from chronically hypoxic rats,[22] confirming profound changes in Ca^{2+} regulation (Fig. 14.1c). This increase in $[Ca^{2+}]_i$ was rapidly normalized by removal of extracellular Ca^{2+}, consistent with active Ca^{2+} entry into the cells as a requirement to maintain elevated $[Ca^{2+}]_i$. Somewhat surprisingly, VDCC blockers had no effect on resting $[Ca^{2+}]_i$,[22] providing

evidence against voltage-dependent enhancement of Ca^{2+} channels during CH. Similarly, removal of extracellular Ca^{2+} relaxed arteries from chronically hypoxic rats, while blockade of VDCCs had no effect on tone.[22] These data confirmed that the CH-induced increase in resting $[Ca^{2+}]_i$ was responsible for maintaining sustained CH-induced pulmonary vasoconstriction but eliminated a role for VDCCs.

In addition to VDCCs, Ca^{2+} influx in PASMCs can occur via nonselective cation channels (NSCCs), which include both receptor-operated Ca^{2+} channels and store-operated Ca^{2+} channels. Unlike VDCCs, NSCCs are not activated by depolarization. Instead, receptor-operated channels are activated by ligand binding to membrane receptors, while store-operated Ca^{2+} channels are activated by depletion of intracellular stores. The Ca^{2+} influx through NSCCs induced by store depletion serves to replenish stores and has thus been termed *capacitative Ca^{2+} entry* (CCE). CCE is present in PASMCs,[10,23] and studies using inhibitors of NSCCs, which inhibit CCE, revealed a role for these channels in PASMC contraction and growth.[10,11]

CCE can be measured as the change in $[Ca^{2+}]_i$ in response to readdition of extracellular Ca^{2+} following depletion of intracellular stores in the presence of VDCC blockers.[23] One caveat of this approach is that the increase in $[Ca^{2+}]_i$ following readdition of Ca^{2+} can be influenced by both Ca^{2+} influx through NSCCs and Ca^{2+} efflux, through plasmalemmal Ca^{2+}-ATPases (adenosine triphosphatases) and Na^+/Ca^{2+} exchange. Therefore, many investigators also use Mn^{2+} quenching of fura-2 at the isobesic point (360 nm), at which the intensity of fura-2 fluorescence is the same for Ca^{2+}-bound and Ca^{2+}-free fura-2, as a more direct evaluation of Ca^{2+} entry. Mn^{2+} readily passes through Ca^{2+}-conducting channels and binds fura-2, reducing fluorescence intensity in proportion to the rate of Mn^{2+} entry. Measuring CCE using these methods revealed that Ca^{2+} influx through NSCCs was greater in PASMCs from chronically hypoxic rats compared to that measured in PASMCs from normoxic rats.[24]

Once it was established that Ca^{2+} influx via NSCCs was present in PASMCs and enhanced in the pulmonary vasculature of hypoxic animals, the question arose regarding whether enhanced CCE might contribute to the elevated basal $[Ca^{2+}]_i$ observed in these cells. Consistent with the Mn^{2+}-quenching experiments, which revealed that Ca^{2+} entry through NSCCs occurred in PASMCs from hypoxic, but not normoxic, animals, the nonselective cation channel inhibitors SKF-96362 and $NiCl_2$ decreased baseline $[Ca^{2+}]_i$ in PASMCs isolated from chronically hypoxic, but not normoxic, rats.[24] These data indicated that activation of NSCCs contributed to the maintenance of elevated basal $[Ca^{2+}]_i$ in PASMCs during CH.

As noted, the increase in resting $[Ca^{2+}]_i$ observed in PASMCs from chronically hypoxic animals has a functional consequence. That removal of extracellular Ca^{2+} causes an immediate decrease in isometric tension in pulmonary arteries from chronically hypoxic rats[22,24] indicates that Ca^{2+} influx is required not only for the CH-induced elevation in $[Ca^{2+}]_i$ but also for maintenance of active vasoconstriction. Similar to results from experiments evaluating the effect of NSCC inhibitors on basal $[Ca^{2+}]_i$, these inhibitors had little effect on tension in arteries from normoxic rats but caused a marked relaxation of intrapulmonary arteries from chronically hypoxic rats that was similar in magnitude to that observed in response to removal of extracellular calcium.[24]

A possible explanation for enhanced CCE following exposure to CH is that the expression of NSCCs or store-operated Ca^{2+} channels is increased. Ca^{2+}-permeable NSCCs are believed to be composed of mammalian homologs of transient receptor potential (TRP) proteins and, in the case of store-operated Ca^{2+} channels, may complex with the recently identified Orai and STIM1 (stromal interacting molecule 1) proteins. The exact molecular identity of the proteins encoding NSCCs remains unclear, although isoforms in the canonical TRP (TRPC) subfamily are the leading candidates. We[23–25] and others[10,26] have demonstrated that STIM1 and TRPC proteins are expressed in PASMCs. Most labs have found that, of the seven isoforms identified to date, TRPC1 and TRPC6 are highly abundant in PASMCs,[10,23–26] with some labs reporting demonstrable levels of TRPC3[26] and TRPC4[23,24] expression. Comparison of TRPC expression in pulmonary vascular smooth muscle from normoxic and chronically hypoxic rats revealed that, following exposure to CH, the expression of TRPC1 and TRPC6, but not TRPC4, increased in both the rat and murine models.[24] These results are in agreement with findings from other labs.[26]

During development of hypoxic pulmonary hypertension in the intact animal, pulmonary arteries are subjected to numerous stimuli in addition to decreased oxygen tension, including increased pressure and altered exposure to circulating factors; however, the effect of CH exposure on TRPC expression does not appear to be a result of these changes since PASMCs isolated from normoxic rats and cultured under hypoxic conditions (4% O_2; 60 h) exhibited similar increases in TRPC1 and TRPC6 mRNA and protein levels, as well as an increase in basal $[Ca^{2+}]_i$.[24] These results suggest that CH might upregulate TPRC expression through a direct effect on gene expression in PASMCs. Possible mediators of this response are discussed in other portions of this chapter.

4 Chronic Hypoxia and PASMC Intracellular pH

Most mammalian systems possess three mechanisms by which intracellular pH (pH_i) homeostasis is maintained. These include the Na^+-dependent Cl^-/HCO_3 exchange, Na^+-independent Cl^-/HCO_3 exchange, and Na^+/H^+ exchange (NHE). Although all of these exchangers have been shown to exist in vascular smooth muscle,[27,28] NHE is the primary mechanism regulating PASMC pH_i.[28] NHE resides in the plasma membrane and uses the transmembrane Na^+ gradient to extrude protons. Regulation of NHE and pH_i is vital for maintaining cell viability, as pH_i modulates a number of important cell functions, including volume regulation, signal transduction pathways involved in cell proliferation, and mediator release. A role for alkaline pH_i in hypoxic regulation of vascular caliber is indicated by studies demonstrating that acute hypoxia induces an increase in pulmonary vascular smooth muscle pH_i[27], and late hypoxic contraction is accompanied by an increase in pH_i.[29] Moreover, activation of NHE and alkalinization are required for PASMC proliferation in response to growth factors,[30] and pulmonary vascular remodeling during CH can be prevented by inhibiting NHE.[31]

In initial studies using the pH-sensitive fluorescent dye BCECF-AM (acetoxymethyl ester), basal pH_i was found to be elevated in PASMCs from chronically hypoxic animals (Fig. 14.1d), and that the elevation in basal pH_i was accompanied by an increase in NHE activity, measured as the Na^+-dependent rate of recovery from ammonium-induced acidosis.[32] Moreover, inhibitors of NHE reduced basal pH_i in PASMCs from chronically hypoxic animals. While there is ample evidence to suggest that changes in phosphorylation and regulation of NHEs could account for the increase in NHE activity during hypoxia, the fact that the change in pH_i and NHE activity in PASMCs isolated from chronically hypoxic mice were observed for several days after returning to normoxic conditions suggested instead that the increase in NHE activity most likely resulted from an increase in NHE protein expression.

Nine genes have been identified that encode nine isoforms of the Na^+/H^+ exchanger (NHE1–NHE9). NHEs 1–3 have been the best characterized. NHE1 is ubiquitously expressed, whereas NHE2 and NHE3 are found predominantly in the gastrointestinal epithelium, although low-level expression of NHE2 in the lung has been reported.[33,34] Little is known about the function and localization of NHE4 and 5, which are not present in lung[35,36], and NHE6–NHE9 are thought to be localized in mitochondria and other organlles.[37–40] Using reverse-transcription polymerase chain reaction (RT-PCR) with primers specific for mouse NHE1–NHE3, the presence of NHE1, but not NHE2 or NHE3, was demonstrated in mouse PASMCs. The presence of NHE1 protein in mouse pulmonary vascular smooth muscle was confirmed via immunoblot. Consistent with the effect of CH on NHE activity and basal pH_i, NHE1 gene and protein expression was increased significantly in PASMCs isolated from chronically hypoxic mice.[32] The importance of NHE1 in the CH-induced changes in the pulmonary vasculature was verified in a study using mice deficient for NHE1, which exhibit reduced pulmonary vascular remodeling and decreased pulmonary hypertension in response to CH.[41] These data indicate that CH has dramatic effects on PASMC function and pH homeostasis via induction of NHE1.

5 Hypoxia-Inducible Factor 1

In the preceding sections, results from several studies were presented indicating that CH-induced changes in PASMC ion channel/transporter expression. In the search for a possible mediator of hypoxic regulation of these genes, the transcription factor HIF-1 became a leading candidate. A member of the basic helix-loop-helix family of proteins, HIF-1 plays a critical role in mediating adaptive responses to hypoxia and regulates the expression of dozens of genes important in growth, vascular development, and metabolism. The expression of HIF-1 is tightly regulated by O_2 availability.[42] HIF-1 exists as a heterodimer, consisting of HIF-1α and HIF-1β subunits. HIF-1β is ubiquitously overexpressed, whereas HIF-1α is found in very low levels under normoxic conditions. Regulation of HIF-1α expression occurs at several levels. In mouse lung, HIF-1α mRNA is rapidly increased within

30 min of exposure to 7% oxygen.[43] However, HIF-1α is primarily regulated post-transcriptionally. Under normoxic conditions, HIF-1α protein is ubiquinated and subjected to proteosomal degradation. During hypoxia, induction of HIF-1 correlates with decreased ubiquination and rapid stabilization of the protein, followed by accumulation in the nucleus, where HIF-1α dimerizes with HIF-1β and binds to the core DNA sequence 5′-RCGTG-3″, resulting in the transactivation of numerous target genes. The O_2 sensitivity of HIF-1α protein expression has been reported in the lung,[44] where acute exposure of cultured rat and human smooth muscle and rat microvascular and sheep arterial endothelial cells to hypoxia resulted in an increase in HIF-1α protein levels. The increase in HIF-1α protein correlated with an increase in DNA-binding activity, showing both induction and activation of HIF-1. Thus, HIF-1α confers sensitivity and specificity for hypoxic induction.

HIF-1 activates transcription of genes encoding proteins that mediate adaptive responses to hypoxia. Mouse embryonic stem cells homozygous or heterozygous for a null allele at the *Hif1α* locus encoding HIF-1α were generated exhibiting complete ($Hif1\alpha^{-/-}$) and partial deficiency ($Hif1\alpha^{+/-}$) for HIF-1α, respectively. Transgenic $Hif1\alpha^{-/-}$ and $Hif1\alpha^{+/-}$ mice were subsequently generated. $Hif1\alpha^{-/-}$ embryos died midgestation, whereas $Hif1\alpha^{+/-}$ mice were viable and phenotypically indistinguishable from their wild-type ($Hif1\alpha^{+/+}$) littermates.[45] Studies utilizing these transgenic mice found $Hif1\alpha^{+/+}$ mice exposed to 10% O_2 for 3 weeks exhibited right heart hypertrophy, elevated pulmonary artery pressure, polycythemia, and vascular remodeling.[46] These changes were markedly attenuated in chronically hypoxic $Hif1\alpha^{+/-}$ mice, demonstrating that HIF-1α plays a pivotal role in development of hypoxic pulmonary hypertension.

5.1 HIF-1 and K_v Channels

Using mice with complete or partial expression of HIF-1α, the role of HIF-1 in mediating the effect of CH on K_v channels could be tested. A CH-induced decrease in K_v current was observed in PASMCs from $Hif1\alpha^{+/+}$mice, whereas CH had no effect on K_v current in $Hif1\alpha^{+/-}$ mice,[47] indicating that HIF-1α is required for the hypoxia-induced reduction in PASMC K_v channel activity. Depolarization was also attenuated in PASMCs from chronically hypoxic $Hif1\alpha^{+/-}$ mice.[47] Later studies[48] demonstrated that the loss of CH-induced reduction in K_v currents in mice partially deficient for HIF-1α was likely due to the fact that K_v channel expression was not altered in pulmonary vascular smooth muscle from chronically hypoxic $Hif1\alpha^{+/-}$ mice (Fig. 14.2). The ability of HIF-1 to repress K_v channels was further demonstrated by the finding that overexpression of HIF-1 under normoxic conditions, using AdCA5, an adenovirus that encodes a constitutively active form of HIF-1α,[49] was able to cause downregulation of $K_v1.5$ and $K_v2.1$.[48] Although HIF-1 has typically been shown to induce transcription of numerous genes, the possibility exists that HIF-1 could bind to, and repress transcription of, genes encoding K_v channels. Alternatively, HIF-1 could induce transcription of genes whose products participate in regulation of K_v channel activity or expression.

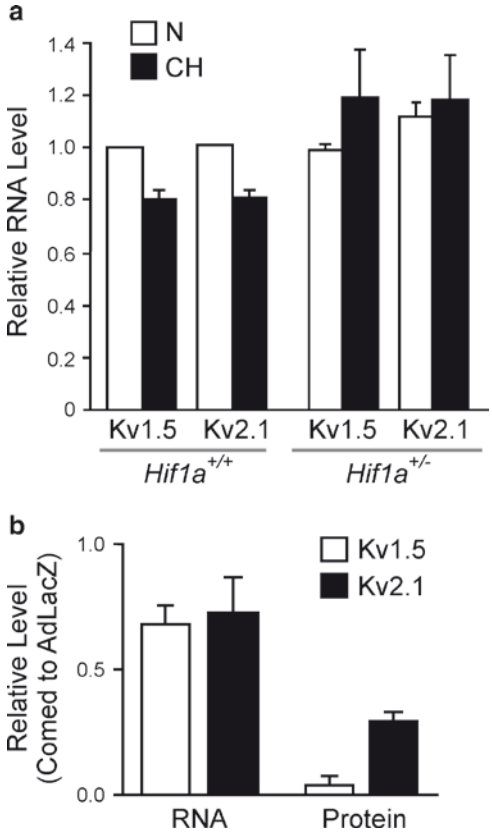

Fig. 14.2 (**a**) $K_v1.5$ and $K_v2.1$ RNA levels in endothelium-denuded intrapulmonary arteries isolated from mice with full (*Hif1α*$^{+/+}$) and partial (*Hif1α*$^{+/-}$) expression of HIF-1α exposed to normoxia (N) or chronically hypoxia (CH). Values are expressed as the fraction of the normoxic *Hif1α*$^{+/+}$ value. (**b**) Effect of overexpression of HIF-1α on $K_v1.5$ and $K_v2.1$ gene and protein expression in rat pulmonary arterial smooth muscle cells. Values are expressed relative to those in cells infected with the control construct (AdLacZ)

In support of the latter possibility, endothelin 1, a downstream target of HIF-1, was able to repress K_v channel expression. Moreover, antagonists of endothelin 1 receptors prevented hypoxic downregulation of K_v channels.[48] These results strongly suggest that hypoxic activation of HIF-1, perhaps through induction of endothelin-1, is responsible for the downregulation of K_v channel expression and activity observed in PASMCs following CH.

5.2 HIF-1 and $[Ca^{2+}]_i$

Little is known about the regulation of TRPC gene transcription, although the genes encoding the isoforms upregulated by hypoxia, TRPC1 and TRPC6, both contain

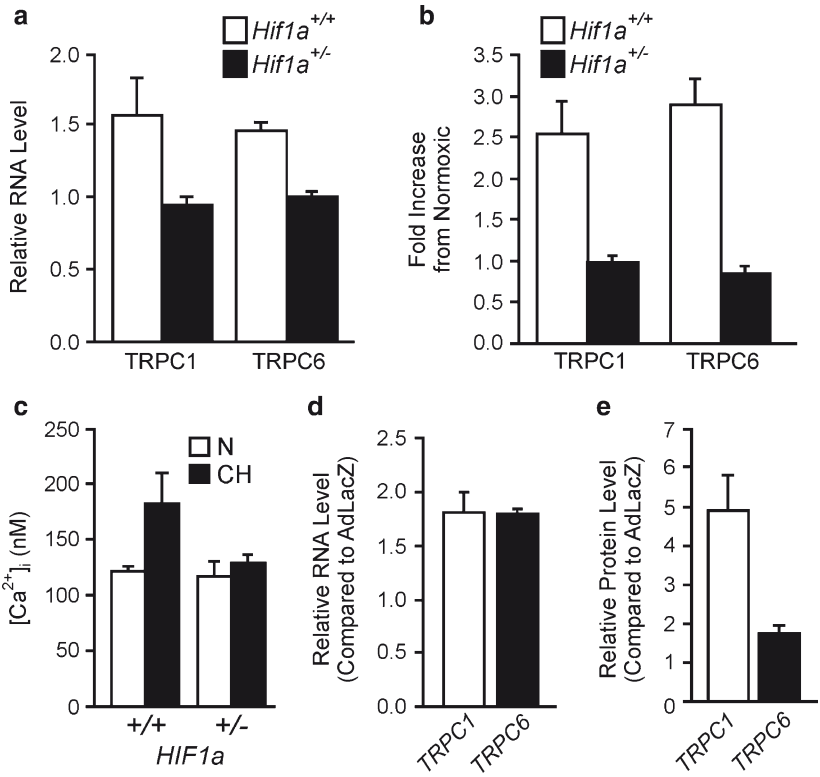

Fig. 14.3 Effect of chronic hypoxia on TRPC1 and TRPC6 (**a**) gene and (**b**) protein expression in endothelium-denuded intrapulmonary arteries isolated from wild-type mice (*Hif1α*$^{+/+}$) and mice partially deficient for HIF-1α (*Hif1α*$^{+/-}$). Values are expressed as the fraction of the normoxic *Hif1α*$^{+/+}$ value. (**c**) Intracellular calcium concentration ($[Ca^{2+}]_i$) in cells isolated from normoxic (N) and chronically hypoxic (CH) *Hif1α*$^{+/+}$ and *Hif1α*$^{+/-}$ mice. (**d**, **e**) Effect of HIF-1α overexpression on TRPC1 and TRPC6 gene and protein expression. Data are normalized to values in cells infected with the control construct (AdLacZ)

putative HIF-1-binding sites. Since previous work demonstrated a crucial role for HIF-1 in the pathogenesis of hypoxic pulmonary hypertension,[46] it was hypothesized that HIF-1 might be involved in the hypoxic induction of TRPC proteins. Consistent with data from the chronically hypoxic rat model, pulmonary arteries from *Hif1α*$^{+/+}$ mice exhibited increased TRPC1 and TRPC6 expression.[24] Likewise, PASMCs from these animals displayed elevated $[Ca^{2+}]_i$ (Fig. 14.3). In mice with partial HIF-1α deficiency, both the elevation in basal $[Ca^{2+}]_i$ and the hypoxic induction of TRPC1 and TRPC6 were lost.[24] Conversely, expression of a constitutively active form of HIF-1α under nonhypoxic conditions increased TRPC1 and TRPC6 expression.[24] These data provided strong evidence that HIF-1 was both necessary and sufficient for induction of TRPC proteins and plays a critical role in regulating alterations in Ca^{2+} homeostasis during CH.

5.3 HIF-1 and Intracellular pH

The transcriptional regulation of NHEs is just beginning to be explored, and much is yet to be learned with respect to the factors involved in the process. Initial examination of the gene encoding NHE1 indicated that the promoter contained putative HIF-1-binding sites, suggesting that hypoxic regulation of NHE1 in pulmonary vascular smooth muscle might be mediated by HIF-1. Exposure to CH markedly increased NHE activity in PASMCs isolated from *Hif1α*$^{+/+}$ mice (Fig. 14.4), whereas the CH-induced increase in NHE activity was absent in PASMCs isolated from mice partially deficient for HIF-1α.[50] As anticipated, NHE1 mRNA expression was increased in endothelium-denuded pulmonary arteries isolated from *Hif1α*$^{+/+}$ mice exposed to CH compared to mRNA levels in pulmonary arteries isolated from normoxic *Hif1α*$^{+/+}$. Immunoblot analysis revealed a similar increase in NHE1 protein

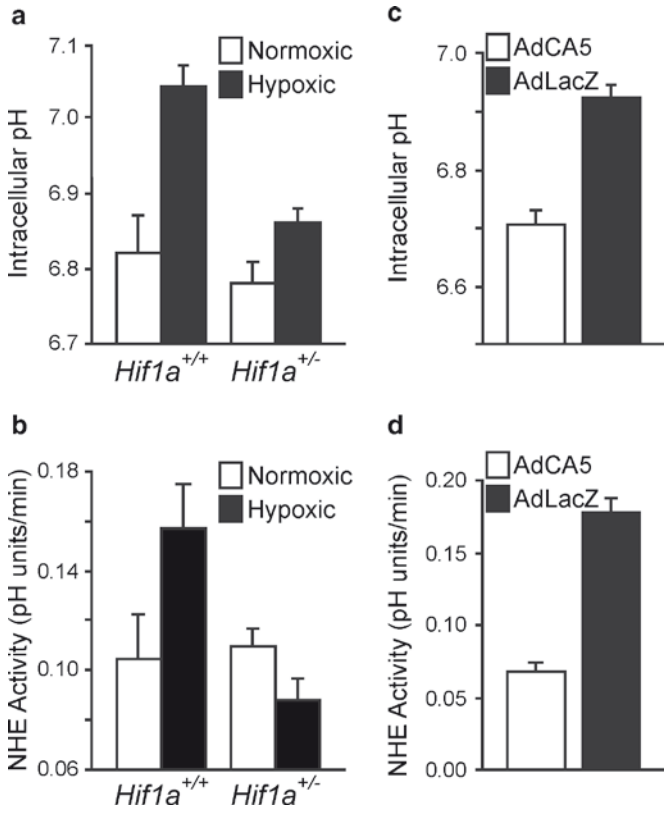

Fig. 14.4 Effect of normoxia (N) and chronic hypoxia (CH) on (**a**) intracellular pH and (**b**) Na^+/H^+ exchange (NHE) activity in pulmonary arterial smooth muscle cells isolated from wild-type mice (*Hif1α*$^{+/+}$) and mice partially deficient for HIF-1α (*Hif1α*$^{+/-}$). (**c**, **d**) Effect of HIF-1α overexpression on intracellular pH and NHE activity

expression in these animals. Neither mRNA nor protein expression of NHE1 increased in response to CH in pulmonary arteries from *Hif1α*$^{+/-}$ mice.

To verify that the hypoxia-induced increase in NHE1 expression was due to activation of HIF-1 and not an unrelated aspect of hypoxic exposure, HIF-1α was overexpressed in rat PASMCs isolated from normoxic animals and cultured under nonhypoxic conditions. Following transfection with AdCA5, basal pH_i, NHE activity, and NHE1 expression were significantly increased.[50] Taken together, data from the loss-of-function and gain-of-function models indicated that HIF-1 is required for the hypoxic regulation of NHE1 and the CH-induced alterations in PASMC pH homeostasis.

6 Summary

For patients with chronic pulmonary disease, hypoxia-induced structural and functional changes in the pulmonary vasculature correlate with the development of pulmonary hypertension and increased mortality. Although previous studies have characterized some of the morphologic and functional changes that occur in the pulmonary vasculature in response to CH, much work remains to completely understand the cellular mechanisms underlying these changes. In this chapter, work from our lab and others has been presented demonstrating an important role for alterations in PASMC ion channel/transporter activity and expression in mediating changes in PASMC function during CH. In particular, the role of HIF-1 in regulating many of these changes was described (Fig. 14.5). To date, no other gene product has been

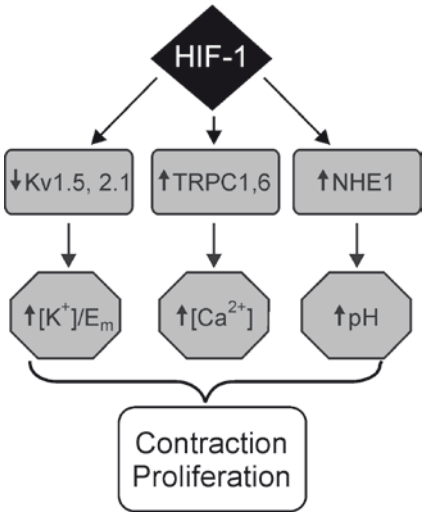

Fig. 14.5 Schematic summarizing the role of HIF-1 in mediating changes in pulmonary arterial smooth muscle cell function

shown to have such a profound effect on pulmonary vascular and, specifically, PASMCs, responses to CH and these findings provide compelling evidence that HIF-1 plays a critical role in mediating the physiological responses to hypoxia and development of pulmonary hypertension. We expect that elucidating the factors involved in this disease process, and further delineating the exact role of HIF-1, will lead to improved methods of pharmacological prevention and treatment of this lethal complication of chronic lung disease.

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References

1. Davies P, Maddalo F, Reid L (1985) Effects of chronic hypoxia on structure and reactivity of rat lung microvessels. J Appl Physiol 58:795–801
2. Meyrick B, Reid L (1978) The effect of continued hypoxia on rat pulmonary arterial circulation. An ultrastructural study. Lab Invest 38:188–200
3. Nagaoka T, Fagan KA, Gebb SA et al (2005) Inhaled Rho kinase inhibitors are potent and selective vasodilators in rat pulmonary hypertension. Am J Respir Crit Care Med 171:494–499
4. Jones K, Higenbottam T, Wallwork J (1989) Pulmonary vasodilation with prostacyclin in primary and secondary pulmonary hypertension. Chest 96:784–789
5. Archer SL, Souil E, Dinh-Xuan AT et al (1998) Molecular identification of the role of voltage-gated K^+ channels, Kv1.5 and Kv2.1, in hypoxic pulmonary vasoconstriction and control of resting membrane potential in rat pulmonary artery myocytes. J Clin Invest 101:2319–2330
6. Yuan XJ (1995) Voltage-gated K^+ currents regulate resting membrane potential and $[Ca^{2+}]_i$ in pulmonary arterial myocytes. Circ Res 77:370–378
7. McMurtry IF, Davidson AB, Reeves JT, Grover RF (1976) Inhibition of hypoxic pulmonary vasoconstriction by calcium antagonists in isolated rat lungs. Circ Res 38:99–104
8. Rodman DM, Yamaguchi T, O'Brien RF, McMurtry IF (1989) Hypoxic contraction of isolated rat pulmonary artery. J Pharmacol Exp Ther 248:952–959
9. Salvaterra CG, Goldman WF (1993) Acute hypoxia increases cytosolic calcium in cultured pulmonary arterial myocytes. Am J Physiol 264:L323–L328
10. Golovina VA, Platoshyn O, Bailey CL et al (2001) Upregulated TRP and enhanced capacitative Ca^{2+} entry in human pulmonary artery myocytes during proliferation. Am J Physiol Heart Circ Physiol 280:H746–H755
11. Sweeney M, Yu Y, Platoshyn O, Zhang S, McDaniel SS, Yuan JX-J (2002) Inhibition of endogenous TRP1 decreases capacitative Ca^{2+} entry and attenuates pulmonary artery smooth muscle cell proliferation. Am J Physiol Lung Cell Mol Physiol 283:L144–L155
12. Kruse HJ, Bauriedel G, Heimerl J, Hofling B, Weber PC (1994) Role of L-type calcium channels on stimulated calcium influx and on proliferative activity of human coronary smooth muscle cells. J Cardiovasc Pharmacol 24:328–335
13. Suzuki H, Twarog BM (1982) Membrane properties of smooth muscle cells in pulmonary hypertensive rats. Am J Physiol 242:H907–H915
14. Smirnov SV, Robertson TP, Ward JP, Aaronson PI (1994) Chronic hypoxia is associated with reduced delayed rectifier K^+ current in rat pulmonary artery muscle cells. Am J Physiol 266:H365–H370

15. Wang J, Juhaszova M, Rubin LJ, Yuan XJ (1997) Hypoxia inhibits gene expression of voltage-gated K^+ channel α-subunits in pulmonary artery smooth muscle cells. J Clin Invest 100: 2347–2353
16. Pozeg ZI, Michelakis ED McMurtry MS et al (2003) In vivo gene transfer of the O_2-sensitive potassium channel Kv1.5 reduces pulmonary hypertension and restores hypoxic pulmonary vasoconstriction in chronically hypoxic rats. Circulation 107:2037–2044
17. Hong Z, Weir EK, Nelson DP, Olschewski A (2004) Subacute hypoxia decreases voltage-activated potassium channel expression and function in pulmonary artery myocytes. Am J Respir Cell Mol Biol 31:337–343
18. Wang J, Weigand L, Wang W, Sylvester JT, Shimoda LA (2005) Chronic hypoxia inhibits Kv channel gene expression in rat distal pulmonary artery. Am J Physiol Lung Cell Mol Physiol 288:L1049–L1058
19. Weigand L, Foxson J, Wang J, Shimoda LA, Sylvester JT (2005) Inhibition of hypoxic pulmonary vasoconstriction by antagonists of store-operated Ca^{2+} and nonselective cation channels. Am J Physiol Lung Cell Mol Physiol 289:L5–L13
20. Johnson DC, Joshi RC, Mehta R, Cunnington AR (1986) Acute and long term effect of nifedipine on pulmonary hypertension secondary to chronic obstructive airways disease. Eur J Respir Dis Suppl 146:495–502
21. Moinard J, Manier G, Pillet O, Castaing Y (1994) Effect of inhaled nitric oxide on hemodynamics and VA/Q inequalities in patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med 149:1482–1487
22. Shimoda LA, Sham JS, Shimoda TH, Sylvester JT (2000) L-type Ca^{2+} channels, resting $[Ca^{2+}]_i$, and ET-1-induced responses in chronically hypoxic pulmonary myocytes. Am J Physiol Lung Cell Mol Physiol 279:L884–L894
23. Wang J, Shimoda LA, Sylvester JT (2004) Capacitative calcium entry and TRPC channel proteins are expressed in rat distal pulmonary arterial smooth muscle. Am J Physiol Lung Cell Mol Physiol 286:L848–L858
24. Wang J, Weigand L, Lu W, Sylvester JT, Semenza GL, Shimoda LA (2006) Hypoxia inducible factor 1 mediates hypoxia-induced TRPC expression and elevated intracellular Ca^{2+} in pulmonary arterial smooth muscle cells. Circ Res 98:1528–1537
25. Lu W, Wang J, Shimoda LA, Sylvester JT (2008) Differences in STIM1 and TRPC expression in proximal and distal pulmonary arterial smooth muscle are associated with differences in Ca^{2+} responses to hypoxia. Am J Physiol Lung Cell Mol Physiol 295:L104–L113
26. Lin MJ, Leung GP, Zhang WM et al (2004) Chronic hypoxia-induced upregulation of store-operated and receptor-operated Ca^{2+} channels in pulmonary arterial smooth muscle cells: a novel mechanism of hypoxic pulmonary hypertension. Circ Res 95:496–505
27. Madden JA, Ray DE, Keller PA, Kleinman JG (2001) Ion exchange activity in pulmonary artery smooth muscle cells: the response to hypoxia. Am J Physiol Lung Cell Mol Physiol 280:L264–L271
28. Quinn DA, Honeyman TW, Joseph PM, Thompson BT, Hales CA, Scheid CR (1991) Contribution of Na^+/H^+ exchange to pH regulation in pulmonary artery smooth muscle cells. Am J Respir Cell Mol Biol 5:586–591
29. Leach RM, Sheehan DW, Chacko VP, Sylvester JT (2000) Energy state, pH, and vasomotor tone during hypoxia in precontracted pulmonary and femoral arteries. Am J Physiol Lung Cell Mol Physiol 278:L294–L304
30. Quinn DA, Dahlberg CG, Bonventre JP et al (1996) The role of Na^+/H^+ exchange and growth factors in pulmonary artery smooth muscle cell proliferation. Am J Respir Cell Mol Biol 14:139–145
31. Quinn DA, Du HK, Thompson BT, Hales CA (1998) Amiloride analogs inhibit chronic hypoxic pulmonary hypertension. Am J Respir Crit Care Med 157:1263–1268
32. Rios EJ, Fallon M, Wang J, Shimoda LA (2005) Chronic hypoxia elevates intracellular pH and activates Na^+/H^+ exchange in pulmonary arterial smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 289:L867–L874

33. Wang Z, Orlowski J, Shull GE (1993) Primary structure and functional expression of a novel gastrointestinal isoform of the rat Na/H exchanger. J Biol Chem 268:11925–11928
34. Brant SR, Yun CH, Donowitz M, Tse CM (1995) Cloning, tissue distribution, and functional analysis of the human Na^+/H^+ exchanger isoform, NHE3. Am J Physiol 269:C198–C206
35. Attaphitaya S, Park K, Melvin JE (1999) Molecular cloning and functional expression of a rat Na^+/H^+ exchanger (NHE5) highly expressed in brain. J Biol Chem 274:4383–4388
36. Orlowski J, Kandasamy RA, Shull GE (1992) Molecular cloning of putative members of the Na/H exchanger gene family. cDNA cloning, deduced amino acid sequence, and mRNA tissue expression of the rat Na/H exchanger NHE-1 and two structurally related proteins. J Biol Chem 267:9331–9339
37. Miyazaki E, Sakaguchi M, Wakabayashi S, Shigekawa M, Mihara K (2001) NHE6 protein possesses a signal peptide destined for endoplasmic reticulum membrane and localizes in secretory organelles of the cell. J Biol Chem 276:49221–49227
38. Nakamura N, Tanaka S, Teko Y, Mitsui K, Kanazawa H (2005) Four Na^+/H^+ exchanger isoforms are distributed to Golgi and post-Golgi compartments and are involved in organelle pH regulation. J Biol Chem 280:1561–1572
39. Numata M, Orlowski J (2001) Molecular cloning and characterization of a novel (Na^+, K^+)/H^+ exchanger localized to the trans-Golgi network. J Biol Chem 276:17387–17394
40. Numata M, Petrecca K, Lake N, Orlowski J (1998) Identification of a mitochondrial Na^+/H^+ exchanger. J Biol Chem 273:6951–6959
41. Yu L, Quinn DA, Garg HG, Hales CA (2008) Deficiency of the NHE1 gene prevents hypoxia-induced pulmonary hypertension and vascular remodeling. Am J Respir Crit Care Med 177:1276–1284
42. Wang GL, Semenza GL (1993) Characterization of hypoxia-inducible factor 1 and regulation of DNA binding activity by hypoxia. J Biol Chem 268:21513–21518
43. Wiener CM, Booth G, Semenza GL (1996) In vivo expression of mRNAs encoding hypoxia-inducible factor 1. Biochem Biophys Res Commun 225:485–488
44. Yu AY, Frid MG, Shimoda LA, Wiener CM, Stenmark K, Semenza GL (1998) Temporal, spatial, and oxygen-regulated expression of hypoxia-inducible factor-1 in the lung. Am J Physiol 275:L818–L826
45. Iyer NV, Kotch LE, Agani F et al (1998) Cellular and developmental control of O_2 homeostasis by hypoxia-inducible factor 1α. Genes Dev 12:149–162
46. Yu AY, Shimoda LA, Iyer NV et al (1999) Impaired physiological responses to chronic hypoxia in mice partially deficient for hypoxia-inducible factor 1α. J Clin Invest 103:691–696
47. Shimoda LA, Manalo DJ, Sham JS, Semenza GL, Sylvester JT (2001) Partial HIF-1α deficiency impairs pulmonary arterial myocyte electrophysiological responses to hypoxia. Am J Physiol Lung Cell Mol Physiol 281:L202–L208
48. Whitman EM, Pisarcik S, Luke T et al (2008) Endothelin-1 mediates hypoxia-induced inhibition of voltage-gated K^+ channel expression in pulmonary arterial myocytes. Am J Physiol Lung Cell Mol Physiol 294:L309–L318
49. Kelly BD, Hackett SF, Hirota K et al (2003) Cell type-specific regulation of angiogenic growth factor gene expression and induction of angiogenesis in nonischemic tissue by a constitutively active form of hypoxia-inducible factor 1. Circ Res 93:1074–1081
50. Shimoda LA, Fallon M, Pisarcik S, Wang J, Semenza GL (2006) HIF-1 regulates hypoxic induction of NHE1 expression and alkalinization of intracellular pH in pulmonary arterial myocytes. Am J Physiol Lung Cell Mol Physiol 291:L941–L949

CLC-3 Chloride Channels in the Pulmonary Vasculature

Joseph R. Hume, Ge-Xin Wang, Jun Yamazaki, Lih Chyuan Ng, and Dayue Duan

Abstract Volume-sensitive outwardly rectifying anion channels (VSOACs) are expressed in pulmonary artery smooth muscle cells (PASMCs) and have been implicated in cell proliferation, growth, apoptosis and protection against oxidative stress. In this chapter, we review the properties of native VSOACs in PASMCs, and consider the evidence that ClC-3, a member of the ClC superfamily of voltage dependent Cl- channels, may be responsible for native VSOACs in PASMCs. Finally, we examine whether or not native VSOACs and heterologously expressed ClC-3 channels function as bona fide chloride channels or as chloride/proton antiporters.

Keywords Chloride channels • cell volume • pulmonary artery • ClC-3

1 Introduction

Volume-sensitive outwardly rectifying anion channels (VSOACs) are ubiquitously expressed in mammalian cells and play a vitally important physiological role in a variety of cellular functions, including cell volume homeostasis, proliferation, apoptosis, and the regulation of electrical activity.[1] VSOACs have been implicated in a number of these functions in vascular smooth muscle cells (SMCs) as well. For example, the magnitude of VSOAC currents in actively growing SMCs is higher than in growth-arrested or differentiated SMCs, suggesting that VSOACs may be important for SMC proliferation.[2] There is evidence that pressure-induced depolariza-

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tion and contraction of cerebral artery smooth muscle may be partially mediated by VSOACs.[3]

Although the exact identification of the proteins responsible for native VSOACs has proven to be elusive, the short isoform of ClC-3 (sClC-3), a member of the ClC superfamily of voltage-dependent chloride channels, has been proposed to be the molecular correlate of the native VSOAC in some cells, including cardiac myocytes and vascular SMCs.[4,5] This hypothesis has been corroborated by a series of other independent studies from different laboratories.[6–11] Despite these data, the role of ClC-3 as a constituent of native VSOACs remains controversial.[12–14] Much of this controversy comes from results reported from the first transgenic ClC-3 global knockout (*ClCn3*$^{-/-}$) mouse produced by Jentsch and coworkers.[15] They reported the apparent presence of native VSOACs in at least two different cell types from *ClCn3*$^{-/-}$ mice. However, later experiments using global *ClCn3*$^{-/-}$ transgenic mice revealed that the properties of native VSOACs were actually altered in heart, and there appeared to be significant compensatory changes in expression of a variety of other membrane proteins (including upregulation of two other members of the ClC chloride channel family), raising fundamental questions about the usefulness of the global *ClCn3*$^{-/-}$ mouse model to assess ClC-3 function.[16] It has been demonstrated that transgenic mice with cardiac-specific overexpression of the human short ClC-3 (hsClC-3) isoform exhibit enhanced VSOAC currents and accelerated regulatory volume decreases,[17] which is consistent with a molecular role for sClC-3 in native VSOAC function.

It has been demonstrated that ClC-3 is expressed in human aortic SMCs[18] and pulmonary artery SMCs (PASMCs).[5] It was demonstrated that antisense oligonucleotide-mediated downregulation of ClC-3 dramatically inhibits cell proliferation of rat aortic SMCs.[19] The *ClCn-3* gene appears to be upregulated in rat pulmonary artery and heart in response to monocrotaline-induced pulmonary hypertension and in canine cultured PASMCs incubated with inflammatory mediators. PASMCs infected to overexpress ClC-3 exhibited enhanced viability against H_2O_2, thus suggesting that ClC-3 may improve the resistance of VSMCs to reactive oxygen species (ROS) in an environment of elevated inflammatory cytokines in hypertensive pulmonary arteries.[20] These and other studies suggested that activation of ClC-3 channels may indeed play a role in proliferation, growth, volume regulation, and apoptosis of vascular SMCs (see Ref. [21] for review).

2 Properties of Native VSOACs in PASMCs

Quantitative reverse-transcription polymerase chain reaction (RT-PCR) has been used to test for molecular expression of ClC-3 in canine pulmonary smooth muscle. Primers were designed to be specific for ClC-3 and do not cross hybridize to other members of the ClC gene family. The competitive "mimic" strategy of quantitative PCR was employed.[12] As shown in Fig. 15.1a, quantitative RT-PCR detected significant levels of ClC-3 transcriptional expression from pulmonary arteries. The figure

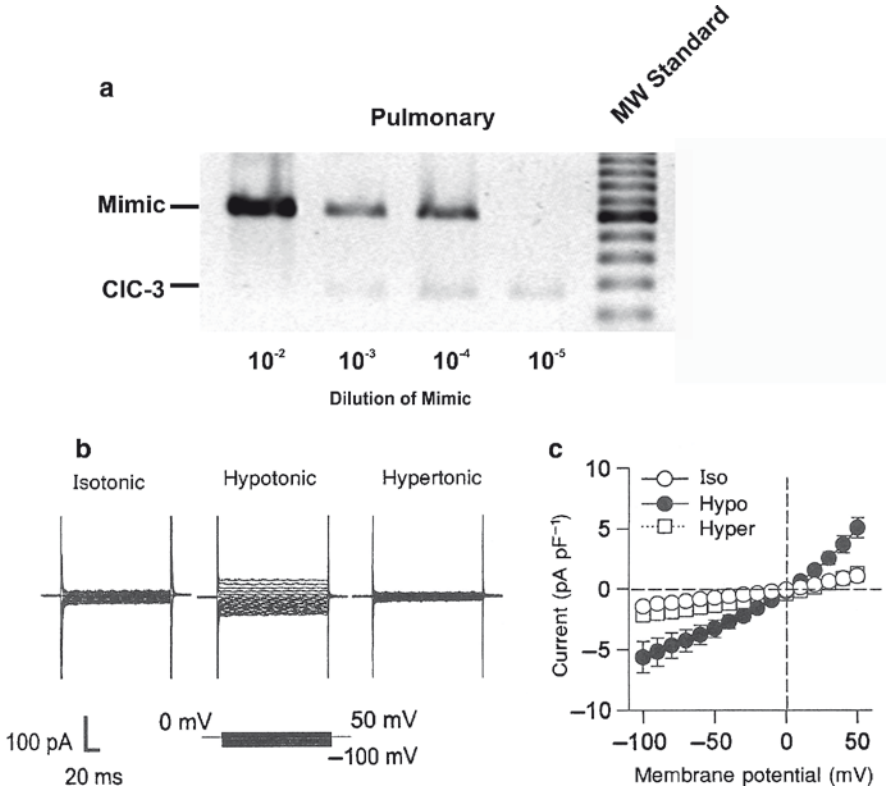

Fig. 15.1 ClC-3 expression and native VSOACs in canine PASMCs. (**a**) Representative gel of quantitative RT-PCR for ClC-3 in canine pulmonary arteries; competitive PCR products were resolved on 2% ethidium bromide agarose gels. Tenfold serial dilutions of mimic DNA were included in the PCRs, while target cDNA (ClC-3) concentration remained constant. The actual concentrations of target complementary DNA (cDNA) were calculated and expressed as percent of β-actin RNA concentration. (**b**) Raw VSOAC currents activated by hypotonic solutions during 150-ms voltage steps from 0 mV to potentials ranging from –100 to 50 mV. The cell was first equilibrated with the isotonic solution and then exposed to the hypotonic solution. Activations of VSOACs were reversed by exposure to hypertonic solutions. (**c**) Current-voltage relations for volume-regulated currents in the isotonic, hypotonic, and hypertonic solutions with 115 m*M* $[Cl^-]_o$ and 115 m*M* $[Cl^-]_i$ ($n = 4$). Modified with permission[5]

illustrates a representative gel used in digital analysis and comparison of mimic and ClC-3 amplification. Digital analysis and comparison of mimic and ClC-3-specific amplification products was performed on the 10^{-4} dilution of mimic DNA and was repeated on three independently generated samples. ClC-3 expression was 48.0% of β-actin in pulmonary artery. Figure 15.1b, c illustrate the activation of native VSOACs in canine PASMCs by exposure to hypotonic (230 mOsm) extracellular solutions. Exposure to a hypotonic solution causes cells to swell, which results in the delayed activation of VSOACs. Membrane currents at –100 and +100 mV were almost negligible in the isotonic solution but began to increase following a delay of some 3–4 min after changing to the hypotonic solution. Figure 15.1b shows raw

current traces evoked by step pulses, which developed during exposure to hypotonic solution, and these were completely abolished by a 10-min perfusion with a hypertonic solution. In these experiments, possible contamination by cation currents was prevented using impermeant cations and appropriate blockers. Figure 15.1c is a plot of the current–voltage relations obtained from several cells in solutions with different osmolarities. In these experiments, both bath and pipet solutions contained 115 m*M* Cl^-, and the currents activated during exposure to hypotonic solution exhibited clear outward rectification. The reversal potential was approximately 0 mV, which is the predicted equilibrium potential for Cl^- (0 mV). The hypotonically activated currents were reduced by subsequent exposure to hypertonic solutions at each membrane potential. Membrane currents activated by exposure to hypotonic solutions were also markedly inhibited by the stilbene compound DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid).

Figure 15.2 illustrates the effects of intracellular dialysis with an anti-ClC-3 carboxyl terminus antibody ($C_{670–687}$ Ab) on native VSOAC currents in PASMCs. Membrane currents were obtained by applying 100-ms step pulses to ±80 mV from a holding potential of –40 mV every 30 s. In Fig. 15.2a, b, the time courses of change in the amplitudes of membrane currents measured at ±80 mV are shown, and original current traces obtained at the time points indicated by small letters are depicted in the insets. In a cell dialyzed with 10 μg mL^{-1} $C_{670–687}$ Ab for over 10 min, basal membrane currents gradually declined in isotonic bath solution. Subsequent hypotonic cell swelling failed to induce any increase of the current amplitude (Fig. 15.2a). To know whether the observed inhibitory effects of $C_{670–687}$ Ab on VSOACs were due to specific binding, similar experiments were repeated with the

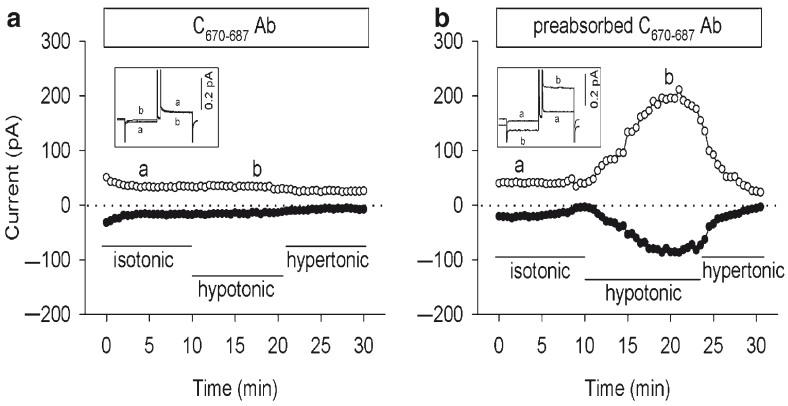

Fig. 15.2 Inhibition of native VSOACs in canine PASMCs by anti-ClC-3 $C_{670–687}$ Ab intracellular dialysis. Membrane currents were induced by repetitive 100-ms voltage steps to ±80 mV from a holding potential of –40 mV every 30 s. (**a**, **b**) Time courses of change in current amplitude measured at both –80 mV (*filled circles*) and +80 mV (*open circles*) in two representative cells intracellular dialyzed with either 10 μg mL^{-1} ClC-3 $C_{670–687}$ Ab (**a**) or the antigen-preabsorbed $C_{670–687}$ Ab (**b**). The *bars* underneath the current traces indicate different bath solutions. Original current recordings obtained at the time points indicated by *small letters* are shown in the corresponding *insets*. Modified with permission[32]

antigen-preabsorbed $C_{670–687}$ Ab. Figure 15.2b shows a representative experiment. In contrast to the effects observed with the $C_{670–687}$ Ab alone, dialysis with 10 μg mL^{-1} antigen-preabsorbed $C_{670–687}$ Ab for over 10 min did not prevent activation of VSOACs on hypotonic cell swelling. Subsequent exposure of the cell to hypertonic bath solution totally reversed the swelling-induced VSOAC currents.

3 Anion Selectivity of Native VSOACs in PASMCs and sClC-3 Heterologously Expressed in NIH/3T3 Cells

To examine Cl^- dependence, the reversal potentials E_{rev} for the volume-sensitive currents were measured using either voltage steps or ramps in hypotonic solutions containing six different concentrations of external Cl^- ($[Cl^-]_o$), replaced with aspartate. As shown in Fig. 15.3a, reducing $[Cl^-]_o$ from 115 to 28 and 9 m*M* shifted E_{rev} rightward, indicating a strong Cl^- dependence of the volume-sensitive conductance. The inset shows the relationship between $[Cl^-]_o$ and E_{rev} of the volume-sensitive conductance obtained from a number of pulmonary cells. The straight line represents a theoretical slope of 57 mV per tenfold decrease in $[Cl^-]_o$, which is predicted from the Nernst equation assuming that Cl^- is the only permeable ion. The slope of the relationship measured experimentally closely followed the predicted slope of 57 mV per tenfold change in $[Cl^-]_o$ for changes in $[Cl^-]_o$ greater than 40 m*M* but deviated

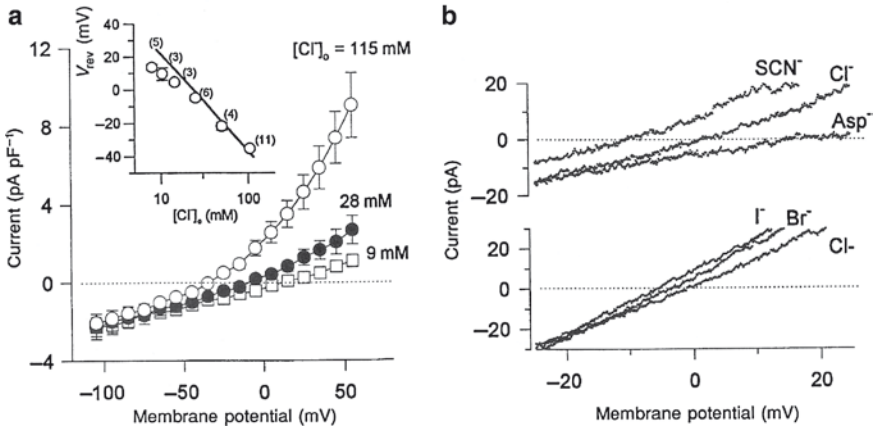

Fig. 15.3 Effect of $[Cl^-]_o$ (**a**) and anion substitution (**b**) on current-voltage relations of VSOAC currents elicited in canine pulmonary arterial smooth muscle cells by voltage steps or ramps. (**a**) The pipet solution contained 24 m*M* Cl^- ($n = 5$). *Inset*, relation between the reversal potential (E_{rev}) and $[Cl^-]_o$. Each *circle* indicates the mean value of E_{rev} with standard errors of the means (SEM) from *N* observations indicated in the *parentheses*. The *straight line* indicates the theoretical slope of 57 mV per tenfold decrease in $[Cl^-]_o$, which is predicted from the Nernst equation. (**b**) NaCl (115 m*M*) in the bath solution was entirely replaced with the same concentration of NaI, NaBr, NaSCN, or Na aspartate. The pipet solution contained 115 m*M* Cl^-. Modified with permission[5]

from the predicted slope at $[Cl^-]_o$ less than 40 m*M*, suggesting that the replacement anion, Asp^-, may exhibit some limited permeability through these channels.

Relative anion selectivity was determined by total replacement of Cl^- with other anions in the hypotonic solutions. Figure 15.3b shows typical hypotonically activated membrane currents elicited in a pulmonary cell by voltage ramps applied in the presence of different extracellular anions. I^- appeared to be slightly more permeable, compared to Br^-, which was more permeable than Cl^- in this example. Likewise, SCN^- appeared to be more permeable compared to Cl^-, which was more permeable than $aspartate^-$. The mean data accumulated from a group of cells gave E_{rev} values (in mV) of –8.29 ± 0.92 (n = 7), –4.67 ± 1.49 (n = 12), –3.30 ± 1.16 (n = 10), 0.46 ± 1.01 (n = 13) and 12.40 ± 1.69 (n = 5) for SCN^-, I^-, Br^-, Cl^- and $aspartate^-$, respectively. Accordingly, the relative permeability for each anion (X^-) to Cl^- (P_{X^-} / P_{Cl^-}) was estimated using the Goldman-Hodgkin-Katz equation. The sequence of (P_{X^-} / P_{Cl^-}) was SCN^- (1.36 ± 0.06) > I^- (1.19 ± 0.06) > Br^- (1.09 ± 0.04) > Cl^- (1.00) > $aspartate^-$ (0.63 ± 0.05). These data indicate that these membrane currents activated by hypotonic cell swelling in PASMCs can be identified as VSOACs.

sClC-3 has been successfully expressed in NIH/3T3 cells[4] and in A10 vascular SMCs.[11] In both cell types, sClC-3 transfection gives rise to larger volume-sensitive Cl^- currents, compared to untransfected cells, with properties resembling those of native VSOACs. VSOACs in A10 sClC-3-transfected cells are completely abolished by intracellular dialysis of an anti-ClC-3 antibody and by ClC-3 antisense oligonucleotides.

We have examined the relative anion selectivity of expressed sClC-3 currents in NIH/3T3 cells to determine if it is similar to the anion permeability properties of native VSOACs in canine PASMCs (cf. Fig. 15.3). The results of these experiments are summarized in Table 15.1. To measure relative whole-cell anion permeability, external NaCl was replaced by the sodium salt of various anions. Reversal potentials were measured for each Cl^- substitute (X) and the relative shifts in reversal potential (E_X - E_{Cl}) were used to calculate the relative permeability ratio (P_X/P_{Cl}) for each anion. The relative anion permeability (P_X/P_{Cl}) of expressed sClC-3 currents activated by hypotonic cell swelling was SCN^-(1.50) > I^-(1.34) > NO_3^-(1.27) > Br^-(1.15) > Cl^-(1.00) > F^-(0.57) > isethionate(0.25) > gluconate(0.09). These

Table 15.1 Relative anion selectivity of sClC-3 channels expressed in NIH 3T3 cells

Anions	$E_X - E_{Cl}$	P_X/P_{Cl}	n
SCN^-	–9.30 ± 2.03	1.50 ± 0.14	5
I^-	–6.89 ± 0.86	1.34 ± 0.05	5
NO_3^-	–5.50 ± 1.22	1.27 ± 0.07	5
Br^-	–3.36 ± 0.42	1.15 ± 0.02	5
Cl^-	0	1.0	
F^-	13.03 ± 0.99	0.57 ± 0.02	5
Isethionate	32.27 ± 4.29	0.25 ± 0.05	5
Gluconate	49.61 ± 4.15	0.09 ± 0.02	5

results demonstrate that sClC-3 channels exhibit a lyotropic anion permeability similar to native VSOACs in PASMCs and most mammalian cells.[1]

4 Do Native VSOACs in PASMCs and Heterologously Expressed sClC-3 Behave as Chloride/Proton Antiporters?

The molecular structure of the ClC family of voltage-gated proteins has been determined by X-ray crystallography of the bacterial homologue EcClC.[22, 23] EcClC is a homodimer with each subunit consisting of 18 α-helical transmembrane-spanning domains and a cytoplasmic domain containing two cystathionine β-synthetase (CBS) subdomains. A selectivity filter has been identified that contains three selective anion-binding sites.[24] Surprisingly, a study[25] demonstrated that the bacterial homologue EcClC functions as an H^+–Cl^--exchange transporter, not as an ion channel. This was convincingly demonstrated since membrane currents associated with EcClC are relatively voltage independent and changes in proton gradients produced easily measurable shifts in current reversal potential, as predicted for an exchange transport mechanism. Efforts have been made to extend these results to the mammalian family of ClC Cl^- channels. Conservation of a putative "proton" glutamate (Glu-203) in the selectivity region between EcClC and the mammalian homologues ClC-3–ClC-7 has suggested the possibility that these proteins may also function as proton exchange transporters.[26] The major evidence for this proposal is indirect and comes from studies heterologously expressing ClC-4 and ClC-5. The difficulty is that expressed ClC-4 and ClC-5 currents exhibit strong outward rectification, making it impossible to measure reversal potentials or shifts in reversal potentials with changes in extracellular anion or proton concentrations. As an alternative approach, changes in intracellular pH were measured in ClC-4- and ClC-5-transfected tsA201 cells[27] or changes in extracellular pH in ClC-4- and ClC-5-transfected oocytes[28] to monitor proton fluxes attributable to electrogenic Cl^-/H^+ exchange.

We have examined whether native VSOACs in cultured canine PASMCs behave as Cl^- channels or as Cl^-/H^+ antiporters. As shown in Fig. 15.4a, VSOAC currents activated by hypotonic cell swelling in canine PASMCs were strongly inhibited at both positive and negative membrane potentials when the extracellular hypotonic solution pH was changed from 7.3 to 4.5. Raw traces of VSOAC currents recorded over the voltage range –100 to +100 mV are illustrated in Fig. 15.4b, c. Figure 15.4d shows the current-voltage relationships of VSOACs recorded in the presence of hypotonic (pH 7.3) solutions and hypotonic (pH 4.5) solutions for a number of cells. Both inward and outward VSOAC currents were inhibited by extracellular acidification; significantly, there was no observed change in membrane current reversal potential.

If the transport mechanism involves Cl^-/H^+ exchange, the membrane current reversal potential V_r is defined by the following equation[25]:

$$V_r = (E_{Cl} + rE_H) / (1 + r)$$

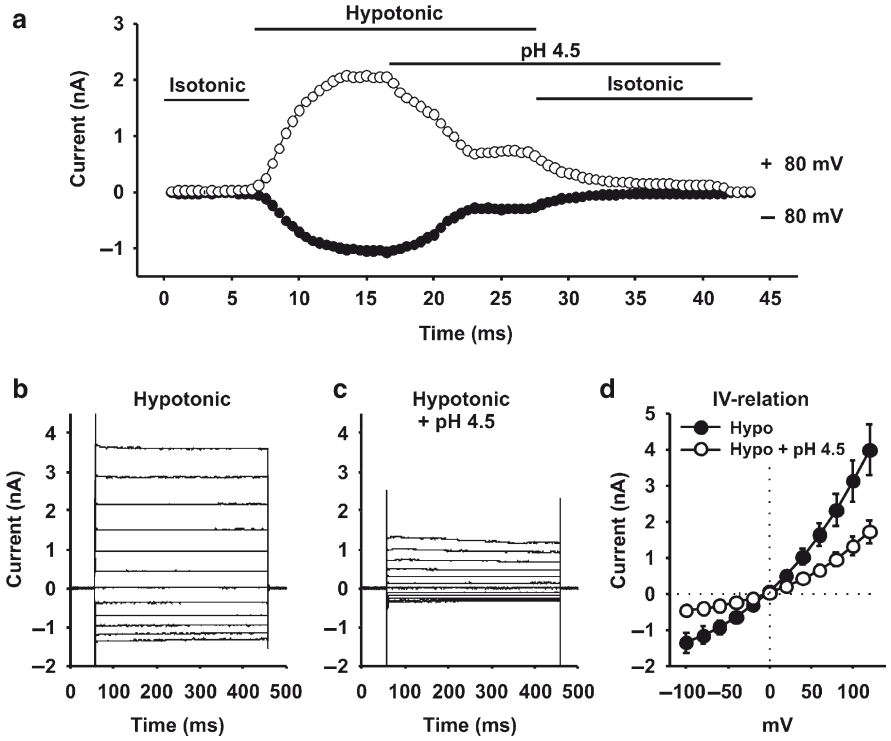

Fig. 15.4 Inhibition of VSOACs by extracellular acidification in cultured canine PASMCs. (**a**) Time course of membrane currents activated at ±80 mV by hypotonic (230 mOsm, pH 7.4) solution. Following maximal activation, the extracellular solution was changed to a hypotonic (230 mOsm, pH 4.5) solution at the time indicated by the horizontal bars. External acidification inhibited both outward and inward VSOAC currents, and the remaining currents were reversed by reexposure to isotonic solution. Traces of membrane currents elicited by voltage steps over the range -100 to +125 mV in hypotonic (230 mOsm, pH 7.4) solution (**b**) and after changing to a hypotonic (230 mOsm, pH 4.5) solution (**c**). (**d**) Current-voltage (IV) relationships of membrane currents shown in (**b**, **c**). Extracellular acidification inhibited VSOACs without inducing any significant change in current reversal potential (G.-X. Wang and J.R. Hume unpublished data)

where E_{Cl} is the equilibrium potential for Cl^-, E_H is the equilibrium potential for protons, and r is the proton-anion coupling ratio. For a Cl^-/H^+ exchange transport protein, significant changes in the proton gradient should produce measurable changes in the membrane current reversal potential, which we failed to observe (Fig. 15.4d) for native VSOACs in PASMCs. This is quite different from the findings on the bacterial homologue EcClC, for which extracellular acidification produced a negative shift in measured reversal potentials.[25]

We have also examined whether hsClC-3 currents measured in transfected NIH/3T3 cells behave as Cl^- channels or as Cl^-/H^+ antiporters. As shown in Fig. 15.5a, b, hypotonically activated hsClC-3 currents were significantly inhibited by extracellular acidification. Extracellular acidification produced no measurable change in hsClC-3 current reversal potential (Fig. 15.5c), suggesting that hsClC-3

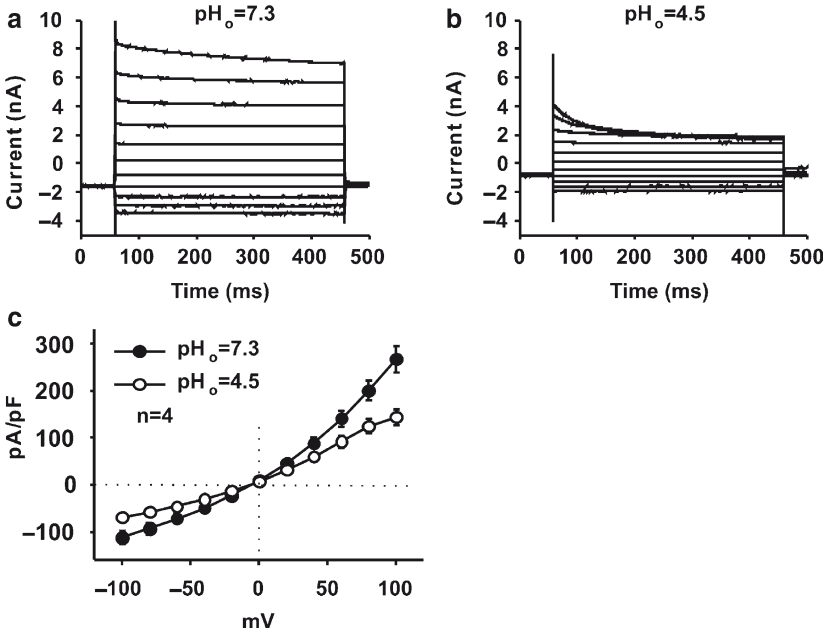

Fig. 15.5 Effect of extracellular acidification on hsClC-3 currents in transfected NIH/3T3 cells. Traces of membrane currents elicited by voltage steps over the range ±100 mV in hypotonic (230 mOsm, pH 7.4) solution (**a**) and after changing to a hypotonic (230 mOsm, pH 4.5) solution (**b**). (**c**) Current–voltage (IV) relationships of membrane currents shown in (**a**, **b**). hsClC-3 currents were inhibited by extracellular acidification, but acidification had no effect on membrane current reversal potential (G.-X. Wang and J.R. Hume unpublished data)

exhibits properties more consistent with a Cl^- channel electrodiffusion mechanism compared to a Cl^-/H^+ countertransport mechanism.

5 Summary and Conclusions

Native VSOACs in cardiac and SMCs share many properties with recombinant sClC-3 channels expressed in heterologous expression systems, including outwardly rectifying Cl^- currents activated by cell swelling and inhibited by cell shrinkage; current inhibition at strong positive membrane potentials; and inhibition by extracellular nucleotides, stilbene derivatives such as DIDS and 4-Acetamido-4'-isothiocyanato-2,2'-stilbenedisulfonic acid (SITS), intracellular dialysis by anti-ClC-3 antibodies and by the antiestrogen compound tamoxifen.[29] Moreover, as shown in this chapter, native VSOACs in PASMCs and expressed recombinant cClC-3 channels exhibit a similar lyotrophic permeability selectivity of $SCN^->I^->NO_3^->Br^->Cl^->F^->$ isethionate > gluconate. Despite suggestions that some members of the mammalian ClC Cl^- channel family may function as chloride/proton

antiporters like the bacterial homologue EcClC, membrane current reversal potential measurements on native VSOACs in PASMCs and recombinant sClC-3 currents failed to provide any evidence supporting a role for chloride/proton antiporter function.

Native VSOACs and ClC-3 have been implicated in a wide variety of SMC functions, including proliferation, growth, apoptosis, and protection against oxidative stress. It is possible that all of these might be explained by the role that VSOACs and ClC-3 normally play in normal cell volume homeostasis. However, it is possible that an important physiological function of these channels may extend beyond their normal role in cell volume regulation.[30] For example, it has been demonstrated[31] that ClC-3 Cl^- channels in the endothelial cell plasma membrane may be a prominent route for transmembrane flux of ROS. Future studies will certainly focus on such a possibility in PASMCs.

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References

1. Nilius B, Droogmans G (2003) Amazing chloride channels: an overview. Acta Physiol Scand 177:119–147
2. Voets T, Wei L, De Smet P et al (1997) Downregulation of volume-activated Cl^- currents during muscle differentiation. Am J Physiol 272:C667–C674
3. Nelson M, Conway MA, Knot HJ, Brayden JE (1997) Chloride channel blockers inhibit myogenic tone in rat cerebral arteries. J Physiol 502(2):259–264
4. Duan D, Winter C, Cowley S, Hume JR, Horowitz B (1997) Molecular identification of a volume-regulated chloride channel. Nature 390:417–421
5. Yamazaki J, Duan D, Janiak R, Kuenzli K, Horowitz B, Hume JR (1998) Functional and molecular expression of volume-regulated chloride channels in canine vascular smooth muscle cells. J Physiol 507:729–736
6. Do CW, Lu W, Mitchell CH, Civan MM (2005) Inhibition of swelling-activated Cl^- currents by functional anti-ClC-3 antibody in native bovine non-pigmented ciliary epithelial cells. Invest Ophthalmol Vis Sci 46:948–955
7. Jin NG, Kim JK, Yang DK et al (2003) Fundamental role of ClC-3 in volume-sensitive Cl^- channel function and cell volume regulation in AGS cells. Am J Physiol Gastrointest Liver Physiol 285:G938–G948
8. Petrunkina AM, Harrison RA, Ekhlasi-Hundrieser M, Topfer-Petersen E (2004) Role of volume-stimulated osmolyte and anion channels in volume regulation by mammalian sperm. Mol Hum Reprod 10:815–823
9. Vessey JP, Shi C, Jollimore CA et al (2004) Hyposmotic activation of I_{Cl}, swell in rabbit non-pigmented ciliary epithelial cells involves increased ClC-3 trafficking to the plasma membrane. Biochem Cell Biol 82:708–718
10. Wang L, Chen L, Jacob TJ (2000) The role of ClC-3 in volume-activated chloride currents and volume regulation in bovine epithelial cells demonstrated by antisense inhibition. J Physiol 524:63–75
11. Zhou JG, Ren JL, Qiu QY, He H, Guan YY (2005) Regulation of intracellular Cl^- concentration through volume-regulated ClC-3 chloride channels in A10 vascular smooth muscle cells. J Biol Chem 280:7301–7308

12. Jentsch TJ, Stein V, Weinreich F, Zdebik AA (2002) Molecular structure and physiological function of chloride channels. Physiol Rev 82:503–568
13. Li X, Shimada K, Showalter LA, Weinman SA (2000) Biophysical properties of ClC-3 differentiate it from swelling-activated chloride channels in Chinese hamster ovary-K1 cells. J Biol Chem 275:35994–35998
14. Weylandt KH, Valverde MA, Nobles M et al (2001) Human ClC-3 is not the swelling-activated chloride channel involved in cell volume regulation. J Biol Chem 276:17461–17467
15. Stobrawa SM, Breiderhoff T, Takamori S et al (2001) Disruption of ClC-3, a chloride channel expressed on synaptic vesicles, leads to a loss of the hippocampus. Neuron 29:185–196
16. Yamamoto-Mizuma S, Wang GX, Liu LL et al (2004) Altered properties of volume-sensitive osmolyte and anion channels (VSOACs) and membrane protein expression in cardiac and smooth muscle myocytes from ClCn3$^{-/-}$ mice. J Physiol 557:439–456
17. Xiong D, Wang G-X, Burkin D et al (2008) Cardiac specific overexpression of the human short ClC-3 chloride channel isoform in mice. Clin Exp Pharmacol Physiol 36:386–393, 2009
18. Lamb FS, Clayton GH, Liu BX, Smith RL, Barna TJ, Schutte BC (1999) Expression of CLCN voltage-gated chloride channel genes in human blood vessels. J Mol Cell Cardiol 31:657–666
19. Wang GL, Wang XR, Lin MJ, He H, Lan XJ, Guan YY (2002) Deficiency in ClC-3 chloride channels prevents rat aortic smooth muscle cell proliferation. Circ Res 91:e28–e32
20. Dai Y-P, Bongalon S, Hatton WJ, Hume JR, Yamboliev IA (2005) ClC-3 chloride channel is upregulated by hypertrophy and inflammation in rat and canine pulmonary artery. Br J Pharmacol 145:5–14
21. Guan YY, Wang GL, Zhou JG (2006) The ClC-3 Cl^- channel in cell volume regulation, proliferation and apoptosis in vascular smooth muscle cells. Trends Pharmacol Sci 27:290–296
22. Arianzi EA, Gpuld MN (1996) Identifying differential gene expression in monoterpene-treated mammary carcinomas using subtractive display. J Biol Chem 271:29286–29294
23. Dutzler R, Campbell EB, MacKinnon R (2002) X-ray structure of a ClC chloride channel at 3.0 Å reveals the molecular basis of anion selectivity. Nature 415:287–294
24. Dutzler R (2006) The ClC family of chloride channels and transporters. Curr Opin Struct Biol 16:1–8
25. Accardi A, Miller C (2004) Secondary active transport mediated by a prokaryotic homologue of ClC Cl^-channels. Nature 427:803–807
26. Zdebik AA, Zifarelli G, Bersforf E-Y et al (2008) Determinants of anion-proton coupling in mammalian endosomal CLC proteins. J Biol Chem 283:4219–4227
27. Scheel O, Zdebik AA, Lourdel S, Jentsch TJ (2005) Voltage-dependent electrogenic chloride/proton exchange by endosomal CLC proteins. Nature 436:424–427
28. Picollo A, Pusch M (2005) Chloride/proton antiporter activity of mammalian CLC proteins ClC-4 and ClC-5. Nature 436:420–423
29. Hume JR, Duan D, Collier ML, Yamazaki J, Horowitz B (2000) Anion transport in heart. Physiol Rev 80:31–81
30. Remillard CV, Yuan X-J (2005) ClC-3: more than just a volume-sensitive Cl^- channel. Br J Pharmacol 145:1–2
31. Hawkins BJ, Madesh M, Kirkpatrick CJ, Fisher AB (2007) Superoxide flux in endothelial cells via the chloride channel-3 mediated intracellular signaling. Mol Biol Cell 18:2002–2012
32. Wang G-X, Hatton WJ, Wang GL et al (2003) Functional effects of novel anti-ClC-3 antibodies on native volume-sensitive osmolyte and anion channels (VSOACs) in cardiac and smooth muscle cells. Am J Physiol 285:H1453–H1463

Part IV
Receptors and Signaling Cascades in Pulmonary Arterial Hypertension

Role of Bone Morphogenetic Protein Receptors in the Development of Pulmonary Arterial Hypertension

Nicholas W. Morrell

Abstract The identification of mutations in the bone morphogenetic protein (BMP) type II receptor in the majority of cases of familial pulmonary arterial hypertension (PAH) has provided a focus for researchers studying the complex pathobiology of this condition. Mutations are also found in a proportion of idiopathic PAH cases and it is now emerging that dysfunctional BMP signaling plays a role in other more common forms of PAH, even in the absence of mutations in the gene. Study of the role of BMP signaling in endothelial, smooth muscle cell, progenitor cell and inflammatory cell biology may reveal novel pathways lending themselves to therapeutic intervention in PAH. This chapter summarizes the present status of our understanding of the role of BMPR-II mutations in PAH and indicates future directions for research.

Keywords Bone morphogenetic proteins • pulmonary hypertension • genetics • signal transduction • vascular biology

1 Introduction

Idiopathic pulmonary arterial hypertension (PAH) is a rare but devastating condition. It affects approximately two or three individuals per million per year and typically affects young women (female/male ratio, 2.3:1). The occlusion of small, peripheral pulmonary arteries leads to a persistently elevated pulmonary vascular resistance, a raised pulmonary arterial pressure, and ultimately death from right heart failure. Before the modern treatment era, therapy was usually symptomatic, and life expectancy was short. The term *pulmonary arterial hypertension* refers to a variety of forms of precapillary pulmonary hypertension. PAH is known to be associated with

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systemic diseases such as collagen vascular disease, HIV infection, or the ingestion of drugs or toxins and may also be found in association with congenital heart disease. Idiopathic PAH remains a diagnosis of exclusion. In 6–10% of cases of idiopathic PAH, there is more than one affected family member, and the condition is referred to as *familial* or *heritable PAH*. Despite the diverse aetiology of PAH in these subdivisions, the group may share a common pathogenesis and as such may be amenable to new advances in therapeutic approaches to the disease.

2 The Pathology of PAH

The pathology of PAH is characterised by vascular remodelling, in situ thrombus formation, and varying degrees of inflammation. Vascular remodelling occurs as a result of abnormal proliferation and survival of vascular smooth muscle cells and myofibroblasts, leading to medial hypertrophy and concentric intimal lesions; the adventitia becomes thickened by increased numbers of activated fibroblasts. Altered proliferation and survival of endothelial cells contributes to the formation of so-called plexiform lesions. Widespread pulmonary endothelial dysfunction also leads to changes in the release of mediators of vascular tone, which in turn has an impact on smooth muscle/myofibroblast function. In the normal pulmonary circulation, there is a finely regulated balance between locally released and circulating vasoconstrictive agents and vasodilators. In contrast, pulmonary hypertension is associated with increased levels of the vasoconstrictors endothelin 1 and serotonin and increased generation of angiotensin II, concomitant with a reduction in vasodilators or their signalling pathways, such as nitric oxide and prostacyclin. The resulting imbalance favours the progression of pulmonary hypertension and has become a useful target for therapy.

Despite the development of several new therapies for PAH, the majority of these drugs primarily serve to redress the imbalance between vasoconstrictor and vasodilator pathways. They may also, but to a lesser extent, have an impact on the process of vascular remodelling. The clinical course of the majority of patients remains one of eventual deterioration, suggesting that vascular remodelling eventually progresses. Genetic studies identified a major growth factor pathway involved in the pathogenesis of familial PAH that is likely to have important implications for our understanding of disease pathobiology and in the design of novel therapeutic strategies.

3 Genetics of Familial PAH

Approximately 10% of cases of idiopathic PAH have an affected relative.[1] In these families, the disease segregates in an autosomal dominant pattern, often with markedly reduced penetrance. True estimates of penetrance are yet to be reported, and will probably vary with the nature of the underlying mutation, but on average is of the order of 20–30%.[2,3] Thus, many patients who carry the disease gene will not manifest clinical PAH. Nevertheless, the relative risk for PAH in someone carrying a *BMPR2*

mutation is on the order of 10^5 compared with a carrier of the wild-type allele. The often markedly reduced penetrance in families with PAH provides evidence that some form of 'second hit' is required in addition to the mutation to lead to the manifestation of clinical disease. Following localisation of the disease gene to the long arm of chromosome 2 (2q33),[4] two independent groups identified heterozygous germline mutations in the bone morphogenetic protein type II receptor (BMPR-II), a receptor for the transforming growth factor β (TGF-β) superfamily, in patients with familial PAH.[5,6] Mutations in the *BMPR2* gene have been found in approximately 80% of families.[7] In addition, 15–40% of patients with apparently sporadic idiopathic PAH have also been found to harbour similar mutations.[8] At least a proportion of these are examples of familial PAH, in which the condition has not manifested in relatives due to low penetrance,[2] whereas others are examples of de novo mutation. To date, some 144 distinct mutations have been identified in 210 independent patients with familial PAH.[7] A number of studies have looked for *BMPR2* mutations in other forms of PAH. Mutations have been identified in some subjects with appetite suppressant-related PAH and in children with PAH and congenital heart disease. They have also been reported in a few patients with pulmonary veno-occlusive disease. Intriguingly, mutations in the TGF-β type I receptor, activin receptor-like kinase (ALK) 1, which is usually found in patients with type 2 hereditary hemorrhagic telangiectasia, have also been identified in patients with severe PAH.

Approximately 30% of mutations are missense mutations occurring in highly conserved amino acids with predictable effects on receptor function. For example, many of these involve the serine-threonine kinase domain of BMPR-II or the extracellular ligand-binding domain. However, the majority (approximately 70%) of *BMPR2* coding mutations are frame-shift and nonsense mutations, many of which would be expected to produce a transcript susceptible to nonsense-mediated mRNA decay (NMD). Thus, haploinsufficiency for BMPR-II represents the predominant molecular mechanism underlying inherited predisposition to familial PAH. Further genetic analysis, including the identification of gene deletions and rearrangements, is revealing an increasing number of families in which BMPR-II mutation is implicated.[9,10] The genetics of familial PAH and BMPR-II mutations has been reviewed in detail.[7]

4 Normal BMPR-II Signalling

Bone morphogenetic proteins (BMPs) are the largest group of cytokines within the TFG-β superfamily and were originally identified as molecules regulating growth and differentiation of bone and cartilage.[11] BMPs are now known to regulate growth, differentiation, and apoptosis in a diverse number of cell lines, including mesenchymal and epithelial cells, acting as instructive signals during embryogenesis and contributing to the maintenance and repair of adult tissues.[11–13] TGF-β superfamily type II receptors are constitutively active serine/threonine kinases and form homodimers, existing either constitutively or recruited to receptor complexes on ligand stimulation.[14] BMPR-II is distinguished from other TGF-β superfamily type II receptors by a long carboxyl-terminal sequence following the intracellular kinase domain.[15] Both long and short forms

of BMPR-II have been isolated, with the short-form splice variant lacking most of exon 12.[16] Although the short form of the receptor is widely expressed in human tissues, it is not known whether this form of the receptor serves a differential function compared to the long form. BMPR-II initiates intracellular signalling in response to specific ligands: BMP2, BMP4, BMP6, BMP7, BMP9, BMP10, GDF5 [growth and differentiation factor], and GDF6.[15] Ligand specificity for different components of the receptor complex are emerging that may have functional significance to the tissue-specific nature of BMP signalling. The extreme tissue specificity of BMP signalling is highlighted by the diverse human diseases associated with mutations in type I and II receptors. Whereas BMPR-II mutation is associated with familial PAH, BMPRIA mutation causes familial juvenile colonic polyposis,[17] and BMPRIB mutation causes hereditary brachydactyly.[18] The majority of ligands (BMP2, -4, -7; GDF5 and -6) bind with high affinity to the type I receptors, predominantly BMPRIA (ALK-3) or BMPRIB (ALK-6) and with very low affinity to BMPR-II. GDF5 demonstrates specificity for BMPRIB.[19] In contrast, BMP6 binds with high affinity to BMPR-II. BMP9 and BMP10 have been identified as ligands for a receptor complex comprising BMPR-II and ALK-1, providing new insight into the link between PAH and hereditary hemorrhagic telangiectasia. BMP9 and BMP10 are expressed in the heart, so they may be key cardiac-derived factors regulating pulmonary vascular function. BMP9 stimulates Smad1/5/8 phosphorylation and, in contrast to BMP2 and BMP4, inhibits angiogenic processes in endothelial cells.[20] Furthermore, BMP9, at circulating concentrations, is a vascular quiescence factor, suggesting that mutations in these receptors may lead to an activated endothelial state.[21] However, BMPR-II small-interfering RNA (siRNA) does not alter BMP9/10 signalling as activin type II receptors (ActR-II) compensates for the functional loss of BMPR-II. Loss of endoglin or ALK-1 function in hereditary hemorrhagic telangiectasia (HHT) may have a greater impact on BMP9/10 functions, providing some explanation for the differing pathologies of HHT and PAH. Unlike ALK-1 and endoglin, BMPR-II is also expressed by smooth muscle cells. This differential distribution, combined with alternative ligand–receptor responses, may contribute to the differences between HHT and PAH.

Following ligand binding, the type II receptor phosphorylates a glycine-serine-rich domain on the proximal intracellular portion of an associated type I receptor. Conformational changes that occur in the ligand–receptor complex when both receptor types contact ligand are required for cross-linking of the ligand to BMPR-II and intracellular signal transduction.

BMP signalling is regulated at many different levels, for example, by endogenous inhibitors of BMP binding/signalling (e.g. Noggin, Chordin, Follistatin, BAMBI, and Smad ubiquitination and regulatory factor [Smurf] 1); the levels of expression of specific BMPs; activation of the inhibitory Smads (Smad6 and -7); interactions with other growth signalling pathways, possibly such as those downstream of serotonin receptors, and nuclear co-activators and co-repressors. In addition, BMP signalling may be regulated by the type and density of type I and II receptors available for dimerisation, dictating the relative levels of pre-formed vs. ligand-induced receptor complexes and therefore the differential activation of downstream signalling pathways. Such diverse levels of regulation may be responsible for the tissue specificity of BMP signalling and may, for example, underlie the pulmonary specific pathology seem in PAH.

In the presence of ligand, activated type I receptors in turn phosphorylate cytoplasmic signalling proteins known as Smads, which are responsible for TGF-β superfamily signal transduction.[22] BMPs signal via a restricted set of receptor-mediated Smads (R-Smads) (Smad1, -5, and -8), which must complex with the common partner Smad (Co-Smad), Smad4, to translocate to the nucleus. TGF-β and activins signal via a different set of R-Smads, Smad2 and -3. Target gene transcription is regulated by a variety of mechanisms, including direct binding of the Smad complex to DNA, interaction with other DNA proteins, such as activator receptor (AP-1) and transcription factor E3 (TFE-3), and recruitment of transcriptional co-activators or co-repressors.[22] Switching off Smad signalling in the cell is achieved via Smurfs[23] and by recently identified Smad phosphatases.[24]

Although Smad signalling has been recognised as the canonical BMP signalling pathway, there is mounting evidence that the mitogen-activated protein kinases (MAPKs), including $p38^{MAPK}$, $p42/44^{MAPK}$ (extracellular signal-regulated kinase [ERK] 1/2), and c-Jun N-terminal kinases and stress activated protein kinases are regulated by BMPs and TGF-βs in certain cell types.[25,26] MAPK signalling has been reported to positively and negatively regulate Smad signalling, depending on the cell type and system studied.

5 Interactions Between MAPK and Smad Signalling

R-Smads consist of a conserved globular Mal homology (MH)1 and an MH2 domain connected by a linker region. The MH1 domain is involved in DNA binding and the MH2 domain in binding to cytoplasmic retention factors, activated receptors, nucleoporins in the nuclear pore, and DNA-binding co-factors, co-activators, and co-repressors in the nucleus. Receptor-mediated phosphorylation occurs at the carboxy-terminal sequence SXS.[22,26] This enables the nuclear accumulation of Smads and their association with the shared partner Smad4 to form transcriptional complexes that are interpreted by the cell as a function of the context. In contrast, the linker region of Smad1 contains four PXSP sites that are susceptible to phosphorylation by MAPK, specifically ERK1/2. This prevents the nuclear localisation of Smad1 and inhibits BMP signalling. These results led to the proposal that the BMP and epidermal growth factor (EGF)/Ras/MAPK pathways converge on Smad1 by phosphorylating the carboxy-terminal tail and the linker region, respectively, with opposite effects.[27,28] The balance of these two inputs would determine the level of Smad1 activity in the nucleus and thus the participation of BMP signalling in the control of cell fate (Fig. 16.1).

6 The Consequences of *BMPR2* Mutation for BMP/TGF-β Signalling

Two studies have shown that the mechanism by which BMPR-II mutants disrupt BMP/Smad signalling is heterogeneous and mutation specific.[29,30] Of the missense mutations, substitution of cysteine residues within the ligand-binding or kinase domain of BMPR-II leads to reduced trafficking of the mutant protein to the cell surface, a process that may also interfere with BMP type I receptor trafficking. In

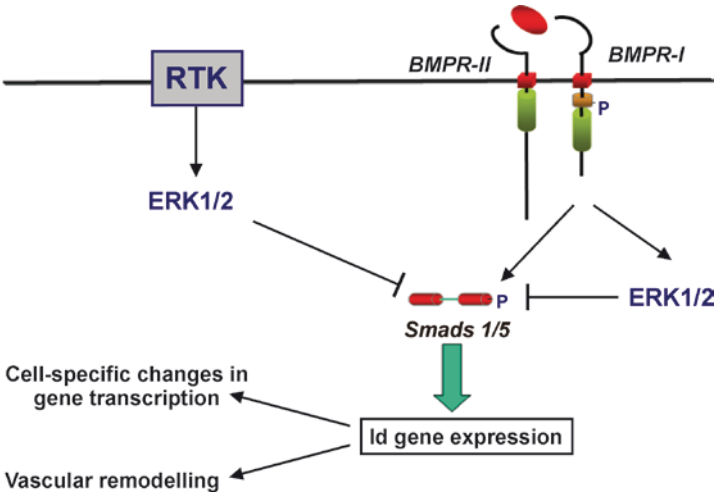

Fig. 16.1 Pathways potentially involved in the regulation of BMP receptor mRNA expression, receptor protein expression and Smad signalling. *RTK* receptor tyrosine kinases; *ERK* extracellular signal-regulated kinases; *HHV-8* human herpes virus 8; *HIV* human immunodeficiency viruses

contrast, non-cysteine mutations within the kinase domain reach the cell surface but fail to activate Smad-responsive luciferase reporter genes due to an inability to phosphorylate BMP type I receptors. Mutations in the ligand-binding and kinase domains exhibit a dominant negative effect on wild-type receptor function in terms of Smad signalling. We have shown that the ligand-binding domain mutants are partially functional, and trafficking of mutant BMPR-II to the cell membrane can be restored with chemical chaperones, such as sodium 4-phenylbutyrate, with rescue of BMP signalling.[31] Interestingly, BMPR-II mutants with missense mutations involving the cytoplasmic tail are able to traffic to the cell surface and are capable of activating Smad-responsive luciferase reporter genes to some extent but are almost certainly relatively deficient in their ability to transduce signals via Smads. In addition, pulmonary artery smooth muscle cells (PASMCs) from mice heterozygous for a null mutation in the *BMPR2* gene are also deficient in Smad signalling.[32,33] Thus, haploinsufficiency or missense mutation leads to a loss of signalling via the Smad1/5 pathway via the majority of BMP ligands. In contrast, marked siRNA knockdown (>90%) of BMPR-II leads to increased Smad signalling in response to some ligands, for example, BMP7.[32] This effect appears to be mediated by increased signalling via ActR-II in the absence of BMPR-II. The significance of this finding remains to be determined since studies in human mutant cells and animal models are consistent with reduced BMP signalling in lung cells.

Our group has shown that PASMCs isolated from patients with idiopathic or familial PAH exhibit an exaggerated growth response to TGF-β1.[34] TGF-β1 is not a ligand for the BMP receptors. In addition, the abnormal response to TGF-β does not seem to be due to alterations in the expression of TGF-β type I, II, or III receptors.[34] The molecular basis of this observation remains unclear but may be due to reciprocal regulation of Smad1/5 and Smad2/3 signalling as reported in endothelial cells.[35–37]

7 Studies in Cells and Tissues from PAH Patients

BMPR-II is widely expressed in normal tissues and cells.[15] In the lung, BMPR-II is highly expressed on the vascular endothelium of the pulmonary arteries.[38] The receptor is also expressed, albeit at a lower level, in PASMCs and fibroblasts. The expression of BMPR-II is markedly reduced in the pulmonary vasculature of patients with mutations in the BMPR-II gene.[38] Notably, BMPR-II expression is also significantly reduced in the pulmonary vasculature of patients with idiopathic PAH in whom no mutation in the *BMPR2* gene was identified. These studies suggest that a critical reduction in the expression of BMPR-II may be important to the pathogenesis of PAH whether or not there is a mutation in the gene. In addition, since the level of BMPR-II expression in familial cases was considerably lower than predicted from the state of haploinsufficiency, this suggests that some additional environmental or genetic factor may be necessary to further reduce BMPR-II expression below the threshold, which triggers profound vascular remodelling (Fig. 16.2). Reduced expression of BMPR1A receptor has also been reported in PAH of diverse aetiologies.[39] Studies of *BMPR2* gene promoter activity may reveal important regulatory elements responsible for expression of BMPR-II transcripts. To date, 3-hydroxy-3-methyl-glutasyl tat protein has been shown to inhibit expression of BMPR-II, which is of interest given the increased prevalence of PAH in HIV-infected patients.[40] Conversely, the HMG coenzyme A (CoA) reductase inhibitor simvastatin enhances BMPR-II promoter activity and prevents pulmonary hypertension in rat models.[41]

In addition to the receptors, phosphorylation of Smad1/5 is reduced in the pulmonary arterial wall of patients with underlying *BMPR2* mutations and patients

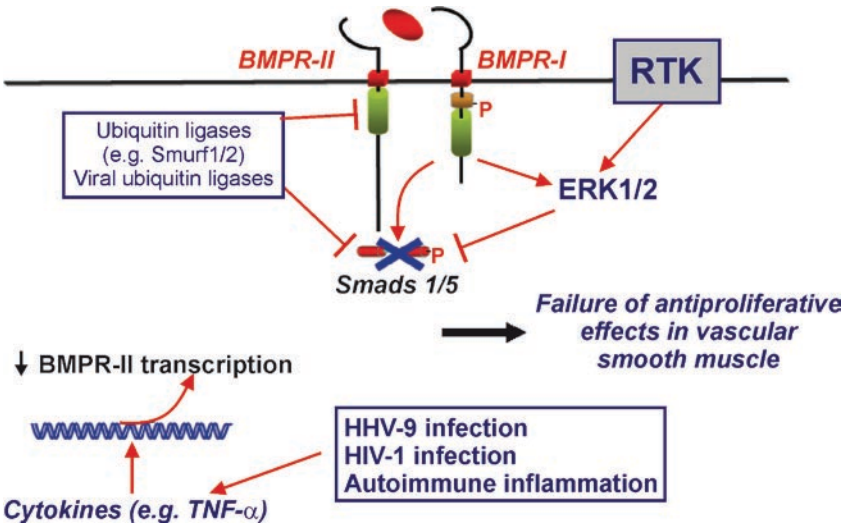

Fig. 16.2 Cross talk between receptor tyrosine kinases (RTKs), ERK1/2, and BMP/Smad signalling and their integration at the level of Id gene expression

with idiopathic PAH with no identifiable mutation.[42] Thus, reduction occurs not only of BMPR-II expression but also of activation of the main downstream signalling pathway in patients with familial and idiopathic PAH.

Animal models of pulmonary hypertension, such as that induced by high flow in pigs,[43] or chronic hypoxia,[44] or monocrotaline in rats, have now also demonstrated reduced BMPR-II expression. Whether these changes in BMPR-II expression observed in animal models are a cause or the consequence of pulmonary vascular remodelling remains to be determined. One study has demonstrated increased expression of phospho-Smad2 in small pulmonary arteries of patients with idiopathic PAH, lending support for the concept that a reduction in BMPR-II/Smad1, -5 signalling can lead to increased signalling via TGF-β/ALK-5/Smad2, -3.[45] Earlier studies in idiopathic PAH lung had confirmed increased expression of TGF-β isoforms in remodelling arteries.[46]

Several groups have explored the response of pulmonary vascular cells from patients and controls in vitro. The response of PASMCs to BMP ligands depends to some extent on the anatomical origin of cells. The serum-stimulated proliferation of cells harvested from the main or lobar pulmonary arteries tends to be inhibited by TGF-β1 and BMP-2, -4, and -7.[42] Indeed, BMPs may induce apoptosis in these cells.[47] Using a dominant negative Smad1 construct, the growth inhibitory effects of BMPs have been shown to be Smad1 dependent.[42] In contrast, in PASMCs isolated from pulmonary arteries of 1- to 2-mm diameter, BMP-2 and -4 stimulate proliferation.[42] This pro-proliferative effect of BMPs in peripheral cells is dependent on the activation of ERK1/2 and p38MAPK. Both Smad and MAPK pathways are activated to a similar extent in cells from both locations, but the integration of these signals by the cell seems to differ. As discussed, MAPK activation has an inhibitory effect on Smad1/5 signalling by Smad linker region phosphorylation. We have shown that this cross talk is active and important in PASMCs in terms of Smad signalling, transcriptional regulation of BMP responsive genes, and cell proliferation.[48]

The response of vascular endothelial cells to the majority of BMPs in vitro is in complete contrast to PASMCs. Endothelial cells proliferate, migrate, and form tubular structures in response to BMP4.[49] The proliferation is driven via Smad1/5 activation and dependent on the induction of the inhibitors of DNA binding (Id) family of transcription factors. In addition, BMPs protect endothelial cells from apoptosis.[50] One caveat to these studies is that BMP9 appears to inhibit endothelial cell (EC) proliferation. Knockdown of BMPR-II with siRNA increases the susceptibility of pulmonary artery endothelial cells to apoptosis.

The contrasting effects of BMPs in the pulmonary vascular endothelium and the underlying PASMCs provide a compelling model for pulmonary vascular damage and remodelling in familial PAH. A critical reduction in BMPR-II function in the endothelium may promote increased endothelial apoptosis, which compromises the integrity of the endothelial barrier and contributes to endothelial dysfunction. This would allow ingress of serum factors to the underlying intima and stimulate activation of vascular elastases. It is conceivable that high rates of apoptosis in the endothelium would favour the development of apoptosis-resistant clones of

endothelial cells and lead to plexiform lesion formation. In addition, apoptosis and engulfment of apoptotic cells is known to be accompanied by the robust release of TGF-β. In the underlying media, PASMCs already compromised in their ability to respond to the growth-suppressive effects of BMPs are exposed to TGF-β, which because of a deficient Smad1/5 pathway causes an exaggerated growth response, as described. This emerging hypothesis is now open to direct testing both in vitro and in vivo.

8 Studies in Transgenic and Knockout Mice

Studies of knockout mice reveal the critical role of the BMP pathway in early embryogenesis and vascular development. Homozygosity for a null mutation in *BMPR2* is lethal prior to gastrulation.[51] Mice deficient in Smad5, one of the BMP-restricted Smads, die due to defects in angiogenesis, specifically failure to recruit vascular smooth muscle to endothelial structures.[52] Heterozygous BMPR-II$^{+/-}$ mice survive to adulthood and breed normally with no readily discernable phenotype. This mouse, at least at the genetic level, mimics the state of haploinsufficiency underlying the majority of families with PAH. In general, the mouse pulmonary vascular bed seems resistant to extensive and severe vascular remodelling seen in human disease. Heterozygous *BMPR2*$^{+/-}$ mice have been shown to have no,[33] or little,[53] resting elevation of pulmonary arterial pressure under normal conditions. However, when heterozygotes are exposed to lung overexpression of interleukin (IL) 1β[54] or infused chronically with serotonin,[33] they develop a greater elevation of pulmonary artery pressure compared with wild-type littermate controls. These observations support the hypothesis that BMPR-II dysfunction increases the susceptibility to pulmonary hypertension when exposed to another environmental stimulus. However, this response depends on the stimulus because chronic hypoxia, a commonly used animal model, does not increase susceptibility to pulmonary hypertension in *BMPR2*$^{+/-}$ mice.[33] The relatively low penetrance of the PAH within families supports a 'two-hit' hypothesis in which the vascular abnormalities characteristic of idiopathic PAH are triggered by accumulation of genetic or environmental insults in a susceptible individual. Environmental injury, such as the ingestion of appetite suppressants that results in an increase in serotonin signalling, may impose an additional burden predisposing to disease. Acquired somatic mutations in the TGF-β type II receptor and Smad4 are well-recognised associations with certain gastrointestinal cancers,[55] a disease process in which such a two-hit paradigm is well recognised.

There is some evidence that increasing the level of BMPR-II dysfunction will cause pulmonary hypertension in mice. Thus, transgenic overexpression of a dominant negative kinase domain mutant BMPR-II in vascular smooth muscle causes increased pulmonary vascular remodelling and pulmonary hypertension.[56] Interestingly, transgenic mice expressing a hypomorphic BMPR-II survive gastrulation but die at midgestation with cardiovascular and skeletal defects, including

defects in the outflow tract of the heart.[57] This study demonstrated the importance of gene dosage in BMP signalling. In addition, cardiac defects have been recognised in some individuals with *BMPR2* mutations.[58] Further studies are needed with conditional knockout mice to overcome the essential requirement of BMPR-II during early embryogenesis and to examine the importance of endothelial vs. smooth muscle expression of mutant BMPR-II. A study showed that conditional deletion of BMPR-II in the endothelium led to pulmonary vascular remodelling associated with inflammation and in situ thrombosis in a subset of mice.[59] If a more robust model of PAH could be established, this would clearly benefit the search for targeted therapies and would provide a means of searching for genetic modifiers of disease expression.

Two studies have evaluated the utility of increasing expression of BMPR-II by gene therapy in rat models of PAH. In the monocrotaline model, adenoviral delivery of BMPR-II via the airways failed to prevent monocrotaline-induced pulmonary hypertension.[60] However, targeted gene delivery of BMPR-II to the pulmonary vascular endothelium did significantly reduce pulmonary arterial pressure and right ventricular hypertrophy in chronically hypoxic rats.[61] Further evaluation of targeted delivery of BMPR-II is warranted in these models.

9 BMPs as Inhibitors of Tissue Remodelling

Evidence shows that BMP2 inhibits serum-stimulated and growth factor-induced proliferation of human aortic smooth muscle cells and induces the expression of smooth muscle cell differentiation markers.[62] Adenovirus-mediated overexpression of BMP2 has been shown to inhibit injury-induced intimal hyperplasia in a rat carotid artery balloon injury model.[63] BMP7, but not BMP4, ameliorates renal fibrosis induced by TGF-β in rat models of glomerular sclerosis.[64,65] In addition, adenoviral delivery of BMP7, Id2, or Id3 suppressed the epithelial-to-mesenchymal-cell transition in the injured mouse lens.[66] Remarkably few, if any, studies have addressed the role of BMP pathways in lung fibrosis, injury, or remodelling. BMPs clearly play a major role during lung morphogenesis,[23,67,68] and there is some evidence that they afford protection against lung fibrosis. Since TGF-β signalling plays such a dominant role in lung remodelling, studies of the interaction with the BMP pathway in airway and parenchymal lung disease are clearly needed.

10 Inhibiting TGF-β Signalling

There is evidence for enhanced TGF-β signalling in the setting of human and experimental models of pulmonary hypertension.[69] In addition, PASMCs from patients with idiopathic PAH are resistant to the antiproliferative effects of TGF-β.

This is also apparent in PASMCs harbouring mutations in BMPR-II or cells in which BMPR-II expression has been knocked down by RNA interference. The precise mechanisms behind these observations are under investigation, but they raise the possibility that blocking the TGF-β type I receptor ALK-5 may be of potential therapeutic benefit. Indeed, three studies have now shown that the small molecule inhibitor of ALK-5 in the rat monocrotaline model is effective in preventing the development of pulmonary hypertension when treatment is initiated early and inhibits progression of disease if administered when disease is already established. ALK-5 inhibition seems less effective in the chronically hypoxic rat model, although deficiency of the TGF-βRII receptor in mice has been shown to ameliorate the development of PH.[69] Monocrotaline-induced PH in the rat is associated with increased expression of TGF-β1, increased Smad2/3 phosphorylation, and increased expression of TGF-β target genes. ALK-5 inhibition effectively targeted these pathways in vivo.[69]

11 Summary and Future Directions

The role of BMPs and BMP signalling in lung disease remains at an early stage. Although clearly of direct relevance to PAH, this pathway is likely to contribute to other lung pathologies characterised by tissue remodelling, such as lung fibrosis and chronic obstructive pulmonary disease. Further exploration of the contribution of BMPs and the functional antagonism with the TGF-β pathway may reveal new targets for therapeutic intervention. Well-planned and large-scale genetic studies are now required to identify additional genetic factors that increase susceptibility to PAH. Such factors may have a further impact on the state of BMPR-II dysfunction. Future functional studies need to identify the cell- and tissue-specific abnormalities in gene expression and cell growth/survival that are responsible for pulmonary vascular remodelling. In addition, studies are required to determine the molecular mechanism of the interaction between BMP and TGF-β pathways in lung cells. Further refinement of animal models using conditional cell-specific transgenic and knockout mice will also be necessary to understand the lung specificity of familial PAH.

References

1. Loyd JE, Primm RK, Newman JH (1984) Familial primary pulmonary hypertension: clinical patterns. Am Rev Respir Dis 129:194–197
2. Newman JH, Wheeler L, Lane KB et al (2001) Mutation in the gene for bone morphogenetic protein receptor II as a cause of primary pulmonary hypertension in a large kindred. N Engl J Med 345:319–324
3. Newman JH, Trembath RC, Morse JA et al (2004) Genetic basis of pulmonary arterial hypertension: current understanding and future directions. J Am Coll Cardiol 43:S33–S39

4. Nichols WC, Koller DL, Slovis B et al (1997) Localization of the gene for familial primary pulmonary hypertension to chromosome 2q31–32. Nat Genet 15:277–280
5. The International PPH Consortium, Lane KB, Machado RD, Pauciulo MW et al (2000) Heterozygous germ-line mutations in BMPR2, encoding a TGF-β receptor, cause familial primary pulmonary hypertension. Nat Genet 26:81–84
6. Deng Z, Morse JH, Slager SL et al (2000) Familial primary pulmonary hypertension (Gene PPH1) is caused by mutations in the bone morphogenetic protein receptor-II gene. Am J Hum Genet 67:737–744
7. Machado RD, Aldred MA, James V et al (2006) Mutations of the TGF-β type II receptor BMPR2 in pulmonary arterial hypertension. Hum Mut 27:121–132
8. Thomson JR, Machado RD, Pauciulo MW et al (2000) Sporadic primary pulmonary hypertension is associated with germline mutations of the gene encoding BMPR-II, a receptor member of the TGF-β family. J Med Genet 37:741–745
9. Aldred M, Vijayakrishnan J, James V et al (2006) BMPR2 gene rearrangements account for a significant proportion of mutations in familial and idiopathic pulmonary arterial hypertension. Hum Mut 27:212–213
10. Cogan JD, Vnencak-Jones CL, Phillips JA et al (2005) Gross BMPR2 gene rearrangements constitute a new cause for primary pulmonary hypertension. Genet Med 7:169–174
11. Miyazono K, Maeda S, Imamura T. (2005) BMP receptor signaling: transcriptional targets, regulation of signals, and signaling cross-talk. Cytokine Growth Factor Rev 16:251–263
12. Kawabata M, Imamura T, Miyazano K (1998) Signal transduction by bone morphogenetic proteins. Cytokine Growth Factor Rev 9:49–61
13. Massagué J, Chen Y-G (2000) Controlling TGF-β signaling. Genes Dev 14:627–644
14. Attisano L, Wrana JL (2002) Signal transduction by the TGF-β superfamily. Science 296:1646–1647
15. Rosenzweig BL, Imamura T, Okadome T et al (1995) Cloning and characterization of a human type II receptor for bone morphogenetic proteins. Proc Natl Acad Sci U S A 92:7632–7636
16. Ishikawa T, Yoshioka H, Ohuchi H, Noji S, Nohno T (1995) Truncated type II receptor for BMP-4 induces secondary axial structures in Xenopus embryos. Biochem Biophys Res Commun 216:26–33
17. Howe JR, Bair JL, Sayed MG et al (2001) Germline mutations of the gene encoding bone morphogenetic protein receptor 1A in juvenile polyposis. Nat Genet 28:184–187
18. Lehmann K, Seemann P, Stricker S et al (2003) Mutations in bone morphogenetic protein receptor 1B cause brachydactyly type A2. Proc Natl Acad Sci U S A 100:12277–12282
19. Nickel J, Kotzsch A, Sebald W, Mueller TD (2005) A single residue of GDF-5 defines binding specificity to BMP receptor IB. J Mol Biol 349:933–947
20. David L, Mallet C, Mazerbourg S, Feige JJ, Bailly S (2007) Identification of BMP9 and BMP10 as functional activators of the orphan activin receptor-like kinase 1 (ALK1) in endothelial cells. Blood 109:1953–1961
21. David L, Mallet C, Keramidas M et al (2008) Bone morphogenetic protein-9 is a circulating vascular quiescence factor. Circ Res 102:914–922
22. Massagué J, Seoane J, Wotton D (2005) Smad transcription factors. Genes Dev 19:2783–2810
23. Shi W, Chen H, Sun J, Chen C, Zhao J, Wang YL et al (2004) Overexpression of Smurf1 negatively regulates mouse embryonic lung branching morphogenesis by specifically reducing Smad1 and Smad5 proteins. Am J Physiol Lung Cell Mol Physiol 286:L293–L300
24. Chen HB, Shen J, Ip YT, Xu L (2006) Identification of phosphatases for Smad in the BMP/DPP pathway. Genes Dev 20:648–653
25. Nohe A, Keating E, Knaus P, Petersen NO (2004) Signal transduction of bone morphogenetic protein receptors. Cell Signal 16:291–299
26. Massagué J (2003) Integration of Smad and MAPK pathways: a link and a linker revisited. Genes Dev 17:2993–2997
27. Grimm OH, Gurdon JB (2002) Nuclear exclusion of Smad2 is a mechanism leading to loss of competence. Nat Cell Biol 4:519–522

28. Kretzschmar M, Doody J, Massagu J (1997) Opposing BMP and EGF signalling pathways converge on the TGF-β family mediator Smad1. Nature 389:618–622
29. Rudarakanchana N, Flanagan JA, Chen H et al (2002) Functional analysis of bone morphogenetic protein type II receptor mutations underlying primary pulmonary hypertension. Hum Mol Genet 11:1517–1525
30. Nishihara A, Watabe T, Imamura T, Miyazono K (2002) Functional heterogeneity of bone morphogenetic protein receptor-II mutants found in patients with primary pulmonary hypertension. Mol Biol Cell 13:3055–3063
31. Sobolewski A, Rudarakanchana N, Upton PD et al (2008) Failure of bone morphogenetic protein receptor trafficking in pulmonary arterial hypertension: potential for rescue. Hum Mol Genet 17:3180–3190
32. Yu PB, Beppu H, Kawai N, Li E, Bloch KD (2005) Bone morphogenetic protein (BMP) type II receptor deletion reveals BMP ligand-specific gain of signaling in pulmonary artery smooth muscle cells. J Biol Chem 280:24443–24450
33. Long L, MacLean MR, Jeffery TK et al (2006) Serotonin increases susceptibility to pulmonary hypertension in BMPR2-deficient mice. Circ Res 98:818–827
34. Morrell NW, Yang X, Upton PD et al (2001) Altered growth responses of pulmonary artery smooth muscle cells from patients with primary pulmonary hypertension to transforming growth factor-β1 and bone morphogenetic proteins. Circulation 104:790–795
35. Goumans MJ, Valdimarsdottir G, Itoh S, Rosendahl A, Sideras P, ten Dijke P (2002) Balancing the activation status of the endothelium via two distinct TGF-β receptors. EMBO J 21:1743–1753
36. Goumans MJ, Valdimarsdottir G, Itoh S et al (2003) Activin receptor-like kinase (ALK)1 is an antagonistic mediator of lateral TGFβ/ALK5 signaling. Mol Cell 12:817–828
37. Itoh S, Thorikay M, Kowanetz M et al (2003) Elucidation of Smad requirement in transforming growth factor-β type I receptor-induced responses. J Biol Chem 278:3751–3761
38. Atkinson C, Stewart S, Upton PD et al (2002) Primary pulmonary hypertension is associated with reduced pulmonary vascular expression of type II bone morphogenetic protein receptor. Circulation 105:1672–1678
39. Du L, Sullivan CC, Chu D et al (2003) Signaling molecules in nonfamilial pulmonary hypertension. N Engl J Med 348:500–509
40. Caldwell RL, Gadipatti R, Lane KB, Shepherd VL (2005) HIV-1 TAT represses transcription of the bone morphogenic protein receptor-2 in U937 monocytic cells. J Leukoc Biol 79:192–201
41. Hu H, Sung A, Zhao G et al (2006) Simvastatin enhances bone morphogenetic protein receptor type II expression. Biochem Biophys Res Commun 339:59–64
42. Yang X, Long L, Southwood M et al (2005) Dysfunctional Smad signaling contributes to abnormal smooth muscle cell proliferation in familial pulmonary arterial hypertension. Circ Res 96:1053–1063
43. Rondelet B, Kerbaul F, Van Beneden R et al (2005) Prevention of pulmonary vascular remodeling and of decreased BMPR-2 expression by losartan therapy in shunt-induced pulmonary hypertension. Am J Physiol Heart Circ Physiol 289:H2319–H2324
44. Takahashi H, Goto N, Kojima Y et al (2006) Downregulation of type II bone morphogenetic protein receptor in hypoxic pulmonary hypertension. Am J Physiol Lung Cell Mol Physiol 290:L450–L458
45. Richter A, Yeager ME, Zaiman A, Cool CD, Voelkel NF, Tuder RM (2004) Impaired transforming growth factor-β signaling in idiopathic pulmonary arterial hypertension. Am J Respir Crit Care Med 170:1340–1348
46. Botney MD, Bahadori L, Gold LI (1994) Vascular remodelling in primary pulmonary hypertension: potential role for transforming growth factor-beta. Am J Pathol 144:286–295
47. Zhang S, Fantozzi I, Tigno DD et al (2003) Bone morphogenetic proteins induce apoptosis in human pulmonary vascular smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 285:L740–L754
48. Yang J, Davies RJ, Southwood M et al (2008) Mutations in bone morphogenetic protein type II receptor cause dysregulation of Id gene expression in pulmonary artery smooth muscle cells: implications for familial pulmonary arterial hypertension. Circ Res 102:1212–1221

49. Valdimarsdottir G, Goumans MJ, Rosendahl A et al (2002) Stimulation of Id1 expression by bone morphogenetic protein is sufficient and necessary for bone morphogenetic protein-induced activation of endothelial cells. Circulation 106:2263–2270
50. Teichert-Kuliszewska K, Kutryk MJB, Kuliszewski MA et al (2006) Bone morphogenetic protein receptor-2 signaling promotes pulmonary arterial endothelial cell survival: implications for loss-of-function mutations in the pathogenesis of pulmonary hypertension. Circ Res 98:209–217
51. Beppu H, Kawabata M, Hamamoto T et al (2000) BMP type II receptor is required for gastrulation and early development of mouse embryos. Dev Biol 221:249–258
52. Yang X, Castilla LH, Xin X et al (1999) Angiogenesis defects and mesenchymal apoptosis in mice lacking smad5. Development 126:1571–1580
53. Beppu H, Ichinose F, Kawai N et al (2004) BMPR-II heterozygous mice have mild pulmonary hypertension and an impaired pulmonary vascular remodeling response to prolonged hypoxia. Am J Physiol Lung Cell Mol Physiol 287:L1241–L1247
54. Song Y, Jones JE, Beppu H, Keaney JF Jr, Loscalzo J, Zhang YY (2005) Increased susceptibility to pulmonary hypertension in heterozygous BMPR2-mutant mice. Circulation 112:553–562
55. Miyaki M, Iijima T, Konishi M et al (1999) Higher frequency of Smad4 gene mutation in human colorectal cancer with distant metastasis. Oncogene 18:3098–3103
56. West J, Fagan K, Steudel W et al (2004) Pulmonary hypertension in transgenic mice expressing a dominant-negative BMPRII gene in smooth muscle. Circ Res 94:1109–1114
57. Delot EC, Bahamonde ME, Zhao M, Lyons KM (2003) BMP signaling is required for septation of the outflow tract of the mammalian heart. Development 130:209–220
58. Roberts KE, McElroy JJ, Wong WPK et al (2004) BMPR2 mutations in pulmonary arterial hypertension with congenital heart disease. Eur Respir J 24:371–374
59. Hong K-H, Lee YJ, Lee E et al (2008) Genetic ablation of the Bmpr2 gene in pulmonary endothelium is sufficient to predispose to pulmonary arterial hypertension. Circulation 118:722–730
60. McMurtry MS, Moudgil R, Hashimoto K, Bonnet S, Michelakis ED, Archer SL (2007) Overexpression of human bone morphogenetic protein receptor 2 does not ameliorate monocrotaline pulmonary arterial hypertension. Am J Physiol Lung Cell Mol Physiol 292:L872–L878
61. Reynolds AM, Xia W, Holmes MD et al (2007) Bone morphogenetic protein type 2 receptor gene therapy attenuates hypoxic pulmonary hypertension. Am J Physiol Lung Cell Mol Physiol 292:L1182–L1192
62. Willette RN, Gu JL, Lysko PG, Anderson KM, Minehart H, Yue T (1999) BMP-2 gene expression and effects on human vascular smooth muscle cells. J Vasc Res 36:120–125
63. Nakaoka T, Gonda K, Ogita T et al (1997) Inhibition of rat vascular smooth muscle proliferation in vitro and in vivo by bone morphogenetic protein-2. J Clin Invest 100:2824–2832
64. Zeisberg M, Hanai Ji, Sugimoto H et al (2003) BMP-7 counteracts TGF-β1-induced epithelial-to-mesenchymal transition and reverses chronic renal injury. Nat Med 9:964–968
65. Morrissey J, Hruska K, Guo G, Wang S, Chen Q, Klahr S (2002) Bone morphogenetic protein-7 improves renal fibrosis and accelerates the return of renal function. J Am Soc Nephrol 13:S14–S21
66. Saika S, Ikeda K, Yamanaka O et al (2006) Adenoviral gene transfer of BMP-7, Id2, or Id3 suppresses injury-induced epithelial-to-mesenchymal transition of lens epithelium in mice. Am J Physiol Cell Physiol 290:C282–C289
67. Buckley S, Shi W, Driscoll B, Ferrario A, Anderson K, Warburton D (2004) BMP4 signaling induces senescence and modulates the oncogenic phenotype of A549 lung adenocarcinoma cells. Am J Physiol Lung Cell Mol Physiol 286:L81–L86
68. Warburton D, Bellusci S (2004) The molecular genetics of lung morphogenesis and injury repair. Paediatr Respir Rev 5:S283–S287
69. Long L, Crosby AC, Yang X et al (2009) Altered BMP/TGF-β signaling in rat models of pulmonary hypertension: potential for ALK-5 inhibition in prevention and progression of disease. Circulation (in press)

Cross Talk Between Smad, MAPK, and Actin in the Etiology of Pulmonary Arterial Hypertension

James West

Abstract The gene for the type 2 receptor for the bone morphogenic pathway, BMPR2, is mutated in a large majority of familial pulmonary arterial hypertension (PAH),. However, the mechanisms linking BMPR2 mutation to disease remain obscure. BMPR2 potentially signals through multiple immediate downstream pathways, including Smad, MAPK, LIM domain kinase 1 (LIMK) and dynein, light chain, Tctex-type 1 (TCTEX), v-src sarcoma viral oncogene homolog (SRC), and nuclear factor kappa-B (NFkB). Functional consequences of BMPR2 mutation, largely ascertained from animal models, include a shift from contractile to synthetic phenotype in smooth muscle, probably downstream of Smad signal; alterations in expression of actin organization related genes, possibly related to focal adhesions; alterations in cytokines and inflammatory cell recruitment; increased proliferation and apoptosis; and increased collagen and matrix. A synthesis of the available data suggests that the normal role of BMPR2 in adult animals is to assist in injury repair. BMPR2 is suppressed in injured tissue, which facilitates inflammatory response, shift to a synthetic cellular phenotype, and alterations in migration or permeability of cells in the vascular wall. We thus hypothesize that BMPR2 mutation thus leads to an impaired ability to terminate the injury repair process, leading to strong predisposition to PAH.

Keywords Bone morphogenetic protein receptor • genetics • inflammatory cells • signal transduction • hypoxia

1 Introduction

A subset (6–8%) of idiopathic pulmonary arterial hypertension (PAH) is associated with dominant inheritance within families with incomplete penetrance. Since disease in these patients with familial PAH is functionally indistinguishable from

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disease in patients with sporadic PAH, determination of the predisposing gene ought to provide strong evidence of the molecular etiology of disease in not only familial but also all PAH. This is particularly important since human patients with idiopathic PAH present in end stage, making early events obscure. Existing treatments, primarily consisting of a variety of vasodilators, are capable of extending the life span but are ineffective in stopping or reversing disease. Understanding the molecular etiology of disease ought to make possible treatments that address root causes rather than end-stage manifestations.

In 2000, the type 2 receptor for the bone morphogenetic pathway, BMPR2, was found to be mutated in a large majority of familial PAH cases[1,2] and in 10–20% of sporadic cases.[3] To that point, however, the bone morphogenetic protein (BMP) pathway had primarily been studied in the context of embryonic development,[4] and patients with familial PAH had no apparent developmental abnormalities. The role of BMPR2 in adults, particularly in adult lung, was largely unknown.

The purpose of this chapter is to compile the existing evidence for the function of BMPR2 in the adult pulmonary vasculature, including immediate effectors of BMPR2 signaling, downstream consequences of BMPR2 dysfunction in animal models, connections between these, and an interpretation of the overall meaning of these in the context of genes known to modify penetrance or age of onset.

2 BMPR2 Signaling Pathways

BMPR2 is a highly conserved single-pass transmembrane receptor and exists on the cell surface as part of preformed homomeric (BMPR2/BMPR2) or heteromeric (BMPR2/ALK [activin receptor-like kinase] 3 or ALK6) complexes[5]; the

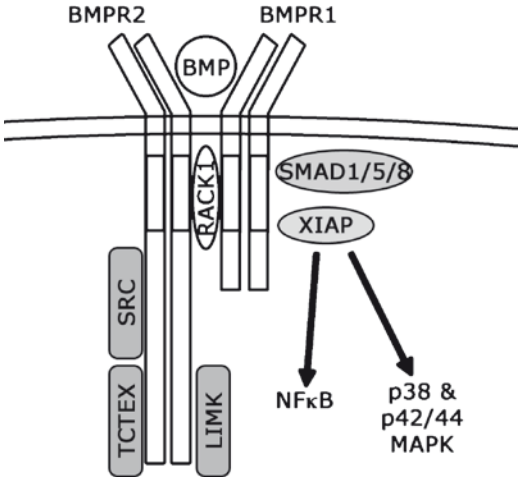

Fig. 17.1 Published signaling pathways downstream of BMPR2. The Smad pathway is best characterized, but others may be important to PAH

nature of these complexes provides some downstream signaling specificity.[6] In the presence of any of the host of BMP ligands, a tetramer consisting of two BMPR2 and two type 1 receptors (ALK3, ALK6, perhaps ALK5) forms. In the canonical Smad signaling pathway, the type 2 receptor then phosphorylates the type 1 receptor, which when phosphorylated can phosphorylate the receptor regulated Smads (Smad1, -5, or -8). These then complex with Smad4, enter the nucleus, and drive transcription targets.

While in the majority of the literature it is assumed that BMPR2 signals primarily through the Smad pathway, several additional signaling pathways downstream of BMPR2 have been reported.[5] Consideration of the role of all potential signaling pathways, and their interaction with each other, is particularly important because many BMPR2 mutations in human familial PAH appear to leave Smad signaling intact.[7] In addition to Smad, BMPR2 has been reported to signal through p38 and p42/44 MAPK (mitogen-activated protein kinase),[8,9] actin organization-related proteins LIM domain kinase 1 (LIMK)[10] and dynein, light chin, Tetex-type 1 (TCTEX),[11] nuclear facter Kappa-B (NFκB),[12] and v-src sarcoma viral oncogene homolog (SRC)[13] (Fig. 17.1). Further, decreased BMP signaling has been predicted to result in increased TGF-β signaling.[14]

2.1 Smad

The Smad transcription factors are most strongly associated with driving the differentiation state in development.[15] Possibly as a side effect of this, Smad signaling is both antiproliferative and antiapoptotic.[16] In addition, Smad signaling is explicitly required to drive smooth muscle differentiation.[17] Further, there is direct cross talk between Smad1 and STAT3, the effector of interleukin (IL) 6,[18] giving Smad signaling a direct role in regulation of inflammatory signaling. Loss of Smad signal could thus be proproliferative, proapoptotic, and proinflammatory and cause loss of terminal differentiation.

2.2 Mapk

Phosphorylation and activation of p42/44 and p38 MAPK are induced both by BMP ligand and by many BMPR2 mutations.[8,9] The data are suggestive of loss of inhibition of activation rather than direct activation; BMP-dependent activation of MAPK may thus be context specific. The mechanism for this is still not entirely clear, although it could be bridging of X-linked Inactivator of Apoptosis (XIAP) by activated BMP receptor complexes, allowing activation of a TGF-beta activated kinase binding protein 1 (TAB1)/TAK1 complex, which itself phosphorylates several MAPKs.[5,19] Phosphorylated MAPK, in cooperation with wingless-type MMTV integration site family (WNT) signaling and Smad specific E3 ubiquitin protein

ligase 1 (SMURF1), marks activated Smad for ubiquitination[20]; it would thus be counterregulatory. Although their targets vary, activation of any of the MAPKs is a stress response, with roles in proliferation, apoptosis, and inflammatory signaling that are highly context specific.[21]

2.3 Actin Organization

LIMK and dynein, light chain, Tctex-type 1 (TCTEX) proteins are both related to actin organization and bind to the long cytoplasmic tail that is unique to BMPR2 among its related receptors.[10,11] LIMK is itself activated by the central actin organization kinase ROCK, and when activated it phosphorylates and inactivates cofilin, preventing cofilin-mediated actin depolymerization. LIMK is bound and inactivated by BMPR2. The R899X mutation of BMPR2 is incapable of binding or inactivating LIMK in vitro,[10] which should lead to increased cofilin phosphorylation. However, in vivo in mice that universally overexpress the R899X mutation of BMPR2, there is an immediate and consistent threefold decrease in cofilin phosphorylation (unpublished data), suggesting that the mechanism of BMPR2 regulation of LIMK is somewhat more complex.

TCTEX is a possibly dispensable[22] light chain of the cytoplasmic motor complex dynein with additional dynein-independent roles, including a role in G protein signaling.[23] Functional consequences of its association with BMPR2 have not yet been investigated, although TCTEX plausibly plays a role in migration.[24]

2.4 SRC and NFκB

v-src sarcoma viral oncogene homolog (SRC) is a tyrosine-protein kinase with roles in development, differentiation, and cell growth, among others. For instance, SRC is heavily implicated in regulating macrophage migration and recruitment to inflammatory sites[25] and cooperates with neuregulin, a LIMK interactor, in cytoskeletal remodeling in myocytes.[26] SRC is inhibited by phosphorylation, and in wild-type cells, BMP signaling results in SRC binding and increased SRC phosphorylation; this activity requires an intact C terminus of BMPR2[13] and may require an intact kinase domain. Thus, most BMPR2 mutations found in familial PAH ought to result in increased SRC activity, with downstream consequences including proliferation, migration, and inflammatory signaling.

NFκB is another potential target of BMP signaling; the same XIAP–TAB1–TGF-beta activated kinase 1 (TAK1) complex through which MAPK is activated can also activate NFκB through IKK.[12] Inducible transgenic mice expressing the R899X BMPR2 mutation universally for 4 weeks, starting as adults, showed substantial increases (three to four times the amount) in p50/p50 or p50/RelA binding to NFκB targets compared to controls (unpublished data). Both these mice and

humans with idiopathic PAH also showed NFκB translocation to the nucleus in cells as yet unidentified within complex lesions (unpublished data). Further, there is evidence that this regulation may be mutual; there are NFκB transcription factor-binding sites conserved in the same location in the BMPR2 promoter in mice, rats, dogs, primates, and humans. NFκB is the "master switch" for inflammatory response[27]; if loss of proper BMP signaling results in increased NFκB activity, this would result in broad increases in inflammatory signaling.

3 *In* Vivo Consequences of BMPR2 Mutation

While immediate effectors of BMPR2 signaling are known, including Smad, MAPK, LIMK, TCTEX, SRC, and possibly NFκB, the consequences of BMP signaling through these pathways is largely unknown in adults. To address this, we created a set of doxycycline-inducible transgenic mouse models. Each mouse requires two transgenes: a tissue-specific promoter driving the reverse tetracycline transactivator (rtTA) and the TetO7 promoter driving a BMPR2 mutation. The TetO7 promoter drives transcription in the presence of both rtTA and doxycycline. These transgenic mice thus only express BMPR2 mutation in specific tissues only when fed doxycycline.

The two models for which we have the most data both use only smooth muscle expression, of either an R899X mutation,[28] referred to as $BMPR2^{R899X}$, or a T base insertion at basepair 504 at the end of coding exon 4,[29] referred to as $BMPR2^{delx4+}$. In addition, we have expressed both of these mutations under control of a universal promoter (Rosa26-rtTA2) and the $BMPR2^{delx4+}$ under the control of an endothelial-specific promoter (Tie2-rtTA). Comparison between the effects of these models allows substantial information about both the roles of different tissues and the relative roles of Smad and non-Smad downstream signaling.

3.1 Smad

The $BMPR2^{delx4+}$ mutation acts as a dominant negative for all BMPR2 functions,[29] while the $BMPR2^{R899X}$ mutation leaves Smad signaling intact.[28] Differences in immediate dysregulation of signaling between these two models are presumably substantially due to Smad signaling. The primary phenotypes seen in $BMPR2^{delx4+}$ animals not seen in $BMPR2^{R899X}$ animals, both expressed only in smooth muscle, were loss of smooth muscle markers combined with increases in specific cytokines and increased expression of complement pathway genes.[28,30] This suggests that loss of Smad contributes to the development of PAH in two ways. First, loss of Smad signaling through BMPR2 results in alteration of smooth muscle from a contractile to a synthetic state, which causes both defects in vasoreactivity and increased propensity for proliferation. Second, loss of Smad signaling results in an increase in cytokine and complement activation, which would predispose to increased injury response.

3.2 Actin Organization

Alterations in regulation of actin organization-related pathways are a common feature to all of our models, regardless of promoter or mutation used. For simplicity, this discussion focuses on universal conditional expression of the BMPR2^{R899X} mutation under the Rosa26-rtTA2 promoter. At Denver altitude, approximately 50% of these develop elevated right ventricular systolic pressure (RVSP) when transgene is activated from age 5–9 weeks (unpublished). While Rosa26-rtTA2 × TetO$_7$-BMPR2^{R899X} mice that develop elevated RVSP have substantial additional changes, even those with normal RVSP have significant overrepresentation of changes in six different actin-related Kyoto encyclopedia of genes and genomes (KEGG) pathways as determined by Affymetrix gene array. These include focal adhesion (19 genes), ECM–receptor interaction (11 genes), leukocyte transendothelial migration (13 genes), cell adhesion molecules (11 genes), regulation of actin cytoskeleton (14 genes), and glycosaminoglycan degradation (3 genes) (unpublished). The functional consequences of these changes have not been directly tested, but the pattern of these changes is appropriate to injury response: increased collagen and fibrin, decreased cell–cell adhesion, increased motility, and increased permeability to inflammatory cells. While mutations capable of separating tail domain functions are unknown, it seems most likely that these changes are downstream of a combination of LIMK, TCTEX, and possibly SRC signaling through BMPR2.

3.3 Mapk

While MAPK activation is a consequence of increased RVSP in all models, in Rosa26-rtTA2 × TetO$_7$-BMPR2^{R899X} mice, there are broad alterations in the MAPK signaling pathway and inflammation-related pathways even before RVSP increases. At the same 4-week time point but with normal RVSP as mentioned, there are nine inflammation-related KEGG pathways that are significantly altered in whole Rosa26-rtTA2 × TetO$_7$-BMPR2^{R899X} mouse lung (unpublished). These include hematopoietic cell lineage (16 genes), B-cell receptor signaling pathway (11 genes), cytokine-cytokine receptor interaction (21 genes), type I diabetes mellitus (7 genes), natural killer cell-mediated cytotoxicity (11 genes), MAPK signaling pathway (18 genes), cell communication (9 genes), adipocytokine signaling pathway (6 genes), and antigen processing and presentation (5 genes).

With several important exceptions, however, gene expression in these pathways appears to point to attempts to downregulate inflammation in this model (which is very distinct from findings in the original SM22-BMPR2^{delx4+} model, in which cytokine expression was universally increased[30]). It is largely unknown whether these changes are a counterregulatory response to increased inflammation through nontranscriptional means, a dilution effect resulting from use of whole lung, or indicative of actual decrease in broad classes of inflammatory signaling. The exceptions to this downregulation include a handful of specific cytokines and markers pointing to increased monocyte/macrophage recruitment. These gene expression

changes are matched by immunohistochemical findings of a large increase in recruitment of monocytes (by 1 week of transgene activation) and later macrophages (by 4 weeks, while RVSP is still normal) to the lung.

It is natural to attribute these changes in inflammatory signaling and inflammatory cell recruitment to activation of MAPK caused by BMPR2 mutation. As yet, however, there is no direct evidence that this is true.

3.4 Summary

Six immediate downstream effectors of BMPR2 are known[8–12] (Fig. 17.2). Transgenic mouse experiments examining the changes caused by BMPR2 mutation before RVSP increases have told us the downstream effects of these effectors (bottom line, Fig. 17.2). While differential analysis of targets of BMPR2 mutations in transgenic mice with and without Smad function allow reasonably definitive understanding of Smad function, one may only make educated guesses of how effectors link to effects based on the literature. The central questions needed to develop effective treatment targeted at the molecular etiology of BMPR2-related PAH are as follows: Which of the effects are necessary for the development of PAH? Can these critical effects be blocked by interventions against specific effectors or the pathways linking the effectors to the effects?

4 The BMP Pathway in Acute Inflammation

The functional in vivo consequences of BMPR2 mutation all point to a role for the BMP pathway in controlling inflammatory response. Loss of Smad leads to a synthetic phenotype in smooth muscle and increased cytokine signal. Loss of LIMK,

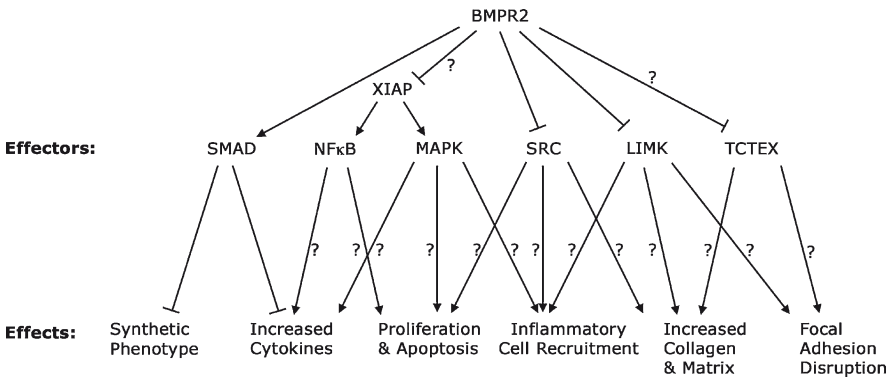

Fig. 17.2 Immediate effectors of BMPR2 signaling and the downstream effects of signaling through these effectors. While the effectors have been well established, and the effects established by transgenic mouse experiments, the links between the effects and the effectors are still in most cases not directly tested

TCTEX, and possibly SRC leads to increases in genes related to motility, permeability, and matrix deposition. Loss of control of (presumably) MAPK leads to broad alterations in inflammatory gene expression and increased recruitment of monocytes and macrophages. In summary, loss of BMP signal appears to cause the pulmonary vasculature to behave as though it had been injured.

We previously published concerning a negative-feedback loop in smooth muscle between the cytokine IL6) and the BMP pathway.[31] Increased IL6 signaling causes induction of the BMP pathway, primarily through inhibition of secreted inhibitors; induction of the BMP pathway causes suppression of IL6 expression. This provides a plausible mechanism by which the BMP pathway drives resolution of inflammation, although it seems likely that this study was excessively narrow: The BMP pathway may inhibit broader classes of inflammatory signaling.

To more directly examine the role of the BMP pathway in pulmonary injury, we injected wild-type mice intraperitoneally with lipopolysaccharide (LPS) and measured BMP pathway activity at 0, 6, 12, and 24 h by Smad phosphorylation, ID1 protein and expression, and expression of 16 BMP pathway genes, including ligands, receptors, and inhibitors. The results of this were highly internally consistent: There was almost complete abrogation of BMP signaling at 6 h, driven by reduced ligand and increased inhibitors, with BMP signaling recovered or increased over control at later time points, primarily driven by reduced inhibitors (unpublished). These data suggest that inhibition of the BMP pathway is a normal part of acute pulmonary injury response, with heightened BMP signaling during recovery.

While recovery of BMP signal was correlated with recovery from LPS-induced injury, we wondered whether it was required for recovery. In preliminary experiments, we tested response to 5 mg/kg LPS in four SM22-rtTA × BMPR2^{delx4+} mice and four SM22-rtTA-only controls. We found response in the SM22-rtTA controls to be equivalent to wild type, as expected: At 24 h, they were ruffled, with some porphyrin staining around the eyes. By 48 h they were substantially recovered, and by 72 h all four had returned to normal. This is a normal time course for response to low-dose LPS in mice. In the SM22-rtTA × BMPR2^{delx4+} mice, the phenotype at 24 h was indistinguishable from controls; however, at 48 h their condition had worsened rather than improved, and by 72 h two had died and the other two were in such poor condition that they were euthanized. Thus, a dose of LPS that is easily resolved in wild-type mice cannot be resolved in presence of BMPR2 mutation. While these results use small numbers and imprecise metrics, they strongly suggest that a functional BMP pathway is required for resolution of inflammation.

5 Context and Conclusions

The literature and studies presented suggest that the function of the BMP pathway in the adult pulmonary circulation is resolution of inflammation; loss of proper BMPR2 function results in inability to resolve pulmonary vascular injury. As a persistent state, this leads to PAH. We believe that this hypothesis is consistent with

data from end-stage human PAH, with known risk factors and modifier genes, and with data derived from other animal models.

5.1 Animal Models

The two nongenetic animal models that most closely resemble human PAH in pathology are monocrotaline in rats and a combination of VEGF receptor inhibition and hypoxia.

Hypoxia alone causes elevation in RVSP with attendant muscularization of the pulmonary vasculature and right heart. However, alone, this does not much resemble human PAH; there is no development of complex lesions, and when removed from hypoxia, animals resolve completely. The addition of a VEGF receptor inhibitor causes small pulmonary capillaries to fill with proliferating endothelial cells, which bears some similarity to human PAH.[32] The relevance of the model to human PAH is probably only to development of late lesions, however: Induction of angiogenesis pathways is a consistent feature of elevation of RVSP.[28,33] Proliferation of endothelial cells is required for angiogenesis; addition of a VEGF receptor inhibitor causes them to lose chemotaxis and fill up existing vessels rather than create new ones. Since generation of these lesions is a late feature in both humans and animals with BMPR2 mutation,[28] VEGF receptor inhibition with hypoxia neither supports nor contradicts our hypothesis but is largely irrelevant to it.

A single injection of the plant alkaloid monocrotaline into rats causes large-scale endothelial injury, with the phenotype worsening over time, resulting in aberrant vasoconstriction and proliferation, leading to mortality in most animals.[34] However, those that do not die resolve. Moreover, many treatments aimed at blocking inflammatory response are capable of substantially improving outcomes in the monocrotaline model, including hemin,[35] sorafenib,[36] and pyrrolidine dithiocarbamate,[37] among others. It is clear that the monocrotaline model causes persistent lung injury, and resolving this injury resolves PAH. This is completely consistent with our working hypothesis, although in the case of BMPR2 mutation the underlying defect in resolution of inflammation may work against treatments that were effective with monocrotaline.

5.2 Risk Factors and Modifiers

There are several classes of risk factors for PAH, aside from BMPR2 mutation: presence of systemic inflammatory conditions such as POEMS, lupus, scleroderma, or HIV infection[34]; female gender and estrogen metabolism[38]; serotonin reuptake inhibition or serotonin receptor promoter polymorphisms[39]; and promoter polymorphism in transforming growth factor, beta (TGF-β)[40] and the potassium channel Kv1.5.[41]

The association of systemic inflammatory conditions with risk of PAH clearly supports the hypothesis that PAH is a disease of unresolved injury; the presence of systemic inflammatory conditions mimics the effects of BMPR2 mutation and provides a comparable relative risk of disease. In scleroderma, for instance, between 10 and 25% of patients develop PAH,[42] although with some differences in presentation as compared to idiopathic PAH. One might hypothesize that scleroderma provides a systemic inflammatory defect, whereas BMPR2 mutation results in a pulmonary-specific persistent inflammatory defect (the reasons for this specificity are as yet unknown). Increased TGF-β expression as a risk factor for PAH[40] fits easily into this framework as it is also a risk factor for scleroderma.[43]

An obvious mechanism by which female gender could predispose to PAH is through the well-characterized association between female gender and higher risk of autoimmune disorders. We have shown a correlation between low expression of estrogen metabolism gene *CYP1B1* and development of PAH in BMPR2 mutation carriers,[38] which suggests that it is estrogen that causes females to develop PAH in greater numbers. Estrogen is associated with increased resistance to sepsis or trauma but decreased resistance to chronic autoimmune diseases.[44]

Dysregulation of serotonin signaling, caused by anorexigens or methamphetamines, is a risk factor for PAH known for decades. Serotonin's primary mechanism of contribution to PAH is still controversial as it has an impact on proliferation, vasoconstriction, and inflammation.[39] It seems likely that, as all three of these mechanisms are clearly implicated in the development of PAH, all three of these serotonin targets are involved in the etiology of PAH, thus its potency as risk for disease. Note that these mimic several of the effects of BMPR2 mutation shown in Fig. 17.2.

Both the finding of Kv1.5 polymorphisms as a risk factor for PAH[41] and the finding of vasoreactivity genes as differentially regulated between disease-affected and unaffected BMPR2 mutation carriers[38] suggest that vasoreactivity defects carry risk for PAH distinct from BMPR2 or proliferation. Further, while the BMPR2-R899X mutation in mice leads to PAH that appears to be primarily related to vascular pruning rather than vasoreactivity,[28] the universally expressing version drops from over 50% penetrance for elevated RVSP after induction of 8 weeks in young adults at Denver altitude (85 kPa) to only about 25% at sea level (100 kPa) (unpublished). These data suggest that, even if the disease is primarily proliferative rather than vasoreactive, there is an important and as yet poorly defined connection with BMP pathway function.

5.3 End-Stage Human Disease

Persistent high pressure in the pulmonary vasculature from any source produces almost all of the central manifestations of end-stage PAH, including angiogenesis, stress markers, and even complex vascular lesions.[45] Thus, almost all of the data on end-stage disease focus on side effects of elevated RVSP rather than on the source of the elevated RVSP. Direct understanding of etiology from end-stage human disease is almost hopelessly compromised by disease and drug effects.

However, while the nature of the data prohibits understanding of causation, if our hypothesis that BMPR2 mutation affecting any of its downstream signaling will result in a proinflammatory state is correct, we ought to be able to continue to see proinflammatory signaling in human patients with end-stage PAH.

Human patients with PAH show extensive signs of ongoing inflammation, including large numbers of circulating cells, both perivascular and throughout developing lesions; elevated circulating levels of proinflammatory cytokines, including IL1 and IL6 and markers of T-cell activation.[34] In addition, in small studies, immunosuppressive therapy has been effective in some patients with PAH.[46,47]

5.4 Conclusions

Effects of dysregulation of Smad, MAPK, and actin organization pathways downstream of BMPR2 mutation combine to drive injury response. This injury response consists of vascular stiffening, shift to a proproliferative phenotype, and induction of inflammatory cytokines and recruitment of inflammatory cells (boxed region, Fig. 17.3). As discussed in Section 17.3, different elements of BMPR2 downstream signaling lead to each of the elements of this response. However, since there are feedback loops between each of these elements, sufficient dysregulation of any one element may be sufficient to drive injury response through all elements. Thus, while there does not seem to be a clear consistency in downstream targets affected by the myriad BMPR2 mutations, there does not need to be: Activation of injury response, through any of several pathways impacted by BMP signaling, predisposes to disease through the same mechanism.

Our overall theory of the etiology of PAH is thus predisposition to chronic injury response through BMPR2 mutation. A combination of other genetic and environmental factors can increase or decrease this predisposition (Fig. 17.3), acting through the same mechanisms. Either the correct combination alone, under the

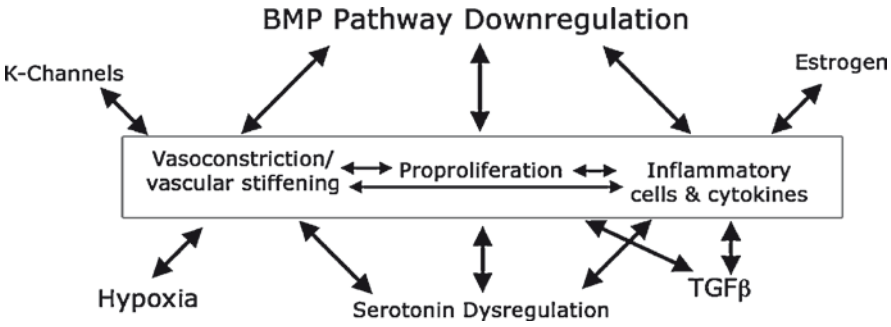

Fig. 17.3 BMP pathway downregulation leads to injury response, with different downstream elements driving vascular stiffening, proproliferation, and inflammatory response. Since these are themselves part of feedback loops, dysregulation of any one element can lead to dysregulation of the whole

theory that the disease develops cryptically over long time spans, or this combination together with a triggering event, under the theory that the disease develops relatively rapidly, leads to the persistent inability to resolve pulmonary vascular injury, which is the central etiology of PAH.

References

1. Deng Z, Morse JH, Slager SL et al (2000) Familial primary pulmonary hypertension (gene PPH1) is caused by mutations in the bone morphogenetic protein receptor-II gene. Am J Hum Genet 67:737–744
2. Lane KB, Machado RD, Pauciulo MW et al (2000) Heterozygous germline mutations in BMPR2, encoding a TGF-β receptor, cause familial primary pulmonary hypertension. The International PPH Consortium. Nat Genet 26:81–84
3. Thomson JR, Machado RD, Pauciulo MW et al (2000) Sporadic primary pulmonary hypertension is associated with germline mutations of the gene encoding BMPR-II, a receptor member of the TGF-β family. J Med Genet 37:741–745
4. Tiedemann H, Asashima M, Grunz H, Knochel W (2001) Pluripotent cells (stem cells) and their determination and differentiation in early vertebrate embryogenesis. Dev Growth Differ 43:469–502
5. Herpin A, Cunningham C (2007) Cross-talk between the bone morphogenetic protein pathway and other major signaling pathways results in tightly regulated cell-specific outcomes. FEBS J 274:2977–2985
6. Nohe A, Keating E, Underhill TM, Knaus P, Petersen NO (2003) Effect of the distribution and clustering of the type I A BMP receptor (ALK3) with the type II BMP receptor on the activation of signalling pathways. J Cell Sci 116:3277–3284
7. Nishihara A, Watabe T, Imamura T, Miyazono K (2002) Functional heterogeneity of bone morphogenetic protein receptor-II mutants found in patients with primary pulmonary hypertension. Mol Biol Cell 13:3055–3063
8. Jeffery TK, Upton PD, Trembath RC, Morrell NW (2005) BMP4 inhibits proliferation and promotes myocyte differentiation of lung fibroblasts via Smad1 and JNK pathways. Am J Physiol Lung Cell Mol Physiol 288:L370–L378
9. Rudarakanchana N, Flanagan JA, Chen H et al (2002) Functional analysis of bone morphogenetic protein type II receptor mutations underlying primary pulmonary hypertension. Hum Mol Genet 11:1517–1525
10. Foletta VC, Lim MA, Soosairajah J et al (2003) Direct signaling by the BMP type II receptor via the cytoskeletal regulator LIMK1. J Cell Biol 162:1089–1098
11. Machado RD, Rudarakanchana N, Atkinson C et al (2003) Functional interaction between BMPR-II and Tctex-1, a light chain of dynein, is isoform-specific and disrupted by mutations underlying primary pulmonary hypertension. Hum Mol Genet 12:3277–3286
12. Lu M, Lin SC, Huang Y et al (2007) XIAP induces NF-κB activation via the BIR1/TAB1 interaction and BIR1 dimerization. Mol Cell 26:689–702
13. Wong WK, Knowles JA, Morse JH (2005) Bone morphogenetic protein receptor type II C-terminus interacts with c-Src: implication for a role in pulmonary arterial hypertension. Am J Respir Cell Mol Biol 33:438–446
14. Newman JH, Phillips JA III, Loyd JE (2008) Narrative review: the enigma of pulmonary arterial hypertension: new insights from genetic studies. Ann Intern Med 148:278–283
15. Lu MM, Yang H, Zhang L, Shu W, Blair DG, Morrisey EE (2001) The bone morphogenic protein antagonist gremlin regulates proximal-distal patterning of the lung. Dev Dyn 222:667–680
16. Morrell NW (2006) Pulmonary hypertension due to BMPR2 mutation: a new paradigm for tissue remodeling? Proc Am Thorac Soc 3:680–686

17. Rajan P, Panchision DM, Newell LF, McKay RD (2003) BMPs signal alternately through a SMAD or FRAP-STAT pathway to regulate fate choice in CNS stem cells. J Cell Biol 161:911–921
18. Yanagisawa M, Nakashima K, Takizawa T, Ochiai W, Arakawa H, Taga T (2001) Signaling crosstalk underlying synergistic induction of astrocyte differentiation by BMPs and IL-6 family of cytokines. FEBS Lett 489:139–143
19. Yamaguchi K, Nagai S, Ninomiya-Tsuji J et al (1999) XIAP, a cellular member of the inhibitor of apoptosis protein family, links the receptors to TAB1-TAK1 in the BMP signaling pathway. EMBO J 18:179–187
20. Fuentealba LC, Eivers E, Ikeda A et al (2007) Integrating patterning signals: Wnt/GSK3 regulates the duration of the BMP/Smad1 signal. Cell 131:980–993
21. Winter-Vann AM, Johnson GL (2007) Integrated activation of MAP3Ks balances cell fate in response to stress. J Cell Biochem 102:848–858
22. Li MG, Serr M, Newman EA, Hays TS (2004) The Drosophila tctex-1 light chain is dispensable for essential cytoplasmic dynein functions but is required during spermatid differentiation. Mol Biol Cell 15:3005–3014
23. Sachdev P, Menon S, Kastner DB et al (2007) G protein βγ subunit interaction with the dynein light-chain component Tctex-1 regulates neurite outgrowth. EMBO J 26:2621–2632
24. Chuang JZ, Yeh TY, Bollati F et al (2005) The dynein light chain Tctex-1 has a dynein-independent role in actin remodeling during neurite outgrowth. Dev Cell 9:75–86
25. Baruzzi A, Caveggion E, Berton G (2008) Regulation of phagocyte migration and recruitment by Src-family kinases. Cell Mol Life Sci 65:2175–2190
26. Kuramochi Y, Guo X, Sawyer DB (2006) Neuregulin activates erbB2-dependent src/FAK signaling and cytoskeletal remodeling in isolated adult rat cardiac myocytes. J Mol Cell Cardiol 41:228–235
27. Ghosh S, Hayden MS (2008) New regulators of NF-κB in inflammation. Nat Rev Immunol 8:837–848
28. West J, Harral J, Lane K et al (2008) Mice expressing $BMPR2^{R899X}$ transgene in smooth muscle develop pulmonary vascular lesions. Am J Physiol Lung Cell Mol Physiol 295:L744–L755
29. West J, Fagan K, Steudel W et al (2004) Pulmonary hypertension in transgenic mice expressing a dominant-negative BMPRII gene in smooth muscle. Circ Res 94:1109–1114
30. Tada Y, Majka S, Carr M et al (2007) Molecular effects of loss of BMPR2 signaling in smooth muscle in a transgenic mouse model of PAH. Am J Physiol Lung Cell Mol Physiol 292:L1556–L1563
31. Hagen M, Fagan K, Steudel W et al (2007) Interaction of interleukin-6 and the BMP pathway in pulmonary smooth muscle. Am J Physiol Lung Cell Mol Physiol 292:L1473–L1479
32. Taraseviciene-Stewart L, Kasahara Y, Alger L et al (2001) Inhibition of the VEGF receptor 2 combined with chronic hypoxia causes cell death-dependent pulmonary endothelial cell proliferation and severe pulmonary hypertension. FASEB J 15:427–438
33. Tada Y, Laudi S, Harral J et al (2008) Murine pulmonary response to chronic hypoxia is strain specific. Exp Lung Res 34:313–323
34. Dorfmuller P, Perros F, Balabanian K, Humbert M (2003) Inflammation in pulmonary arterial hypertension. Eur Respir J 22:358–363
35. Shimzu K, Takahashi T, Iwasaki T et al (2008) Hemin treatment abrogates monocrotaline-induced pulmonary hypertension. Med Chem 4:572–576
36. Klein M, Schermuly RT, Ellinghaus P et al (2008) Combined tyrosine and serine/threonine kinase inhibition by sorafenib prevents progression of experimental pulmonary hypertension and myocardial remodeling. Circulation 118:2081–2090
37. Huang J, Kaminski PM, Edwards JG et al (2008) Pyrrolidine dithiocarbamate restores endothelial cell membrane integrity and attenuates monocrotaline-induced pulmonary artery hypertension. Am J Physiol Lung Cell Mol Physiol 294:L1250–L1259
38. West J, Cogan J, Geraci M et al (2008) Gene expression in BMPR2 mutation carriers with and without evidence of pulmonary arterial hypertension suggests pathways relevant to disease penetrance. BMC Med Genomics 1:45

39. MacLean MR (2007) Pulmonary hypertension and the serotonin hypothesis: where are we now. Int J Clin Pract Suppl 156:27–31
40. Phillips JA III, Poling JS, Phillips CA et al (2008) Synergistic heterozygosity for TGFβ1 SNPs and BMPR2 mutations modulates the age at diagnosis and penetrance of familial pulmonary arterial hypertension. Genet Med 10:359–365
41. Remillard CV, Tigno DD, Platoshyn O et al (2007) Function of Kv1.5 channels and genetic variations of KCNA5 in patients with idiopathic pulmonary arterial hypertension. Am J Physiol Cell Physiol 292:C1837–C1853
42. Wigley FM, Lima JA, Mayes M, McLain D, Chapin JL, Ward-Able C (2005) The prevalence of undiagnosed pulmonary arterial hypertension in subjects with connective tissue disease at the secondary health care level of community-based rheumatologists (the UNCOVER study). Arthritis Rheum 52:2125–2132
43. Ihn H (2008) Autocrine TGF-β signaling in the pathogenesis of systemic sclerosis. J Dermatol Sci 49:103–113
44. Straub RH (2007) The complex role of estrogens in inflammation. Endocr Rev 28:521–574
45. Wagenvoort CA, Wagenvoort N, Draulans-Noe Y (1984) Reversibility of plexogenic pulmonary arteriopathy following banding of the pulmonary artery. J Thorac Cardiovasc Surg 87:876–886
46. Bellotto F, Chiavacci P, Laveder F, Angelini A, Thiene G, Marcolongo R (1999) Effective immunosuppressive therapy in a patient with primary pulmonary hypertension. Thorax 54:372–374
47. Sanchez O, Sitbon O, Jais X, Simonneau G, Humbert M (2006) Immunosuppressive therapy in connective tissue diseases-associated pulmonary arterial hypertension. Chest 130:182–189

Notch Signaling in Pulmonary Hypertension

Patricia A. Thistlethwaite, Xiaodong Li, and Xiaoxue Zhang

Abstract Proteins of the Notch receptor family are cell surface receptors that transduce signals between neighboring cells. The Notch signaling pathway is highly evolutionarily conserved and critical for cell fate determination during embryogenesis and early postnatal life, including many aspects of vascular development. The interaction of Notch receptor with its membrane-bound ligands leads to cleavage of the receptor into an intracellular domain that translocates to the nucleus and activates the transcription factor, C-promoter binding factor 1 (CBF1; also known as Recombination signal-binding protein for immunoglobulin κ J region, RBPJ). To date, four Notch receptors have been characterized in humans. Of these, Notch3 is expressed only in arterial smooth muscle cells in the human. The functional importance of Notch3 signaling in human vascular smooth muscle cells has been recognized. Notch3 receptor signaling has been shown in several model systems to control vascular smooth muscle cell proliferation and maintain smooth muscle cells in an undifferentiated state. This review focuses on recent findings of the role of Notch3 in regulating vascular smooth muscle cell behavior and phenotype and discusses the potential role of Notch3 signaling in the genesis of pulmonary arterial hypertension.

Keywords Pulmonary hypertension • receptor signaling

1 Introduction

Changes in the structure, function, and integrity of blood vessels are necessary to the pathogenesis of many diseases, including pulmonary arterial hypertension (PAH). It has been well established that adult vascular smooth muscle cells are not terminally differentiated and are capable of phenotypic change in response to exogenous stimuli,

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cell-cell interaction, and cell–matrix signaling.[1] However, the genetic pathways that regulate plasticity and proliferation of pulmonary vascular smooth muscle cells have not been well characterized.

PAH is a disease characterized by structural remodeling of small pulmonary arteries and arterioles due to vessel thickening and luminal occlusion by vascular smooth muscle cell and endothelial proliferation.[2] The vasculopathy seen in this disease is progressive and diffuse and eventually results in obliteration of the distal pulmonary arterial tree. From a clinical point of view, PAH manifests as sustained elevation in pulmonary arterial pressures and pulmonary vascular resistance, leading to right heart failure and death. Each year, PAH afflicts approximately 100,000 people and is the cause of death in 20,000 individuals in the United States.[3] Although several stimuli and conditions, such as hypoxia, fenfluramine ingestion, collagen vascular disease, portal hypertension, and intracardiac left-to-right shunting are associated with this disease,[4] the exact mechanism of how the lung remodels its vascular smooth muscle cell architecture in the arteriolar bed in PAH is not known. Increasing evidence suggests that Notch receptors and downstream Notch effectors play important roles during embryonic and postnatal vascular development, as well as in the response of vascular smooth muscle cells to growth factor stimulation and vessel wall injury. This emerging body of literature lays the groundwork for understanding the potential role of Notch signaling in the genesis of PAH.

2 Overview of Notch Signaling

Genes of the Notch family encode single-pass transmembrane receptors that transduce signals through cell–cell interactions (Fig. 18.1). In mammals, four Notch family receptors have been described: Notch1–4.[5] The extracellular domain of Notch family proteins contains up to 36 tandemly repeated copies of an epidermal growth factor-like (EGF-like) motif, involved in ligand interaction, and three juxtamembrane repeats known as Lin-12-Notch repeats, which modulate interactions between the extracellular and the membrane-tethered intracellular domains (ICDs).[6] The intracellular region of Notch includes seven ankyrin repeats flanked by nuclear localization signals; a proline, glutamine, serine, threonine-rich (PEST) domain; and a transactivation domain.[7,8] Notch receptors interact with single-pass transmembrane ligands expressed on adjacent cells. This restricts the Notch pathway to regulating short-range intercellular interactions. Notch cell-bound ligands are encoded by the Jagged (*Jag1*, *Jag2*) and Delta-like (*Dll1*, *Dll3*, and *Dll4*) gene families.[9,10] To date, in adult mammals, including humans, Notch ligands have been found to be principally expressed on vascular endothelium, although reports suggested that these ligands may also have limited expression on vascular smooth muscle cells.[11,12]

An important feature of Notch is that it acts, at the same time, as a transmembrane receptor and as a transcription factor (Fig. 18.2). After ligand binding, Notch receptors undergo a series of proteolytic events, including a final cleavage by a γ-secretase enzyme, that lead to release of the ICD of these receptors. The Notch

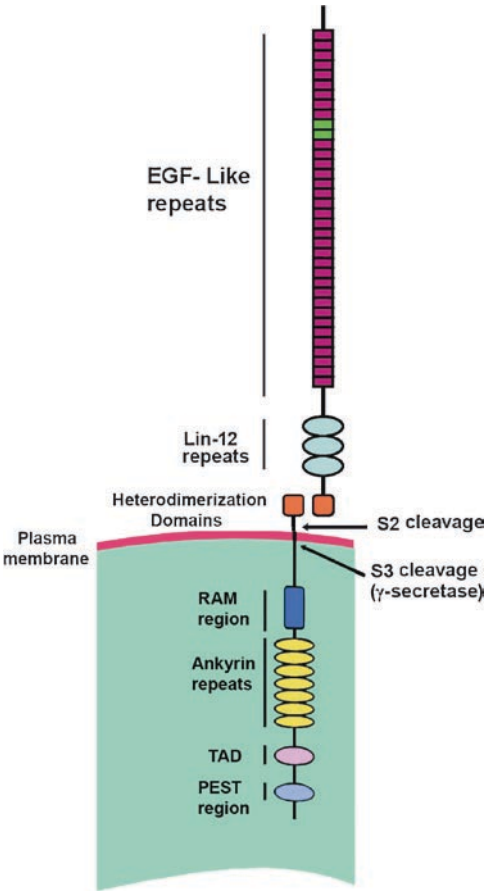


Fig. 18.1 Structure of the Notch receptor. The Notch receptor locates at the cell surface as a heterodimer following protease cleavage (S1 cleavage) during protein maturation. The extracellular domain associates noncovalently with a membrane-tethered intracellular domain (ICD) and is composed of up to 36 epidermal growth factor (EGF)-like repeats and three cysteine-rich Lin-12 repeats. EGF repeats 11 and 12 are sufficient to mediate the interaction between Notch and its ligands. In the ICD, Notch has a region called RAM (RBPJ associate molecule) followed by repeated structural motifs named ankyrin repeats (mediate the action between Notch and CBF1), a transactivation domain (TAD), and a PEST domain. The PEST domain is involved in the degradation of Notch.

ICD fragment translocates to the nucleus because of the presence of nuclear localization signals located within it.[13] Once in the nucleus, the Notch ICD forms an active transcriptional complex with the DNA-binding protein, CBF1 (also known as RBPJ).[14] In the absence of the ICD, RBPJ protein binds to specific DNA sequences in the regulatory elements of various target genes and represses transcription of these genes by recruiting histone deacetylases to form a corepressor complex.

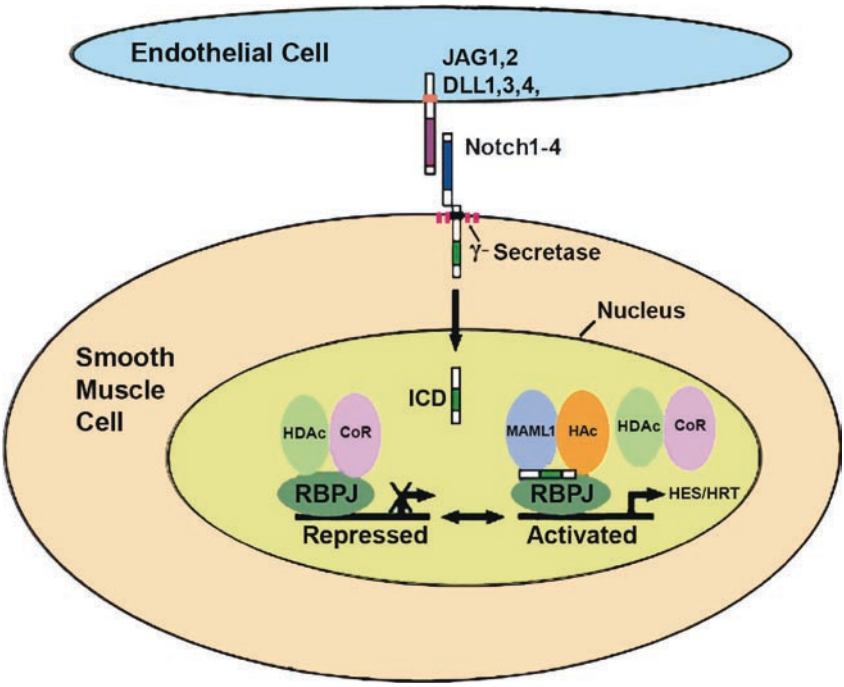


Fig. 18.2 The Notch signaling pathway. Ligands of the Jagged (*Jag1* and *Jag2*) and Delta-like (*Dll1, Dll3, Dll4*) families interact with Notch receptors (Notch1–4) on an adjacent cell. The Notch receptor exists at the cell surface as a proteolytically cleaved heterodimer consisting of a large extracellular domain and a membrane-tethered intracellular domain. The receptor–ligand interaction induces two proteolytic cleavages that free the Notch ICD from the cell membrane: the S2 cleavage mediated by the ADAM metalloproteinase family protein tumor necrosis factor α (TNF-α)-converting enzyme (TACE) and the subsequent S3 cleavage within the transmembrane domain by γ-secretase. The Notch ICD translocates to the nucleus, where it forms a complex with the RBPJ protein, displacing a histone deacetylase (HDAc)-corepressor (CoR) complex from the RBPJ protein. Components of an activation complex, such as MAML1 and histone acetyltransferases (HAc), are recruited to the ICD-RBPJ complex, leading to the transcriptional activation of Notch target genes (*Hes* and *Hrt*).

The Notch ICD displaces the histone deacetylase–corepressor complex from the RBPJ protein. Subsequently, the Notch ICD–RBPJ complex recruits the protein mastermind-like 1 (MAML1) and histone acetyltransferases, leading to the transcriptional activation of Notch target genes.[15] The key downstream genes of Notch signaling that have been identified are the *Hes* (*Hairy/Enhancers of Split*) and *Hrt* (*Hes-related transcriptional factor*) gene families, which when activated by Notch reduce expression of downstream transcriptional effectors like Mash,[16] Myogenic differentiation 1 (myoD),[17] and myocardin,[18] as well as cell cycle regulatory proteins, $p27^{kip1}$,[19] and $p21^{waf/cip1}$.[20] Notch signaling has also been found to directly modulate the expression of the platelet-derived growth factor β (PDGFβ) receptor.[21]

3 Notch3 Regulates Specification of Arterial Smooth Muscle Cells

The *Notch3* gene is expressed solely in vascular smooth muscle cells of arteries and arterioles, and is not seen in vascular smooth muscle cells of veins or venules. Clues regarding the function of Notch3 have been provided by the study of *Notch3*-null mice, which are viable and fertile.[22,23] In the mouse, maturation of arterial vessels occurs from birth to postnatal day 28, with vascular smooth muscle cells undergoing changes in both morphology and orientation. This remodeling process is strongly impaired in *Notch3*$^{-/-}$ mice. Arteries of *Notch3*$^{-/-}$ mice are enlarged with a thinner vascular smooth muscle cell coat than is found in wild-type arteries.[24] Arterial defects were observed in all organs analyzed in homozygous *Notch3* knockout mice but were milder or absent in the major elastic arteries of the trunk, suggesting an important role of Notch3 in small resistance arteries. Morphologically, arterial vascular smooth muscle cells of *Notch3*$^{-/-}$ mice resembled smooth muscle cells surrounding veins in wild-type mice, with elongated cell shape and poorly oriented clusters of smooth muscle cells around lumens. Arteries from *Notch3*$^{-/-}$ mice were found to have smooth muscle cell markers, like myosin heavy chain and α-smooth muscle- (α-SM-) actin at birth, but by the seventh postnatal day, α-SM-actin and myosin heavy chain-positive cells were abnormally aggregated within the vessel wall.

Only a few markers are known to be expressed predominantly in arterial vascular smooth muscle cells, not in venous ones. These include smoothelin[25] and a transgenic line expressing the β-galactosidase protein from arterial-specific regulatory elements of the SM22α promoter.[26] *Notch3*$^{-/-}$ mice demonstrated impaired postnatal arterial differentiation of vascular smooth muscle cells, indicated by a marked reduction in expression of the arterial-specific marker smoothelin and by a reduced ability to stimulate arterial-specific regulatory elements with the *SM22α* gene promoter when this was introduced in the *Notch3*-null background.[24] In the adult *Notch3* knockout mice, some arteries were found to be dilated, covered by a thinner than normal smooth muscle cells coat, and vascular smooth muscle cells failed to properly integrate into the vessel wall. It is of interest that, in arteries of *Notch3*$^{-/-}$ mice, which did not express arterial markers for vascular smooth muscle cells (i.e., smoothelin), normal expression of several endothelial cell arterial markers occurred, including that of ephrin B2, connexin 40, Hes1, Hey1, Hey2.[24] These results demonstrated that, in the systemic circulation, the arterial identity of endothelial cells and vascular smooth muscle cells surrounding them is specified independently.

4 Studies Supporting a Role of Notch3 in Vascular Smooth Muscle Cell Proliferation and Dedifferentiation

Several lines of evidence suggest that Notch3 signaling is crucial for maintenance of arterial vascular smooth muscle cell proliferative capacity. First, *Notch3* expression is restricted to vascular smooth muscle cells of arteries and is not seen in veins

or other tissues.[27] Second, expression of this gene has been linked to modulation of vascular smooth muscle cells into an undifferentiated, proliferative state. Campos et al.[28] described the growth behavior of a Notch3 ICD stable cell line generated from rat embryonic vascular smooth muscle cells, in which the growth rate failed to decelerate at postconfluence. Notch3 ICD-induced proliferation was associated with decreased expression of the cell cycle inhibitor $p27^{kip}$. Overexpression of $p27^{kip1}$ has been shown to result in a dose-dependent rescue of the Notch-induced proliferative phenotype and exit from the cell cycle.[19] Similarly, when smooth muscle cells were forced to express Hrt1, a downstream effector of Notch3 signaling, they displayed postconfluence growth coincident with diminished $p21^{waf1/cip1}$.[20] Sweeney and colleagues[14,29] found that Notch3 promotion of vascular smooth muscle cell proliferation was RBPJ dependent. While serum is known to stimulate vascular smooth muscle cell proliferation, it also upregulates expression of Notch target genes *Hrt1* and *Hrt2*. In addition, cultured vascular smooth muscle cells isolated from *Hrt2* knockout mice have been shown to have a decreased proliferative capacity and diminished migratory response to PDGF.[30] Third, Notch3 signaling has been shown to repress terminal smooth muscle cell differentiation and maintenance of a contractile smooth muscle cell phenotype.[18] Specifically, forced expression of the Notch ICD or Hrt2 in C3H10T1/2 fibroblasts inhibited myocardin-dependent transcription of smooth muscle cell-restricted genes and activity of multiple smooth muscle cell transcriptional regulatory elements. In addition, expression of the constitutively active form of Notch3 ICD has been found to downregulate smooth muscle cell-specific α-actin, myosin, calponin, and smoothelin in human aortic smooth muscle cells.[31] This effect was CBF1/RBPJ dependent and mediated by the Notch target genes *Hrt1*, *-2*, and *-3*. Taken together, these data support a critical role of Notch signaling in repressing expression of genes encoding contractile proteins and promoting cell proliferation.

The importance of Notch3 signaling in arterial vascular disease is further highlighted by the fact that its impairment is responsible for two congenital diseases that affect the vasculature: cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL)[32] and Alagille syndromes (AGS).[33] CADASIL is a hereditary vascular degenerative disorder caused by mutations in the human *Notch3* gene. This syndrome is characterized by arteriopathy that affects mainly the small cerebral arteries/arterioles and leads to stroke and dementia in humans. In fact, CADASIL is the most common single gene disorder associated with ischemic stroke, but because it is underdiagnosed, its true prevalence is not known. The genetic defects in CADASIL are typically missense mutations, most of which translate into amino acid exchanges within the first five EGF-like repeats of the Notch3 ectodomain. Although the disease is characterized by the progressive degeneration of vascular smooth muscle cells, the downstream consequences of *Notch3* mutation (whether mutations lead to gain or loss of Notch signaling) are not known.

AGS is a congenital disorder caused by mutations in the human gene for *Jagged 1* (*Jag1*). Although this syndrome is associated with abnormalities in the liver, heart, eye, and skeleton, the most frequent vascular anomalies in AGS patients are segmental and subsegmental pulmonary artery stenoses, contributing to the development

of severe pulmonary hypertension. AGS is caused by *Jag1* haploinsufficiency. Both CADASIL and AGS, which affect the Notch signaling pathway, manifest pathology in mid- to small-size arteries/arterioles in different target organs (brain and lung) To date, the etiology of CADASIL and AGS remains insufficiently understood, so recent advancements in understanding the role of Notch3 in the vasculature may allow new insight into the pathogenesis of these diseases.

5 Notch3 Is a Marker for PAH and PAH Disease Severity

Recently, Notch3 signaling has been studied in the context of PAH in humans as well as pulmonary hypertension models in rodents.[34] Lung biopsies from patients with nonfamilial PAH undergoing lung transplantation were compared to lung biopsies from patients without PAH undergoing lung resection for benign conditions. Notch3 messenger RNA (mRNA) and ICD protein were found to be expressed in low baseline levels in human lung tissue. Markedly elevated levels of Notch3 mRNA and ICD protein were found in human pulmonary hypertensive lung tissues compared to normotensive lung samples (Fig. 18.3a, b). In a similar fashion, the lungs of mice with hypoxia-induced pulmonary hypertension and the lungs of rats with monocrotaline-induced pulmonary hypertension had elevated levels of Notch3 mRNA and Notch3 ICD protein compared to species- and strain-specific nonpulmonary hypertensive lungs (Fig. 18.3a, b). To characterize whether augmented Notch3 levels were specific to lung tissue in pulmonary hypertension, organ-specific levels of Notch3 between normal and pulmonary hypertensive mice were compared. Notch3 mRNA levels were higher in the lung, brain, heart, and kidney in animals exposed to 10% oxygen for 6 weeks (pulmonary hypertensive animals) compared to normoxic animals (nonpulmonary hypertensive animals). However, levels of Notch3 ICD protein were only minimally detected in most organs, while lung levels of Notch3 ICD protein more than tripled in animals with hypoxia-induced pulmonary hypertension (Fig. 18.3c).

Notch3 expression has been found to correlate with pulmonary hypertension disease progression. Rodents were studied at serial time points during either hypoxia-induced (mouse) or monocrotaline-induced (rat) pulmonary hypertension. An increase in expression of Notch3 was seen in the lung as a function of time and disease severity for both hypoxia- and monocrotaline-induced pulmonary hypertensive animals (Fig. 18.3d, e). Mice subjected to 6 weeks of hypoxia were found to have threefold higher levels of Notch3 expression at both mRNA and protein (ICD) levels in their lungs compared to animals in 21% oxygen and had pulmonary artery pressures consistent with advanced pulmonary hypertension. Rats with monocrotaline-induced pulmonary hypertension had progressive elevation in steady-state levels of Notch3 mRNA and ICD protein in their lungs compared to saline-injected controls over the time span of disease progression. No difference in the expression of Notch1, -2, or -4 was found in the lungs of normotensive vs. pulmonary hypertensive animals. Hes5, a known downstream effector of Notch3 signaling, was also found to be increased in the lungs of rodents with pulmonary hypertension (Fig 18.3e).

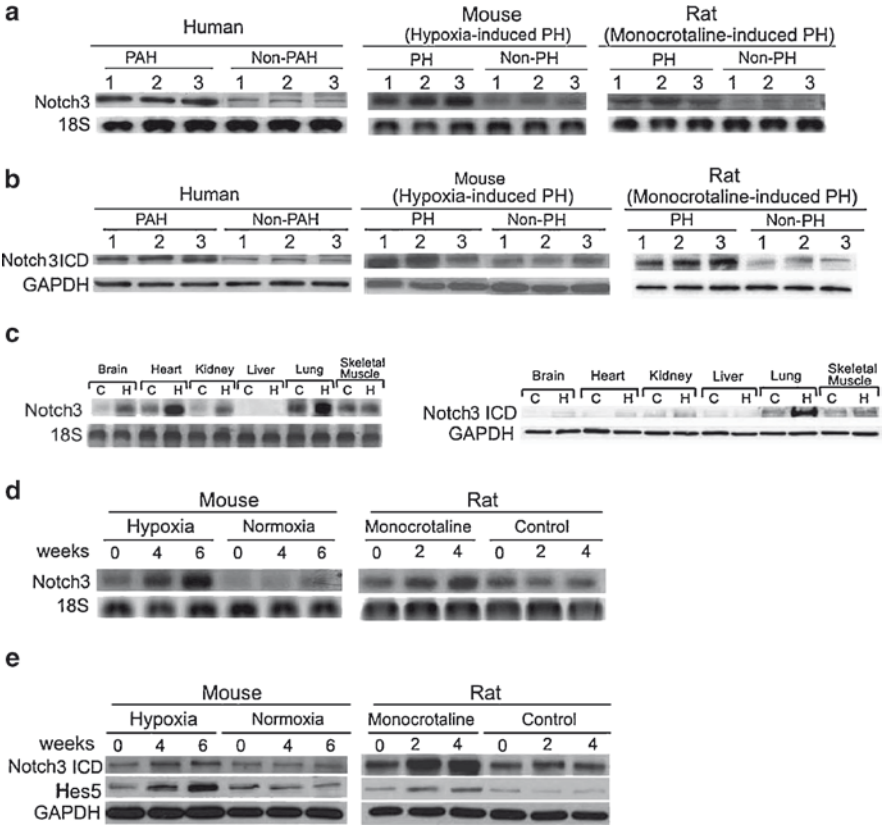


Fig. 18.3 Notch3 expression in the lung as a molecular marker for the severity of pulmonary arterial hypertension (PAH) in humans and pulmonary hypertension (PH) in rodents. (**a**) Northern blot analysis of total RNA from lungs from humans with idiopathic PAH (three patients – *left panel*), mice with hypoxia-induced PH (three animals – *middle panel*), and rats with monocrotaline-induced PH (three animals – *right panel*) compared to normotensive human and rodent lung tissue. (**b**) Western blot analysis of Notch3 ICD relative to GAPDH expression in the same human and rodent lung tissue as in (**a**). (**c**) Northern blot analysis of total RNA from organs of mice exposed to hypoxia (H) or normoxia (C) for 6 weeks (*left panel*), Western blot analysis of Notch3 ICD in tissue from in tissue from the same organs (*right panel*). (**d**) Northern blot analysis of total RNA from the lungs of mice during development of hypoxia-induced PH over 6 weeks compared to control animals (*left panel*) or lung of rats during development of monocrotaline-induced PH over 4 weeks compared to control animals (*right panel*). As disease severity worsens, steady-state levels of Notch3 mRNA increase. (**e**) Western blot analysis of Notch3 ICD relative to GAPDH expression in the same rodent lung tissue as in (**d**). As disease severity worsens, Notch3 ICD and Hes5 protein increase

High levels of Hes5 in lung tissue correlated with worsening disease severity. These results established a link between Notch3 signaling and the magnitude of pulmonary arterial pressures in two animal models of pulmonary hypertension and suggested that Notch3 and Hes5 are sensitive markers for the severity of pulmonary hypertension in rodent models of this disease.

6 Cellular Localization of Notch3 and Hes5 to Vascular Smooth Muscle Cells in the Lung

Studies using immunofluorescent staining have shown that Notch3 and Hes5 expression are confined to vascular smooth muscle cells in pulmonary arteries and arterioles[34] (Fig. 18.4a, b). Interestingly, Notch3 and Hes5 staining has not been detected in vascular smooth muscle cells from pulmonary veins or venules. Higher

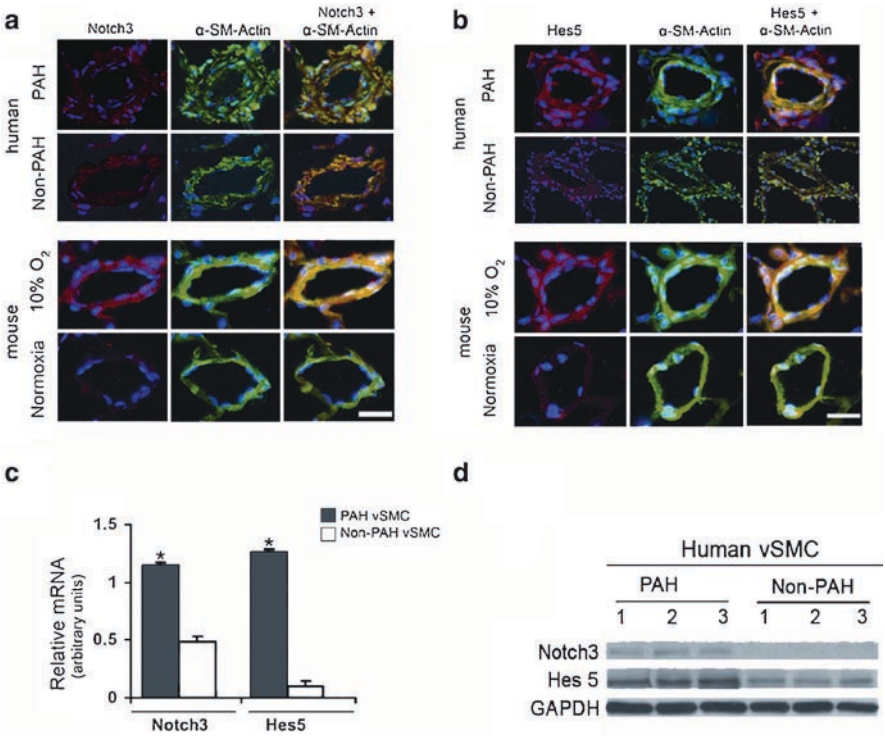


Fig. 18.4 Notch3 and Hes5 expression are specific to pulmonary arteriolar smooth muscle cells (SMCs) in the lung. (**a**) Notch3 (*red*) and α-SM-actin (*green*) immunofluorescence staining in arterioles 75–100 μm in diameter in lung tissue from patients (*top panels*) and mice (*bottom panels*) with and without PAH/PH (pulmonary hypertension). Nuclei are counterstained with DAPI (*blue*). Notch3 staining is confined to pulmonary arteriolar SMCs and predominates in vessels from PAH/PH lung tissue. Scale bar = 50 μm. (**b**) Hes5 (*red*) and α-SM-actin (*green*) immunofluorescence staining in arterioles 75–100 μm in diameter in pulmonary hypertensive and normotensive human (*top panels*) and mice (*bottom panels*) lung tissue. Nuclei are counterstained with DAPI (*blue*). Hes5 staining is confined to pulmonary arteriolar SMCs and predominates in vessels from PAH/PH lung tissue. Scale bar = 50 μm. (**c**) Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis of total RNA from subcultured arteriolar SMCs derived from the lungs of ten patients with and ten patients without PAH. Notch3 and Hes5 values are normalized to 18S ribosomal RNA (rRNA) control and presented as mean ± the standard error of the mean. $*P < 0.01$ vs. control non-PAH subcultures. (**d**) Western blot analysis of Notch3 and Hes5 in subcultured arteriolar SMCs derived from the lungs of three patients with and three patients without PAH

levels of Notch3 and Hes5 vascular smooth muscle cell-specific staining were seen in pulmonary hypertensive lung tissues compared to normotensive and age- and sex-matched control lung tissue in both humans and rodents.

In addition, analysis of subcultured human pulmonary arteriolar smooth muscle cells (isolated from vessels 500–1,500 μm in diameter) from patients with PAH demonstrated that steady-state levels of Notch3 mRNA and protein as well as Hes5 mRNA and protein were increased in these cells compared to arteriolar smooth muscle cells subcultured from the lungs of patients without PAH (Fig. 18.4c, d).

7 Notch3 Increases Proliferation in Pulmonary Arteriolar Smooth Muscle Cells

In aortic smooth muscle cells and smooth muscle precursor cells, constitutive *Notch3* expression has been associated with increased cellular proliferation in vitro.[28] This finding has been extended to smooth muscle cells isolated from pulmonary arterioles of normal human lungs.[34] Using an adenoviral transfection system, Li et al. showed that overexpression of Notch3 in human pulmonary arteriolar smooth muscle cells was associated with significantly increased growth rates at preconfluence, as measured by cell count and ^{3}[H]-leucine incorporation (Fig. 18.5a). Knockdown of Hes5 abolished the proliferative effect of Notch3 ICD in subcultured pulmonary arteriolar smooth muscle cells. Transfection of Hes5 small-interfering RNA (siRNA) into pulmonary arteriolar smooth muscle cells constitutively expressing Notch3 ICD significantly decreased Hes5 expression, cellular proliferation, and ^{3}[H]-leucine incorporation; these parameters were unaffected in vascular smooth muscle cells constitutively expressing Notch3 ICD transfected with a scrambled oligonucleotide (Fig. 18.5b, c). These results suggested that upregulated the Notch3–Hes5 signaling pathway plays an important role in the proliferation of human pulmonary arteriolar smooth muscle cells, and that inhibition of Hes5 can attenuate this process.

8 Increased Proliferation Rate of Human Pulmonary Arteriolar Smooth Muscle Cells from PAH Versus Normal Lungs Is Dependent on Notch3-Hes5 Signaling

The growth rate and gene expression patterns of arteriolar smooth muscle cells isolated from patients with and without PAH have been studied with respect to Notch3-Hes5 signaling.[34] First, pulmonary arteriolar smooth muscle cells from patients with PAH have been shown to have lower levels of differentiated smooth muscle cell markers (smoothelin and myosin heavy chain) compared to pulmonary vascular smooth muscle cells from nonpulmonary hypertensive patients (Fig. 18.6a).

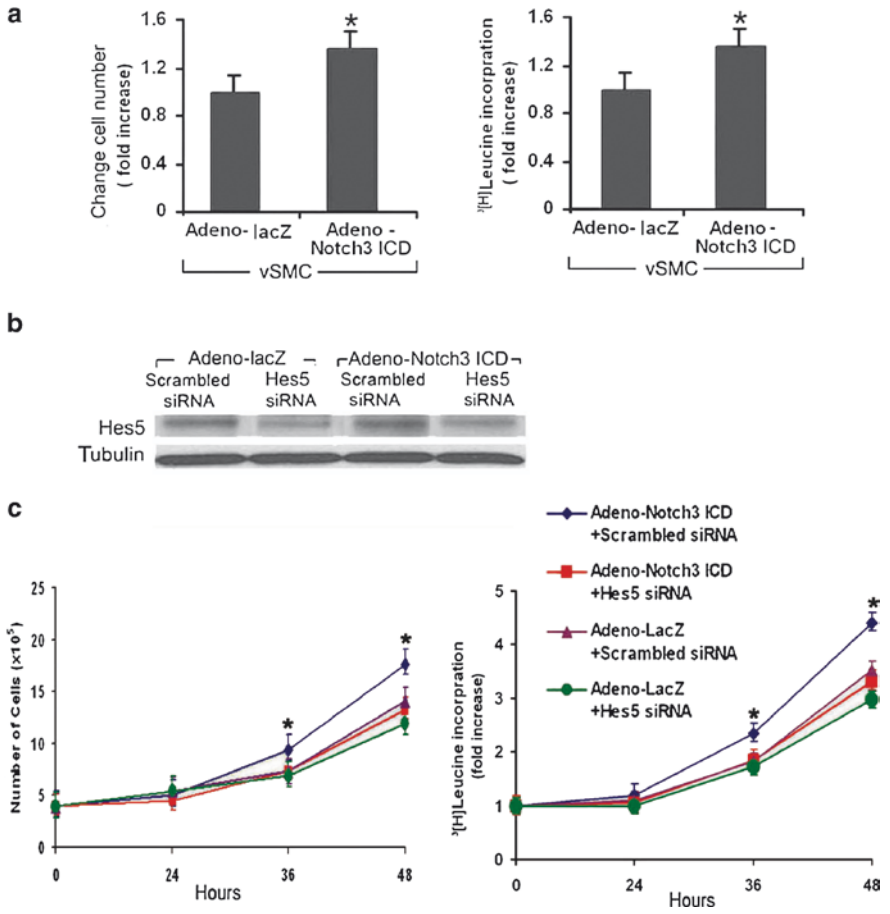


Fig. 18.5 Notch3 increases vSMC proliferative capacity in vitro. (**a**) Stimulation of human pulmonary arteriolar SMC proliferation by constitutive Notch3 expression. Independent subcultures were derived from the lungs of ten patients without PAH and passaged three times before use. *Left panel*: Averaged change in cell number after Adeno-Notch3 ICD or Adeno-lacZ transduction (for both, 12 independent viral infections per patient subculture; multiplicity of infection, 100) and synchronization in 0% serum (6 h) followed by 18 h in 5% serum. $*P < 0.02$ vs. control Adeno-lacZ-infected cells. *Right panel*: ^{3}[H]-leucine incorporation in the same cells as in *left panel*. $*P < 0.02$ vs. control Adeno-lacZ-infected cells. (**b**) Effects of Hes5 siRNA and its scrambled control (30 n*M*). Western blot analysis of protein derived from vSMCs ($n = 3$ independent subcultures from ten patients tested) treated with either Hes5 siRNA or scrambled RNA after either Adeno-lacZ or Adeno-Notch3 ICD transfection. (**c**) *Left panel*: Growth curve of pulmonary arteriolar SMCs from the lungs of patients without PAH treated with either scrambled siRNA or siRNA specific to Hes5, followed by transfection with either Adeno-Notch3 ICD or Adeno-lacZ. $*P < 0.01$ compared to control groups for the given time point. *Right panel*: ^{3}[H]-leucine incorporation for the same cells as in *left panel*. $*P < 0.01$ compared to control groups for the given time point

Second, arteriolar smooth muscle cells from human lungs with PAH demonstrated significantly shorter doubling times and higher rates of ^{3}[H]-leucine incorporation compared to vascular smooth muscle cells from non-PAH lungs (Fig. 18.6b).

Hes5 was found to contribute to the enhanced in vitro proliferative rate of arteriolar smooth muscle cells from human PAH patients compared to those of non-PAH patients; Hes5 siRNA was used to knock down this gene product in PAH and non-PAH arteriolar smooth muscle cells (Fig. 18.6c). Inhibition of endogenous Hes5 expression using siRNA markedly attenuated PAH arteriolar smooth muscle cell proliferation and ^{3}[H]-leucine incorporation (Fig. 18.6d), suggesting that Notch signaling through Hes5 may play a role in the development of pulmonary medial hypertrophy. siRNA knockdown of endogenous Hes5 in human pulmonary arteriolar smooth muscle cells also resulted in increased expression of smooth muscle cell markers, myosin heavy chain, and smoothelin over that seen in scramble-treated PAH arteriolar smooth muscle cells (Fig. 18.6e). These observations suggested that enhanced Notch signaling through Hes5 in PAH vascular smooth muscle cells may contribute to the ability of these cells to selectively proliferate and lose expression of markers of differentiated smooth muscle cells.

9 *Notch3*$^{-/-}$ Mice Are Resistant to the Development of Pulmonary Hypertension

"Proof of concept" that Notch3 signaling is requisite for the development of pulmonary hypertension has been the demonstration that mice with homozygous knockout mutation in Notch3 do not develop pulmonary hypertension in response to hypoxic stimulation.[34] Mice with homozygous deletion of the *Notch3* allele lacking 2.5 kb

Fig. 18.6 (continued) per group, three subcultures per patient) from patients with and without PAH. Average expression values normalized to 18S rRNA ±standard error of the mean are shown. Significant differences in expression ($P < 0.01$) are indicated by an *asterisk* (*). (**b**) *Left panel*: Growth curves of pulmonary arteriolar SMCs isolated from lungs of patients ($n = 10$ patients per group, three subcultures per patient) with and without PAH. *$P < 0.01$ vs. control non-PAH subcultured cells for the given time point. *Right panel*: ^{3}[H]-leucine incorporation for the same cells as in the *left panel*. *$P < 0.01$ vs. control non-PAH subcultured cells for the given time point. (**c**) Effects of Hes5 siRNA and its scrambled control (30 n*M*). Western blot analysis of protein from PAH and non-PAH arteriolar SMCs ($n = 3$ independent subcultures from ten patients per group) treated with Hes5 siRNA or scrambled RNA. (**d**) *Left panel*: Growth curve of pulmonary arteriolar SMCs isolated from the lungs of patients with and without PAH treated with either scrambled siRNA or siRNA specific to Hes5. siRNA knockdown of Hes5 attenuates proliferation of PAH SMCs. *$P < 0.01$ compared to control groups. *Right panel*: ^{3}[H]-leucine incorporation for the same cells as in *left panel*. *$P < 0.01$ compared to control groups. (**e**) Effect of selective knockdown of Hes5 on qRT-PCR measurement of MHC, smoothelin, α-SM-actin, and calponin mRNA in pulmonary arteriolar SMCs from patients with and without PAH ($n = 10$ patients per group, three subcultures per patient). Data were normalized to 18S rRNA levels and represent means ± standard error of the mean from three independent experiments. *$P < 0.02$ vs. expression measured in scrambled siRNA controls

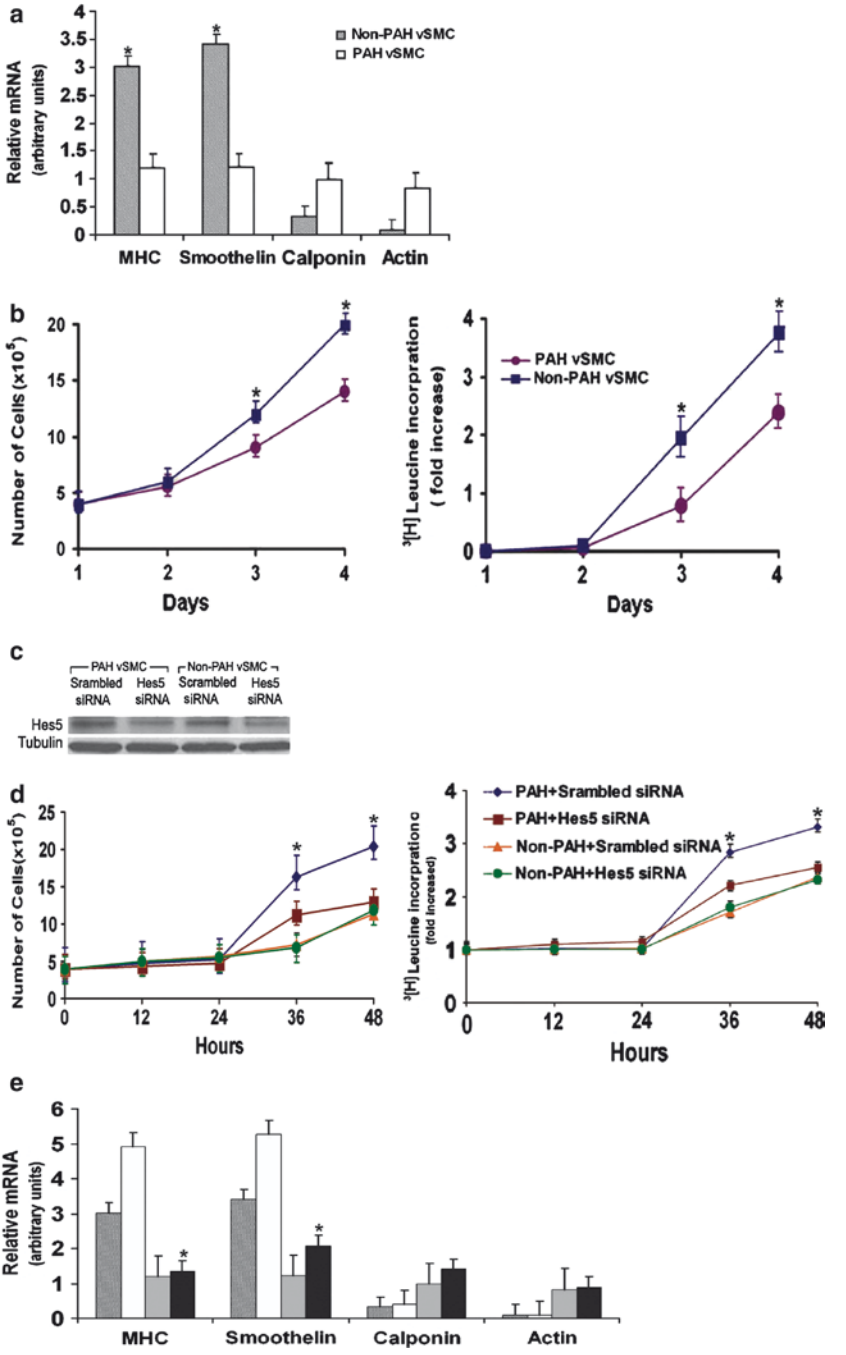


Fig. 18.6 Faster growth rates and lower levels of differentiated SMC markers in pulmonary arteriolar SMCs from PAH patients compared to SMCs from non-PAH patients are dependent on Hes5 expression. (**a**) qRT-PCR analysis of genes associated with SMC differentiation. Complementary DNA (cDNA) generated from human pulmonary arteriolar SMCs ($n = 10$ patients

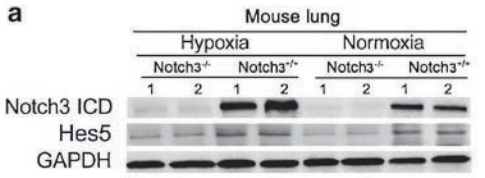
of genomic sequence encoding EGF-like repeats 8–12 in the extracellular domain[22] have been found to have minimal expression of Hes5 in the lung in conditions of hypoxia and normoxia. In contrast, *Notch3*$^{+/+}$ mouse lungs have been found to have elevated levels of Notch3 ICD and Hes5 protein when the animals were subjected to hypoxia (Fig. 18.7a). *Notch3*$^{-/-}$ mice did not develop elevated right ventricular systolic pressures (RVSPs) over a 6-week hypoxic period (Fig. 18.7b). In contrast, wild-type age-matched littermates manifested progressively elevated RVSP over the same time course while in 10% oxygen. *Notch3*$^{+/+}$ animals developed abnormal pulmonary arterial muscularization and luminal narrowing consistent with advanced pulmonary hypertension, while *Notch3*$^{-/-}$ mice had normal-appearing arterioles and small arteries with excessive muscular thickening (Fig. 18.7c). Expression of proliferating cell nuclear antigen (PCNA) was found to be decreased in small pulmonary arteries and arterioles in *Notch3*$^{-/-}$ mice compared to their wild-type counterparts at 4 and 6 weeks in hypoxia (Fig. 18.7c). Changes in wall thickness and morphology were quantified for small pulmonary arteries and arterioles in the range of 50–100 μm in diameter for *Notch3*$^{-/-}$ and *Notch3*$^{+/+}$ mice. Pulmonary arteriolar medial thickening during conditions of hypoxia correlated directly with evidence of cellular proliferation as measured by the number of cells positively stained for PCNA for control wild-type animals. *Notch3*$^{-/-}$ mice had absence of medial thickening of pulmonary arterioles/small pulmonary arteries and had minimally detectable PCNA staining in lung vascular smooth muscle cells.

Notch3$^{-/-}$ mice, maintained in hypoxia for 6 weeks, had normal pulmonary angiograms with diffuse vascular blush, while control wild-type littermates had pulmonary angiograms demonstrating severe small-vessel pruning similar to that seen in human PAH (Fig. 18.7d). Right ventricular hypertrophy, a known consequence of pulmonary hypertension, developed in *Notch3*$^{+/+}$ mice, did not occur in *Notch3*$^{-/-}$ mice over the 6-week period of hypoxic stimulation (Fig. 18.7e).

10 In Vivo Inhibition of Notch3 Cleavage by the γ-Secretase Inhibitor DAPT Reverses Pulmonary Hypertension in Rodents

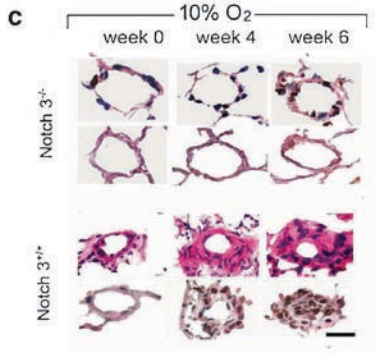
The γ-secretase inhibitor DAPT (*N*-[*N*-(3,5-diflurophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester) has been previously shown to block the in vitro and in vivo cleavage of Notch proteins to ICD peptides.[35] Chemical blockage of Notch3

Fig. 18.7 (continued) using the Microfil cast technique of *Notch3*$^{-/-}$ and *Notch3*$^{+/+}$ animals after 6 weeks of hypoxia. There is normal pulmonary tree morphology in lungs of *Notch3*$^{-/-}$ mice and absence of peripheral vessel blush in the lungs of *Notch 3*$^{+/+}$ animals, which is indicative of small-vessel occlusion. Scale bar = 2 mm. (**e**) Ratio of the weight of the right ventricle (RV) to that of the left ventricle plus septum (LV + S) as an index of RV hypertrophy in *Notch3*$^{-/-}$ ($n = 20$) and *Notch3*$^{+/+}$ mice ($n = 20$) after 6 weeks of hypoxia. *$P < 0.02$ vs. control for same time point



b

Phenotype	10%O_2	SBP*	RVSP*
Notch $3^{-/-}$	0 weeks	85±7	18.9±1.5
	4 weeks	91±5	20.4±2.0
	6 weeks	87±4	22.3±1.2
Notch $3^{+/+}$	0 weeks	90±3	18.6±2.0
	4 weeks	87±4	29.3±1.0
	6 weeks	88±5	32.5±1.4



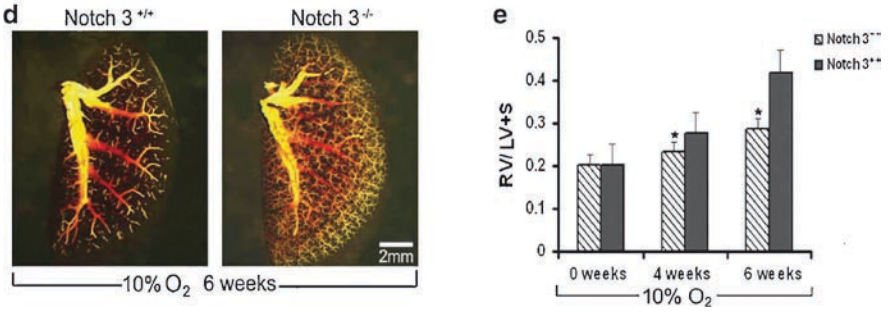


Fig. 18.7 *Notch3*$^{-/-}$ mice are resistant to the development of hypoxic PH. (**a**) Western blot analysis of Notch3 ICD and Hes5 in lung tissues from *Notch3*$^{-/-}$ and *Notch3*$^{+/+}$ mice. *Notch3*$^{-/-}$ mice have minimal expression of Hes5 in the lung in conditions of hypoxia and normoxia. (**b**) Averaged systolic blood pressure (SBP) and right ventricular systolic pressure (RVSP) in *Notch3*$^{-/-}$ mice and *Notch3*$^{+/+}$ littermate controls at serial time points under hypoxic conditions of 10% oxygen (ten readings for each animal over a 1-h period, 20 animals for each group at each time point). Values are reported as number ± standard error of the mean. *Values in mmHg. (**c**) Hematoxylin- and eosin-stained sections (rows 1 and 3) and immunohistochemical analysis of PCNA (rows 2 and 4) of pulmonary arterioles 50 μm in diameter from the lungs of *Notch3*$^{-/-}$ and *Notch3*$^{+/+}$ mice after 4 and 6 weeks of hypoxia. Dark nuclei are PCNA positive. Results are representative sections from at least five animals per group for each time point. Scale bar = 25 μm. (**d**) Pulmonary angiograms

cleavage (and thus activation) to Notch3 ICD using this drug has been shown to effectively treat and reverse pulmonary hypertension in rodents.[34] In these experiments, mice were placed in 10% oxygen for 2 weeks and developed pulmonary hypertension. Subsequent to this, the animals were treated with a daily subcutaneous dose of DAPT from weeks 3 to 6 while they were in 10% oxygen. Verification of inhibition of Notch3 cleavage into its active form in lung tissue was done by Western blotting at weekly intervals during DAPT administration (Fig. 18.8a). Administration of DAPT reversed pulmonary hypertension induced by chronic hypoxia, as measured by RVSPs (Fig. 18.8b), muscularization and morphology of pulmonary arterioles and small pulmonary arteries (Fig. 18.8c), and pulmonary angiography (Fig. 18.8d). Sham-treated animals developed progressive pulmonary arteriolar medial thickening consistent with the usual pattern of pulmonary hypertension development in hypoxic animals, while DAPT-treated animals did not. There were minimal proliferating vascular smooth muscle cells, as measured by PCNA staining, in the pulmonary arteriolar walls of animals treated with DAPT compared to sham-treated controls (Fig. 18.8c). Mice treated with DAPT had minimal elevations in RVSPs and had regression of right ventricular hypertrophy, further indicating that they were effectively treated for pulmonary hypertension (Fig. 18.8e).

11 Linking Notch Signaling to Other Pathways Implicated in Pulmonary Hypertension

Clues regarding why Notch3 may play a role in PAH come from recent studies of its function in the setting of hypoxia and its involvement with bone morphogenetic protein (BMP) signaling. Mutations in the BMP receptor type 2 gene (*BMPR2*) have been found to be associated with the development of a familial form of human PAH.[36] However, 40% of patients with familial PAH do not harbor mutations in *BMPR2*, and most nonfamilial cases of this disease lack association with *BMPR2* mutation.[37] This suggests that alternate or convergent pathways to

Fig. 18.8 (continued) over a 1-h period, 20 animals for each group at each time point). Values are reported as number ± standard error of the mean. $*P < 0.01$ vs. placebo control for same time point. (**c**) Hematoxylin- and eosin-stained sections (rows 1 and 3) and immunohistochemical analysis of PCNA (rows 2 and 4) of pulmonary arterioles 50 μm in diameter from the lungs of mice after treatment with DAPT or placebo (days 15–42) and 6-week course of hypoxia. Dark nuclei are PCNA positive. Results are representative sections from at least five animals per group per time point. Scale bar = 25 μm. (**d**) Pulmonary angiograms using the Microfil cast technique of DAPT- and placebo-treated animals after 6 weeks of hypoxia. DAPT-treated mice have normal angiograms, while placebo-treated mice have diffuse vascular pruning consistent with PH. Scale bar = 2 mm. (**e**) Ratio of the weight of the right ventricle (RV) to that of the left ventricle plus septum (LV + S) as an index of RV hypertrophy in DAPT- ($n = 20$) vs. placebo-treated ($n = 20$) mice after 6 weeks of hypoxia. $*P < 0.01$ vs. control for same time point

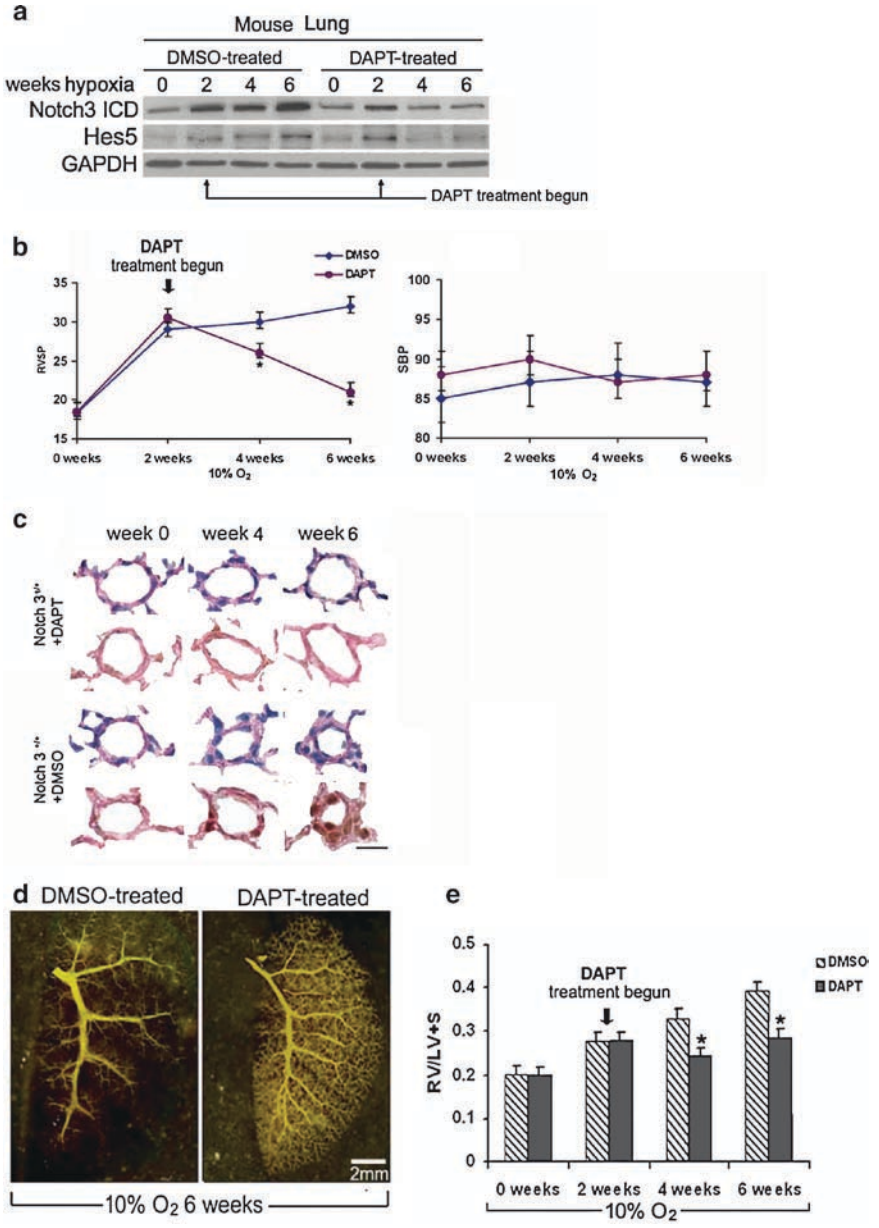


Fig. 18.8 DAPT treatment reverses the development of hypoxic pulmonary hypertension in mice. Animals were exposed to 2 weeks of 10% oxygen, followed by 4 weeks of 10% oxygen and treatment with either subcutaneous DAPT or placebo (dimethyl sulfoxide [DMSO]). (**a**) Western blot analysis of Notch3 ICD and Hes5 in the lungs of mice receiving DAPT as a function of time. DAPT blocks the conversion of full-length Notch3 to Notch3 ICD, causing a reduction in Hes5 expression. (**b**) Averaged systolic arterial pressure and right ventricular systolic pressure (RVSP) in mice at serial time points under hypoxic conditions of 10% oxygen (ten readings for each animal

BMPR signaling may play a role in this disease. Cross talk between Notch and BMP signaling has been found. Activation of BMP signaling has been found to lead to synergistic activation of the Notch target gene *Herp2* (*Hrt1*) through interactions between the intracellular BMP mediator Smad1 with Notch intracellular domain (ICD).[38–40] Smad1was found to bind with Notch ICD and activate Herp2 transcription, although direct binding of Smad1 to the Herp2 promoter was not required for this to take place. Interestingly, Herp2 was then found to efficiently bind to and induce degradation of Id1, whose transcription is induced by BMP signaling. This feedback loop demonstrates that Notch signaling modulates downstream BMPR signaling. Furthermore, functional Notch signaling has been found to be required for BMP4-mediated block of differentiation of muscle stem cells.[39] Collectively, these data suggest the possibility of signal integration between Notch and BMP pathways in modulating the proliferation/differentiation phenotype of vascular smooth muscle cells.

A second line of evidence suggesting that Notch signaling is essential to the development of PAH is its role in hypoxia, a known environmental inducer of this disease. Hypoxia is known to promote the undifferentiated cell state in various stem cell and precursor populations.[41] It has been found that hypoxia requires functional Notch signaling to maintain cells in an undifferentiated state.[42] In a mechanism that bears similarity to the cross talk between BMP and Notch, hypoxia-inducible factor 1α (HIF-1α), an intracellular mediator of oxygen sensing, has been found to interact with Notch ICD to activate Notch-responsive genes under hypoxic conditions. This protein–protein–DNA interaction does not require HIF-1α to bind DNA; rather, HIF-1α potentiates the ability of Notch to stimulate Hrt2/Hes signaling. Thus, Notch ICD is at the convergence point of two different signaling mechanisms: hypoxic HIF-1α signaling and BMP signaling, both of which have been implicated in the development of PAH.

12 Conclusions

The study of Notch signaling as it relates to smooth muscle cell growth and differentiation is in its infancy. Several key findings suggest that Notch signaling may play a role in the pathogenesis of PAH: (1) High steady-state levels of Notch3 and Hes5 are seen in the lungs of patients with PAH, (2) levels of Notch3 and Hes5 protein in the lung are predictive of the severity of pulmonary hypertension in two rodent models of the disease, (3) Notch3 signaling is requisite for the development of hypoxic pulmonary hypertension in mice, (4) Notch3 signaling induces pulmonary arteriolar smooth muscle cells into a proliferative phenotype, and (5) pulmonary hypertensive vascular pathology in vivo can be prevented by treatment with a drug that blocks Notch signaling. These results suggest that inhibition and molecular targeting of the Notch3–Hes5 axis in pulmonary vascular smooth muscle cells may be novel strategies for the treatment of PAH in the future.

References

1. Owens GK, Kumar MS, Wamhoff BR (2004) Molecular regulation of vascular smooth muscle differentiation in development and disease. Physiol Rev 84:767–801
2. Yuan JX-J, Rubin LJ (2005) Pathogenesis of pulmonary arterial hypertension: the need for multiple hits. Circulation 111:534–538
3. Hyduk A, Croft JB, Ayala C et al (2005) Pulmonary hypertension surveillance - United States, 1980–2002. 54(SS05):1–28. http://www.cic.gov.mmwr/preview/mmwrhtml/ss5405a1.htm
4. Simonneau G, Galiè N, Rubin LJ et al (2004) Clinical classification of pulmonary hypertension. J Am Coll Cardiol 43:5S–12S
5. Roca C, Adams RH (2007) Regulation of vascular morphogenesis by Notch signaling. Genes Dev 21:2511–2524
6. Bray SJ (2006) Notch signaling: a simple pathway becomes complex. Nat Rev Mol Cell Biol 7:678–689
7. Fleming RJ (1998) Structural conservation of Notch receptors and ligands. Semin Cell Dev Biol 9:599–607
8. Lubman OY, Korolev SV, Kopan R (2004) Anchoring notch genetics and biochemistry: structural analysis of the ankyrin domain sheds light on existing data. Mol Cell 13:619–626
9. Lissemore JL, Starmer WT (1999) Phylogenetic analysis of vertebrate and invertebrate Delta;Serrate/LAG-2 (DSL) proteins. Mol Phylogenet Evol 11:308–319
10. Lai EC (2004) Notch signaling: control of cell communication and cell fate. Development 131:965–973
11. High FA, Lu MM, Pear WS, Loomes KM, Kaestner KH, Epstein JA (2008) Endothelial expression of the Notch ligand Jagged1 is required for vascular smooth muscle development. Proc Natl Acad Sci U S A 105:1955–1959
12. Shutter JR, Scully S, Fan W et al (2000) Dll4, a novel Notch ligand expressed in arterial endothelium. Genes Dev 14:1313–1318
13. Gridley T (2007) Notch signaling in vascular development and physiology. Development 134:2709–2718
14. Morrow D, Scheller A, Birney YA et al (2005) Notch-mediated CBF-1/RBP-Jkappa-dependent regulation of human vascular smooth muscle cell phenotype in vitro. Am J Physiol Cell Physiol 289:C1188–C1196
15. Iso T, Kedes L, Hamamori Y (2003) HES and HERP families: multiple effectors of the Notch signaling pathway. J Cell Physiol 194:237–255
16. de la Pompa JL, Wakeham A, Correia KM et al (1997) Conservation of the Notch signaling pathway in mammalian neurogenesis. Development 124:1139–1148
17. Kopan R, Nye JS, Weintraub H (1994) The intracellular domain of mouse Notch: a constitutively activated repressor of myogenesis directed at the basic helix-loop-helix region of MyoD. Development 120:2385–2396
18. Proweller A, Pear WS, Parmacek MS (2005) Notch signaling represses myocardin-induced smooth muscle differentiation. J Biol Chem 280:8994–9004
19. Havrda MC, Johnson MJ, O'Neill CF, Liaw L (2006) A novel mechanism of transcriptional repression of $p27^{kip1}$ through Notch/HRT2 signaling in vascular smooth muscle cells. Thromb Haemost 96:361–370
20. Wang W, Prince CZ, Hu X, Pollman MJ (2003) HRT1 modulates vascular smooth muscle cell proliferation and apoptosis. Biochem Biophys Res Commun 308:596–601
21. Jin S, Hansson EM, Tikka S et al (2008) Notch signaling regulates platelet-derived growth factor receptor-β expression in vascular smooth muscle cells. Circ Res 102:1448–1450
22. Krebs LT, Xue Y, Norton CR et al (2003) Characterization of Notch3-deficient mice: normal embryonic development and absence of genetic interactions with a Notch1 mutation. Genesis 37:139–143
23. Kitamoto T, Takahashi K, Takimoto H et al (2005) Functional redundancy of the Notch gene family during mouse embryogenesis: analysis of Notch gene expression in Notch3-deficient mice. Biochem Biophys Res Commun 331:1154–1162

24. Domenga V, Fardoux P, Lacombe R et al (2004) Notch3 is required for arterial identity and maturation of vascular smooth muscle cells. Genes Dev 18:2730–2735
25. van der Loop FT, Gabbiani G, Kohnen G, Ramaekers FC, van Eys GJ (1997) Differentiation of smooth muscle cells in human blood vessels as defined by smoothelin, a novel marker for the contractile phenotype. Arterioscler Thromb Vasc Biol 17:665–671
26. Moessler H, Mericskay M, Li Z, Nagl S, Paulin D, Small JV (1996) The SM 22 promoter directs tissue-specific expression in arterial but not in venous or visceral smooth muscle cells in transgenic mice. Development 122:2415–2425
27. Villa N, Walker L, Lindsell CE, Gasson J, Iruela-Arispe ML, Weinmaster G (2001) Vascular expression of Notch pathway receptors and ligands is restricted to arterial vessels. Mech Dev 108:161–164
28. Campos AH, Wang W, Pollman MJ, Gibbons GH (2002) Determinants of Notch-3 receptor expression and signaling in vascular smooth muscle cells: implications in cell-cycle regulation. Circ Res 91:999–1006
29. Sweeney C, Morrow D, Birney YA et al (2004) Notch1 and 3 receptor signaling modulates vascular smooth muscle cell growth, apoptosis, and migration via a CBF-1/RBP-Jκ-dependent pathway. FASEB J 18:1421–1423
30. Sakata Y, Zhiping X, Yoriko C et al (2004) Transcription factor CHF1/Hey2 regulates neointimal formation in vivo and vascular smooth muscle proliferation and migration in vitro. Arterioscler Thromb Vasc Biol 24:2069–2074
31. Wang T, Baron M, Trump D (2008) An overview of Notch3 function in vascular smooth muscle cells. Prog Biophys Mol Biol 96:499–509
32. Joutel A, Andreux F, Gaulis S et al (2000) The ectodomain of the Notch3 receptor accumulates within the cerebrovasculature of CADASIL patients. J Clin Invest 105:597–605
33. Oda T, Elkahloun AG, Pike BL et al (1997) Mutations in the human Jagged1 gene are responsible for Alagille syndrome. Nat Genet 16:235–242
34. Li X, Leathers R, Makino A, Huang C, Parsa P, Macias J, Yuan JX, Jamieson SW, Thistlethwaite PA. Notch3 signaling promotes the development of pulmonary arterial hypertension. Nat Med 2009 Oct 25, epub ahead of print.
35. Hellstrom M, Phng LK, Hofmann JJ et al (2007) Dll4 signaling through Notch1 regulates formation of tip cells during angiogenesis. Nature 445:776–780
36. Deng Z, Morse JH, Slager SL et al (2000) Familial primary pulmonary hypetension (gene PPH1) is caused by mutations in the bone morphogenetic protein receptor-II gene. Am J Hum Genet 67:737–744
37. Lane KB, Machado RD, Pauciulo MW et al (2000) Heterozygous germline mutations in BMPR2, encoding TGF-β receptor, cause familial primary pulmonary hypertension. The International PPH Consortium. Nat Genet 26:81–84
38. Blokzijl A, Dahlqvist C, Reissmann E et al (2003) Cross-talk between the Notch and TGF-β signaling pathways mediated by interaction of the Notch intracellular domain with Smad3. J Cell Biol 163:723–728
39. Dahlqvist C, Blokzijl A, Chapman G et al (2003) Functional Notch signaling is required for BMP4-induced inhibition of myogenic differentiation. Development 130:6089–6099
40. Kluppel M, Wrana JL (2005) Turning it up a notch: cross-talk between TGF-β and notch signaling. Bioessays 27:115–118
41. Lin Q, Lee YJ, Yun Z (2006) Differentiation arrest by hypoxia. J Biol Chem 281:30678–30683
42. Gustafsson MV, Zheng X, Pereira T et al (2005) Hypoxia requires notch signaling to maintain the undifferentiated cell state. Dev Cell 9:617–628

Rho Kinase-Mediated Vasoconstriction in Pulmonary Hypertension

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Abstract Rho kinase-mediated vasoconstriction rather than fixed arterial wall thickening is responsible for increased pulmonary vascular resistance and pulmonary hypertension in chronically hypoxic and monocrotaline-injected rats. In the absence of vascular tone, the medial and adventitial thickening in these models has only minimal impact on the cross-sectional area of the pulmonary arterial bed. In contrast, increased pulmonary vascular resistance in left-pneumonectomized plus monocrotaline-injected rats and VEGF receptor blocker-injected plus chronic hypoxia rats is attributable to both Rho kinase-mediated vasoconstriction and formation of lumen obliterating lesions in small pulmonary arteries. The upstream signals responsible for activation of RhoA/Rho kinase signaling in hypertensive pulmonary arteries and whether or not they differ in different forms of pulmonary hypertension are unclear. The RhoA/Rho kinase pathway is a convergence point of several different vasoconstrictor signals, including those mediated by G protein-coupled receptors, receptor tyrosine kinases, and integrin clustering. Both isoforms of Rho kinase can also be constitutively activated by cleavage, and cleaved Rho kinase 1 has been detected in the hypertensive lungs of left-pneumonectomized

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plus monocrotaline-injected rats. That such diverse stimuli can lead to activation of Rho kinase, which may cause hypercontraction of smooth muscle by promoting both actomyosin interaction and remodeling of the cytoskeleton, may explain why in various rat models of pulmonary hypertension Rho kinase inhibitors are more effective pulmonary vasodilators than conventional agents such as nitric oxide, prostacyclin, and nifedipine. We suspect the same will be true in at least some forms of human pulmonary arterial hypertension.

Keywords RhoA • Rho kinase • pulmonary vasoconstriction • pulmonary hypertension • pulmonary vascular remodeling • chronic hypoxia • monocrotaline

1 Introduction

The notion of this chapter is that vasoconstriction is an important component of the pathogenesis of pulmonary hypertension in many different rodent models of the disease. This statement may seem so obvious as to be nonsensical. However, many original and review articles on experimental pulmonary hypertension continue to be published with little or no consideration of the possible contribution of vasoconstriction to the increased pulmonary vascular resistance. Most investigators in the field are focused on the role of structural remodeling of the pulmonary arteries. This is because of arterial wall thickening and, in most cases, the cellular and fibrotic luminal obliteration of distal pulmonary arteries in patients with severe pulmonary arterial hypertension.[1] Furthermore, most adult patients with symptomatic pulmonary arterial hypertension respond poorly to either acute or chronic administration of conventional vasodilators.[2,3] This is interpreted to mean the high pulmonary vascular resistance is due largely to fixed structural obstruction or vessel rarefaction rather than to active vasoconstriction. Accordingly, current thinking is that effective treatment of severe pulmonary arterial hypertension will require identification of drugs or other therapeutic strategies that can reverse structural obliteration of the pulmonary vasculature.[4] This may well be true. However, based on findings of significant RhoA/Rho kinase-mediated vasoconstriction in several different rodent models of pulmonary hypertension,[5,6] we believe possible inhibition of this vasoconstrictor mechanism should be considered whenever a new therapy is experimentally evaluated.

2 RhoA/Rho Kinase-Mediated Smooth Muscle Cell Contraction and Vasoconstriction

Our understanding of the regulation of smooth muscle cell contraction is steadily becoming more and more sophisticated.[7–11] While we once believed cytosolic Ca^{2+} concentration, myosin light chain kinase activity, myosin light chain phos-

phorylation, and actomyosin interaction were the determinants of smooth muscle cell contraction, it is now appreciated that several additional signaling pathways and cytoskeletal processes are also important in regulating the force of contraction. The total contraction and tension development of smooth muscle tissues can be due to a combination of effects. These include phosphorylation of myosin regulatory light chains and actomyosin cross-bridge cycling, formation of so-called latch bridges or other cross-links between actin and myosin filaments, actin polymerization and formation of additional actin filaments, and dynamic remodeling of the actin cytoskeleton to reinforce the connection of contractile proteins via focal adhesion complexes to the extracellular matrix. As Kim et al. emphasized, "Smooth muscle 'excitation–contraction coupling' consists of far more than a simple calcium switch."[8]

There are several intracellular signaling pathways that can contribute to the regulation of these processes and smooth muscle contractility.[7–11] It now appears, however, that activation of the small GTPase (guanosine triphosphatase) RhoA and its downstream effector Rho kinase is a major determinant of the intensity and persistence of vascular smooth muscle cell contraction and vasoconstriction in hypertensive pulmonary arteries.[5,6] In Chap. 23, Tom Resta and colleagues review the basic mechanisms of activation of RhoA/Rho kinase signaling and the evidence from their and other laboratories that the signaling pathway mediates "Ca^{2+} sensitization" of spontaneous (myogenic) and stimulus-induced constriction in hypertensive pulmonary arteries of chronically hypoxic rats. RhoA/Rho kinase-mediated Ca^{2+} sensitization is generally attributed to inhibition of myosin light chain phosphatase, increased myosin light chain phosphorylation, and increased vascular tone in face of constant or even declining levels of cytosolic Ca^{2+}. Direct measurements of vessel diameter and smooth muscle cell cytosolic [Ca^{2+}] show that Rho kinase-mediated constriction of rat hypertensive small pulmonary arteries is largely independent of Ca^{2+} signals.[12–14]

It should be noted, however, that there are instances in which RhoA/Rho kinase activation also induces Ca^{2+} signaling, and Ca^{2+} signals can activate RhoA/Rho kinase.[9,15] Thus, while considerable evidence now exists that regulating Ca^{2+} sensitivity is as important as regulating cytosolic [Ca^{2+}] in the control of vascular tone, there can be agonist- and artery segment-dependent interplay between the two pathways. Based on studies with the inhibitors Y-27632 and fasudil (HA-1077), Rho kinase activity has been found to play an important role in the acute pulmonary vasoconstrictor response to several different stimuli, including hypoxia,[16–19] superoxide anion,[13,20] KCl,[17–19,21] endothelin 1,[13,14,22] thromboxane A_2 (U-46619),[23–25] prostaglandin F2α,[19,26] serotonin,[27,28] angiotensin II,[16,17] BAY K8644,[16] isoprostanes,[24] norepinephrine or phenylephrine,[24,29,30] platelet-activating factor,[25] epoxyeicosatrienoic acids,[31] sphingosylphosphorylcholine,[32] and sphingosine-1-phosphate.[33] Whether the Rho kinase-dependent component of the vasoconstriction occurs parallel to or in series with increases in smooth muscle cell cytosolic [Ca^{2+}] seems to vary with agonist and pulmonary artery preparation.

3 RhoA/Rho Kinase-Mediated Vasoconstriction in Animal Models of Pulmonary Hypertension

In 2004, we reported the surprising observation that acute administration of the Rho kinase inhibitors Y-27632 and fasudil, but not normoxic ventilation or the L-type Ca^{2+} channel blocker nifedipine, to chronically hypoxic adult male rats and blood-perfused hypertensive lungs elicited considerable vasodilation and nearly normalized the high pulmonary vascular resistance.[18] The central role of sustained Rho kinase-mediated vasoconstriction in the pulmonary hypertension of chronically hypoxic rats was subsequently confirmed by Hyvelin et al.,[34] and McNamara et al. found that Rho kinase inhibitors, but not inhaled nitric oxide, also acutely and fully reverse chronic hypoxia-induced pulmonary hypertension in neonatal rats.[35]

These findings indicate that in the absence of active vascular tone the pulmonary arterial remodeling that occurs in chronically hypoxic rats (i.e., the medial and adventitial thickening of muscular arteries and muscularization of arterioles) causes minimal fixed reduction of lumen cross-sectional area.[36] This interpretation is supported by direct measurements of increased medial wall thickness but no decrease in lumen diameter or cross-sectional area in histological sections of hypoxic hypertensive rat lungs maximally vasodilated prior to fixation.[34,37,38] The finding of Crossno et al. that chronic treatment of hypoxic rats with rosiglitazone, an agonist of peroxisome proliferator-activated receptor γ, inhibits pulmonary arterial remodeling but not pulmonary hypertension, which was reversed acutely by intravenous fasudil, is also compatible with a minimal direct role of fixed vascular wall remodeling in increased pulmonary vascular resistance.[39] As well, substantial pulmonary arterial muscularization can apparently occur without necessarily causing pulmonary hypertension.[40,41]

In addition to chronically hypoxic rats, we have found, as shown in Fig. 19.1, that sustained Rho kinase-mediated vasoconstriction is also a major component of increased pulmonary vascular resistance and pulmonary hypertension in standard monocrotaline-injected rats,[42] in left-pneumonectomized plus monocrotaline-injected rats,[43] in rats exposed to hypoxia after a single subcutaneous injection of the vascular endothelial growth factor (VEGF) receptor blocker Sugen-5416,[44] and in fawn-hooded rats raised from birth in the mild hypoxia of Denver's altitude.[42,45] Jiang et al. also observed marked pulmonary vasodilation in response to acute oral fasudil in the monocrotaline-injected rat,[46] and just as they did in chronically hypoxic rats, van Suylen et al. found minimal inward remodeling of pulmonary arteries in monocrotaline-induced hypertensive rat lungs vasodilated before fixation.[38] In another rat model, McNamara et al. found that intraperitoneal Y-27632, but not inhaled nitric oxide, acutely normalized pulmonary vascular resistance in neonatal rats with bleomycin-induced pulmonary hypertension.[35] Rho kinase-mediated vasoconstriction is also a major component of the high pulmonary vascular resistance in fetal lambs.[47]

Although Rho kinase-mediated vasoconstriction contributes substantially to the severe pulmonary hypertension in the VEGF receptor blocker plus chronic

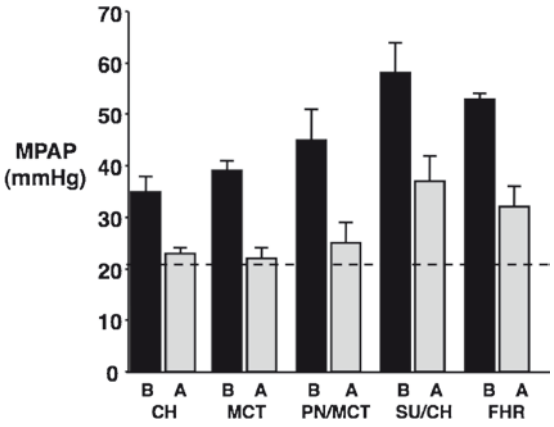

Fig. 19.1 Acute effects of fasudil (10 mg/kg, iv) on mean pulmonary artery pressure (MPAP) in five different rat models of pulmonary hypertension induced by CH, chronic hypoxia (3–4 weeks of exposure to simulated altitude of 18,000 ft); MCT, monocrotaline injection (60 mg/kg, sc); PN/MCT, left pneumonectomy plus MCT; SU/CH, Sugen 5416 injection (20 mg/kg, sc) plus 3-week exposure to chronic hypoxia; FHR, exposure of fawn-hooded rats to mild hypoxia (Denver's altitude of 5,280 ft) from birth. *Dashed line* indicates historical normal MPAP value of male Sprague–Dawley rats at Denver's altitude. Values are means ± SE of before (B) and after (A) fasudil injection (n = 3–5 per group)

hypoxia rat (Fig. 19.1), this model develops obliterative pulmonary arterial lesions in addition to increased medial muscularization, which presumably prevent the near normalization of pulmonary vascular resistance by acute Rho kinase inhibition. In fact, we have observed in these rats that the nadir to which right ventricular systolic pressure is reduced by acute Rho kinase inhibition increases with duration or severity of hypertension and density of obliterative lesions in small, distal pulmonary arteries.[44] Despite the development of severe pulmonary hypertension in Denver-raised fawn-hooded rats, we have not observed obliterative lesions in this model. The relatively higher pulmonary arterial pressure after acute fasudil in this model, as compared to chronically hypoxic and monocrotaline-injected rats, likely reflects lung dysplasia and vascular rarefaction,[45] although a contribution of Rho kinase-independent mechanisms of sustained vasoconstriction has not been ruled out.

4 Evidence of Vasoconstriction in Bone Morphogenetic Protein Type II Receptor-Related Mouse Models of Pulmonary Hypertension

There are several experimental mouse models of pulmonary hypertension in which sustained vasoconstriction, although not necessarily Rho kinase mediated, can also be implicated as an important component of increased pulmonary

vascular resistance. These include transgenic mice with a smooth muscle cell-targeted mutation of bone morphogenetic protein type II receptor (BMPR-II) [SM22-tet-BMPR2(delx4+)], which develop pulmonary hypertension that is acutely reversed by the L-type Ca^{2+} channel blocker nifedipine[48]; mice deficient in BMPR-II ($BMPR2^{+/-}$ mice), which develop more severe serotonin-induced pulmonary hypertension and whose pulmonary arteries show increased contractile responses to serotonin compared to wild-type mice[49]; mice carrying heterozygous hypomorphic Bmpr2 mutations (Bmpr2 delta Ex2/+), which develop more severe hypoxic pulmonary hypertension without an associated increase in pulmonary vascular remodeling and whose pulmonary arteries show defective endothelial-dependent dilation and enhanced KCl- and norepinephrine-induced constrictions compared to wild-type mice[50]; and $BMPR2^{+/-}$ mice challenged with two injections of monocrotaline combined with intratracheal instillation of adenovirus expressing 5-lipoxygenase in which the development of pulmonary hypertension precedes the muscularization of small pulmonary arteries.[51]

5 Evidence of RhoA/Rho Kinase-Mediated Vasoconstriction in Patients with Pulmonary Arterial Hypertension

Whether or not RhoA/Rho kinase-mediated vasoconstriction contributes significantly to the pathogenesis of one or more of the various forms of human pulmonary arterial hypertension remains to be determined. There is, however, growing evidence the signaling pathway is activated in both the smooth muscle and endothelial cells of human hypertensive pulmonary arteries,[52–55] and preliminary reports by Hemnes et al.[54] and Doe et al.[52] suggested the blunted vasodilator responsiveness of hypertensive pulmonary arteries isolated from patients undergoing lung transplantation for severe hypertension is due to high RhoA/Rho kinase activity. Although it has been reported that acute intravenous fasudil elicits only modest decreases in pulmonary vascular resistance in adult patients with pulmonary arterial hypertension,[56,57] both pulmonary arterial pressure and vascular resistance were significantly reduced by the Rho kinase inhibitor in children with pulmonary arterial hypertension associated with congenital heart disease.[58] It should be noted that to avoid systemic vasodilation the dose of fasudil used in these patient studies was much lower than that used in the rat studies.[18,35,44] The doses of Rho kinase inhibitors required to convincingly determine if Rho kinase-mediated vasoconstriction is important in human pulmonary arterial hypertension will likely cause systemic hypotension and will therefore have to be selectively targeted to the lung. We have observed in rats that the vasodilator effects of Rho kinase inhibitors are limited to the hypertensive pulmonary arteries when they are administered via inhalation.[42]

6 Summary

We and others have observed that sustained Rho kinase-mediated vasoconstriction rather than fixed arterial wall thickening is mainly responsible for the increased pulmonary vascular resistance in the two most commonly used experimental models of pulmonary hypertension: chronically hypoxic and monocrotaline-injected rats. It appears that in the absence of active vascular tone, the medial and adventitial thickening that occurs in these models does not directly reduce the cross-sectional area of the pulmonary arterial bed. This suggests to us that any treatment that prevents or reverses pulmonary hypertension in these two models is likely doing so by somehow interfering with the sustained activation of RhoA/Rho kinase signaling and resultant contraction of the pulmonary arterial smooth muscle. In this regard, we have observed that the tyrosine kinase inhibitor imatinib mesylate (Gleevec), which has been found to effectively reverse pulmonary hypertension in both monocrotaline-injected rats and chronically hypoxic mice and interpreted to do so by inhibiting smooth muscle cell proliferation,[59] is acutely a potent pulmonary vasodilator in the VEGF receptor blocker plus chronic hypoxia model (unpublished). We have not yet determined if imatinib elicits pulmonary vasodilation by inhibiting RhoA/Rho kinase signaling.

In contrast to the standard chronically hypoxic and monocrotaline-injected models, increased pulmonary vascular resistance in left-pneumonectomized plus monocrotaline-injected rats and VEGF receptor blocker plus chronic hypoxia rats is attributable to both Rho kinase-mediated vasoconstriction and the formation of lumen-obliterating lesions in small, distal pulmonary arteries. Thus, in these two models, both vasoconstriction and vascular remodeling need to be taken into account. Although it may or may not involve RhoA/Rho kinase signaling, it appears that vasoconstriction is also important in the pathogenesis of pulmonary hypertension in many BMPR-II-related mouse models of pulmonary hypertension and should be considered as a potential target of any therapeutic strategy.

Exactly what upstream signals are responsible for sustained activation of RhoA/Rho kinase signaling in hypertensive pulmonary arteries and whether they differ in different forms of pulmonary hypertension are unclear. The RhoA/Rho kinase signaling pathway is a convergence point downstream of several different vasoconstrictor signals, including those mediated by G protein-coupled receptors, receptor tyrosine kinases, and integrin clustering.[10] Both isoforms of Rho kinase (i.e., ROCK1 and ROCK2) can also be constitutively activated by cleavage of the carboxy-terminal regions of the enzymes, and we have detected cleaved ROCK1 in the hypertensive lungs of left-pneumonectomized plus monocrotaline-injected rats.[43] That such diverse stimuli can lead to activation of Rho kinase, which may cause hypercontraction of smooth muscle by promoting both actomyosin interaction and remodeling of the cytoskeleton,[60] may explain why we and others have found in various rat models of pulmonary hypertension that Rho kinase inhibitors are more effective pulmonary vasodilators than the more conventional agents such as nitric oxide, iloprost, and nifedipine.[18,35,39,44]

We suspect the same will be true in at least some forms of human pulmonary arterial hypertension.

References

1. Pietra GG, Capron F, Stewart S et al (2004) Pathologic assessment of vasculopathies in pulmonary hypertension. J Am Coll Cardiol 43:25S–32S
2. Rich S (2009) The effects of vasodilators in pulmonary hypertension. Pulmonary vascular or peripheral vascular? Circ Heart Fail 2:145–150
3. Sitbon O, Humbert M, Jais X et al (2005) Long-term response to calcium channel blockers in idiopathic pulmonary arterial hypertension. Circulation 111:3105–3111
4. Rabinovitch M (2008) Molecular pathogenesis of pulmonary arterial hypertension. J Clin Invest 118:2372–2379
5. Oka M, Fagan KA, Jones PL, McMurtry IF (2008) Therapeutic potential of RhoA/Rho kinase inhibitors in pulmonary hypertension. Br J Pharmacol 155:444–454
6. Oka M, Homma N, McMurtry IF (2008) Rho kinase-mediated vasoconstriction in rat models of pulmonary hypertension. Methods Enzymol 439:191–204
7. Gunst SJ, Zhang W (2008) Actin cytoskeletal dynamics in smooth muscle: a new paradigm for the regulation of smooth muscle contraction. Am J Physiol Cell Physiol 295:C576–C587
8. Kim HR, Appel S, Vetterkind S, Gangopadhyay SS, Morgan KG (2008) Smooth muscle signalling pathways in health and disease. J Cell Mol Med 12:2165–2180
9. Ratz PH, Berg KM, Urban NH, Miner AS (2005) Regulation of smooth muscle calcium sensitivity: KCl as a calcium-sensitizing stimulus. Am J Physiol Cell Physiol 288:C769–C783
10. Somlyo AP, Somlyo AV (2003) Ca^{2+} sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. Physiol Rev 83:1325–1358
11. Tang DD, Anfinogenova Y (2008) Physiologic properties and regulation of the actin cytoskeleton in vascular smooth muscle. J Cardiovasc Pharmacol Ther 13:130–140
12. Broughton BR, Walker BR, Resta TC (2008) Chronic hypoxia induces Rho kinase-dependent myogenic tone in small pulmonary arteries. Am J Physiol Lung Cell Mol Physiol 294:L797–L806
13. Jernigan NL, Walker BR, Resta TC (2008) Reactive oxygen species mediate RhoA/Rho kinase-induced Ca^{2+} sensitization in pulmonary vascular smooth muscle following chronic hypoxia. Am J Physiol Lung Cell Mol Physiol 295:L515–L529
14. Weigand L, Sylvester JT, Shimoda LA (2006) Mechanisms of endothelin-1-induced contraction in pulmonary arteries from chronically hypoxic rats. Am J Physiol Lung Cell Mol Physiol 290:L284–L290
15. Wang J, Weigand L, Foxson J, Shimoda LA, Sylvester JT (2007) Ca^{2+} signaling in hypoxic pulmonary vasoconstriction: effects of myosin light chain and rho kinase antagonists. Am J Physiol Lung Cell Mol Physiol 293:L674–L685
16. Badejo AM Jr, Dhaliwal JS, Casey DB, Gallen TB, Greco AJ, Kadowitz PJ (2008) Analysis of pulmonary vasodilator responses to the Rho-kinase inhibitor fasudil in the anesthetized rat. Am J Physiol Lung Cell Mol Physiol 295:L828–L836
17. Fagan KA, Oka M, Bauer NR et al (2004) Attenuation of acute hypoxic pulmonary vasoconstriction and hypoxic pulmonary hypertension in mice by inhibition of Rho-kinase. Am J Physiol Lung Cell Mol Physiol 287:L656–L664
18. Nagaoka T, Morio Y, Casanova N et al (2004) Rho/Rho kinase signaling mediates increased basal pulmonary vascular tone in chronically hypoxic rats. Am J Physiol Lung Cell Mol Physiol 287:L665–L672
19. Robertson TP, Dipp M, Ward JPT, Aaronson PI, Evans AM (2000) Inhibition of sustained hypoxic vasoconstriction by Y-27632 in isolated intrapulmonary arteries and perfused lung of the rat. Br J Pharmacol 131:5–9

20. Knock GA, Snetkov VA, Shaifta Y et al (2009) Superoxide constricts rat pulmonary arteries via Rho-kinase-mediated Ca^{2+} sensitization. Free Radic Biol Med 46:633–642
21. Homma N, Nagaoka T, Morio Y et al (2007) Endothelin-1 and serotonin are involved in activation of RhoA/Rho kinase signaling in the chronically hypoxic hypertensive rat pulmonary circulation. J Cardiovasc Pharmacol 50:697–702
22. Barman SA (2007) Vasoconstrictor effect of endothelin-1 on hypertensive pulmonary arterial smooth muscle involves Rho-kinase and protein kinase C. Am J Physiol Lung Cell Mol Physiol 293:L472–L479
23. Alapati VR, McKenzie C, Blair A, Kenny D, MacDonald A, Shaw AM (2007) Mechanisms of U46619- and 5-HT-induced contraction of bovine pulmonary arteries: role of chloride ions. Br J Pharmacol 151:1224–1234
24. Janssen LJ, Premji M, Netherton S, Coruzzi J, Lu-Chao H, Cox PG (2001) Vasoconstrictor actions of isoprostanes via tyrosine kinase and Rho kinase in human and canine pulmonary vascular smooth muscles. Br J Pharmacol 132:127–134
25. Martin C, Goggel R, Ressmeyer AR, Uhlig S (2004) Pressor responses to platelet-activating factor and thromboxane are mediated by Rho-kinase. Am J Physiol Lung Cell Mol Physiol 287:L250–L257
26. Knock GA, Shaifta Y, Snetkov VA et al (2008) Interaction between src family kinases and Rho-kinase in agonist-induced Ca^{2+}-sensitization of rat pulmonary artery. Cardiovasc Res 77:570–579
27. Rodat-Despoix L, Crevel H, Marthan R, Savineau JP, Guibert C (2008) Heterogeneity in 5-HT-induced contractile and proliferative responses in rat pulmonary arterial bed. J Vasc Res 45:181–192
28. Witzenrath M, Ahrens B, Kube SM et al (2006) Allergic lung inflammation induces pulmonary vascular hyperresponsiveness. Eur Respir J 28:370–377
29. Boer C, van der Linden PJ, Scheffer GJ, Westerhof N, de Lange JJ, Sipkema P (2002) RhoA/Rho kinase and nitric oxide modulate the agonist-induced pulmonary artery diameter response time. Am J Physiol Heart Circ Physiol 282:H990–H998
30. Damron DS, Kanaya N, Homma Y, Kim SO, Murray PA (2002) Role of PKC, tyrosine kinases, and Rho kinase in α-adrenoreceptor-mediated PASM contraction. Am J Physiol Lung Cell Mol Physiol 283:L1051–L1064
31. Losapio JL, Sprague RS, Lonigro AJ, Stephenson AH (2005) 5,6-EET-induced contraction of intralobar pulmonary arteries depends on the activation of Rho-kinase. J Appl Physiol 99:1391–1396
32. Thomas GD, Snetkov VA, Patel R, Leach RM, Aaronson PI, Ward JPT (2005) Sphingosylphosphorylcholine-induced vasoconstriction of pulmonary artery: activation of non-store-operated Ca^{2+} entry. Cardiovasc Res 68:56–64
33. Beutz MA, Nagaoka T, Oka M, McMurtry IF (2005) Sphingosine-1-phosphate constricts fawn-hooded rat pulmonary arteries. Proc Am Thorac Soc 2:A706
34. Hyvelin JM, Howell K, Nichol A, Costello CM, Preston RJ, McLoughlin P (2005) Inhibition of Rho-kinase attenuates hypoxia-induced angiogenesis in the pulmonary circulation. Circ Res 97:185–191
35. McNamara PJ, Murthy P, Kantores C et al (2008) Acute vasodilator effects of Rho-kinase inhibitors in neonatal rats with pulmonary hypertension unresponsive to nitric oxide. Am J Physiol Lung Cell Mol Physiol 294:L205–L213
36. Stenmark KR, McMurtry IF (2005) Vascular remodeling versus vasoconstriction in chronic hypoxic pulmonary hypertension: a time for reappraisal? Circ Res 97:95–98
37. Howell K, Preston RJ, McLoughlin P (2003) Chronic hypoxia causes angiogenesis in addition to remodelling in the adult rat pulmonary circulation. J Physiol 547:133–145
38. van Suylen RJ, Smits JF, Daemen MJ (1998) Pulmonary artery remodeling differs in hypoxia- and monocrotaline-induced pulmonary hypertension. Am J Respir Crit Care Med 157:1423–1428
39. Crossno JT Jr, Garat CV, Reusch JE et al (2007) Rosiglitazone attenuates hypoxia-induced pulmonary arterial remodeling. Am J Physiol Lung Cell Mol Physiol 292:L885–L897
40. Daley E, Emson C, Guignabert C et al (2008) Pulmonary arterial remodeling induced by a Th2 immune response. J Exp Med 205:361–372

41. Hopkins N, Cadogan E, Giles S, McLoughlin P (2001) Chronic airway infection leads to angiogenesis in the pulmonary circulation. J Appl Physiol 91:919–928
42. Nagaoka T, Fagan KA, Gebb SA et al (2005) Inhaled Rho kinase inhibitors are potent and selective vasodilators in rat pulmonary hypertension. Am J Respir Crit Care Med 171:494–499
43. Homma N, Nagaoka T, Karoor V et al (2008) Involvement of RhoA/Rho kinase signaling in protection against monocrotaline-induced pulmonary hypertension in pneumonectomized rats by dehydroepiandrosterone. Am J Physiol Lung Cell Mol Physiol 295:L71–L78
44. Oka M, Homma N, Taraseviciene-Stewart L et al (2007) Rho kinase-mediated vasoconstriction is important in severe occlusive pulmonary arterial hypertension in rats. Circ Res 100:923–929
45. Nagaoka T, Gebb SA, Karoor V et al (2006) Involvement of RhoA/Rho kinase signaling in pulmonary hypertension of the fawn-hooded rat. J Appl Physiol 100:996–1002
46. Jiang BH, Tawara S, Abe K, Takaki A, Fukumoto Y, Shimokawa H (2007) Acute vasodilator effect of fasudil, a Rho-kinase inhibitor, in monocrotaline-induced pulmonary hypertension in rats. J Cardiovasc Pharmacol 49:85–89
47. Parker TA, Roe G, Grover TR, Abman SH (2006) Rho kinase activation maintains high pulmonary vascular resistance in the ovine fetal lung. Am J Physiol Lung Cell Mol Physiol 291:L976–L982
48. Young KA, Ivester C, West J, Carr M, Rodman DM (2006) BMP signaling controls PASMC K_v channel expression in vitro and in vivo. Am J Physiol Lung Cell Mol Physiol 290:L841–L848
49. Long L, MacLean MR, Jeffery TK et al (2006) Serotonin increases susceptibility to pulmonary hypertension in BMPR2-deficient mice. Circ Res 98:818–827
50. Frank DB, Lowery J, Anderson L, Brink M, Reese J, de Caestecker M (2008) Increased susceptibility to hypoxic pulmonary hypertension in bmpr2 mutant mice is associated with endothelial dysfunction in the pulmonary vasculature. Am J Physiol Lung Cell Mol Physiol 294:L98–L109
51. Song Y, Coleman L, Shi J et al (2008) Inflammation, endothelial injury, and persistent pulmonary hypertension in heterozygous BMPR2-mutant mice. Am J Physiol Heart Circ Physiol 295:H677–H690
52. Doe Z, Fukumoto Y, Takaki A et al (2008) Evidence for Rho-kinase activation in patients with pulmonary hypertension. Circulation 118:S-446
53. Guilluy C, Eddahibi S, Agard C et al (2009) Rhoa and Rho kinase activation in human pulmonary hypertension - role of 5-HT signaling. Am J Respir Crit Care Med 179(12):1151–1158
54. Hemnes AR, Wigley F, Rodrigues FW, Girgis RE, Yang SC, Conte JV (2005) Pulmonary hypertension is associated with increased expression and activity of phosphodiesterase type 5a. Circulation 112:II-221–II-222
55. Laumanns IP, Fink L, Wilhelm J et al (2009) The non-canonical WNT-pathway is operative in idiopathic pulmonary arterial hypertension. Am J Respir Cell Mol Biol 40(6):683–691
56. Fukumoto Y, Matoba T, Ito A et al (2005) Acute vasodilator effects of a Rho-kinase inhibitor, fasudil, in patients with severe pulmonary hypertension. Heart 91:391–392
57. Ishikura K, Yamada N, Ito M et al (2006) Beneficial acute effects of Rho-kinase inhibitor in patients with pulmonary arterial hypertension. Circ J 70:174–178
58. Li F, Xia W, Yuan S, Sun R (2009) Acute inhibition of Rho-kinase attenuates pulmonary hypertension in patients with congenital heart disease. Pediatr Cardiol 30(3):363–366
59. Schermuly RT, Dony E, Ghofrani HA et al (2005) Reversal of experimental pulmonary hypertension by PDGF inhibition. J Clin Invest 115:2811–2821
60. Gokina NI, Park KM, McElroy-Yaggy K, Osol G (2005) Effects of Rho kinase inhibition on cerebral artery myogenic tone and reactivity. J Appl Physiol 98:1940–1948

The Serotonin Hypothesis of Pulmonary Hypertension Revisited

Margaret R. MacLean and Yvonne Dempsie

Abstract The serotonin hypothesis of pulmonary arterial hypertension (PAH) arose after an outbreak of PAH in patients taking the anorexigenic drugs aminorex and dexfenfluramine. Both of these drugs are serotonin transporter (SERT) substrates and indirect serotinergic agonists. There is now a wealth of evidence to support a role for serotonin in the pathobiology of PAH. Synthesis of serotonin can occur in pulmonary artery endothelial cells by the enzyme tryptophan hydroxylase 1 (TPH1). Serotonin then acts at the 5-HT_{1B} receptor and the SERT to mediate constriction and proliferation of pulmonary artery smooth muscle cells. Downstream signalling molecules which play a role in serotonin-induced constriction and proliferation include reactive oxygen species (ROS), Rho-kinase (ROCK) p38 and extracellular signal-regulated kinase (ERK). There is also evidence to suggest that serotonin may interact with the bone morphogenetic receptor type II (BMPRII) to provide a 'second hit' risk factor for PAH.

Keywords pulmonary arterial hypertension • serotonin • tryptophan hydroxylase • serotonin transporter • serotonin receptors • dexfenfluramine • bone morphogenetic protein

1 Pulmonary Arterial Hypertension

Pulmonary arterial hypertension (PAH) is a condition in which pulmonary vascular pressure rises from 12–16 mmHg to >25 mmHg at rest and more than 30 mmHg at exercise. This increase in pulmonary vascular pressure is due to both constriction

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and remodelling of the pulmonary vasculature. The increased pulmonary vascular pressure eventually leads to right heart failure and death.

PAH was reclassified at the Third World Symposium on Pulmonary Hypertension in 2003 according to the clinical diagnosis,[1] with some minor changes being made at the Fourth World Symposium on Pulmonary Hypertension in 2008. Category I PAH includes idiopathic PAH (IPAH), familial PAH (FPAH), and acquired PAH (APAH). IPAH is a rare form of the disease, with an incidence of around two or three per million, for which there is no known cause. FPAH is rarer still, with an incidence of around 0.2–0.3 per million, and transmits as an autosomal dominant trait that exhibits genetic anticipation. Mutations in the bone morphogenetic protein receptor II (BMPR-II) gene, a member of the transforming growth factor β (TGF-β) superfamily, have been identified in over 75% of patients with FPAH and between 10 and 40% of patients with IPAH. APAH can arise in association with collagen vascular disease, pulmonary arterial shunts, portal hypertension, HIV infection, drugs (such as the indirect serotonergic agonists aminorex and dexfenfluramine [Dfen]), toxins, and other conditions that include thyroid disorders, hemoglobinopathies, and hereditary hemorrhagic telangiectasia. Category II PAH is left-sided heart disease resulting from valvular disorders or myocardial dysfunction. Category III is associated with lung disease or hypoxemia. Category IV is associated with chronic thrombotic or embolic disease. Finally, category V includes a grouping of miscellaneous disorders that are more rarely associated with PAH, including mediastinitis and sarcoid disease.

1.1 Serotonin and the Serotonin Hypothesis

Normally, plasma 5-hydroxytryptamine (5-HT) levels are extremely low (i.e. <1 n*M*) as more than 99% of 5-HT in the blood is stored in platelets via the serotonin 5-HT transporter (SERT). In addition, there is rapid metabolism of 5-HT to 5-hydroxyindoleacetic acid (5-HIAA) by monoamine oxidase. In the 1960s, there was an 'epidemic' of cases of PAH in women taking the indirect serotonergic agonist aminorex. In the 1980s, the new generation of anorexigens, the fenfluramines were also found to be associated with PAH. Aminorex and fenfluramine are substrates for the SERT, and at one time it was believed that they caused PAH by evoking the release of serotonin by acting as SERT substrates and reversing the normal direction of serotonin flux.[2] Once inside the cell, these substrates may also compete with monoamines for vesicular sequestration via the vesicular monoamine transporter (VMAT) and by subsequent disruption of vesicular monoamine storage (Fig. 20.1). Often, the fenfluramines were co-administered with phentermine (the 'Fen-Phen' combination). As phentermine is also a SERT substrate[2] and inhibits monoamine oxidase, it was believed that this led to further accumulation of plasma serotonin.[3] These observations formed the basis of the 'serotonin hypothesis of anorexigen-induced pulmonary hypertension'.

It had been hypothesised that such anorexigen-induced elevations in circulating serotonin, secondary to release from platelets, might be involved in anorexigen-induced PAH. Consistent with this, in the 1990s there were reports of elevated

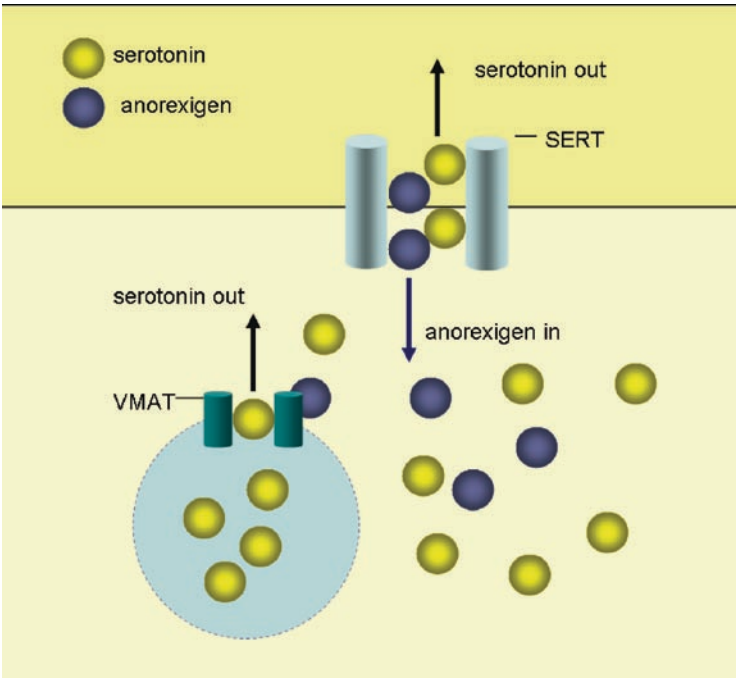

Fig. 20.1 The original serotonin hypothesis of anorexigen-induced pulmonary arterial hypertension. Anorexigens such as aminorex, fenfluramine and chlorphentermine are serotonin transporter (SERT) substrates. They may evoke the release of serotonin by acting as SERT substrates and reversing the normal direction of serotonin flux. Once inside the cell, these drugs may also compete with monoamines for vesicular sequestration via the vesicular monoamine transporter (VMAT) and by subsequent disruption of vesicular monoamine storage

plasma levels in patients with PAH as well as PAH occurring in patients with platelet pool storage pool disease.[4,5] However, fenfluramine can actually lower blood 5-HT and may only slightly elevate plasma serotonin levels to nontoxic levels.[6,7] Alternative, non-serotonergic explanations have been proposed. For example, it has been suggested that the SERT serves as a 'gateway' for the accumulation and concentration of drugs in pulmonary cells.[2] Further, Dfen has been reported to have non-serotonergic and direct effects on pulmonary arteries, including inhibition of potassium channels, increased intracellular calcium, vasoconstriction (albeit with very low potency), and mitogenic effects.[8–11] In addition, the Dfen metabolite nor-Dfen is an agonist at 5-HT_{2A} and 5-HT_{2B} receptors.[12]

There is much evidence, however, that serotonin per se may facilitate FPAH and IPAH via alterations in various components of the serotonin system. Here, we reconsider and redefine the serotonin hypothesis of pulmonary hypertension in light of such recent advances in the field.

2 The Serotonin System in the Pulmonary Arterial Circulation

There are four main components of the serotonin system in the pulmonary arterial bed: the serotonin receptor, the serotonin transporter, the synthesis of serotonin via tryptophan hydroxylase (Tph) 1 and downstream signalling pathways that are activated following SERT or 5-HT receptor activation.

2.1 5-HT Receptors

There are 14 different, structurally distinct 5-HT receptors, which are divided up into seven families (5-$HT_{1–7}$[13)]. It is the 5-HT_{2A} receptor that mediates vasoconstriction in most systemic arteries; prior to 1993, it had been assumed that it was the 5-HT_{2A} that mediated pulmonary arterial vasoconstriction. The 5-HT_{2A} antagonist ketanserin has proved clinically effective in the treatment of systemic hypertension, especially in the elderly.[14] However, in studies on small cohorts of PAH patients, ketanserin at high doses had only a very small effect on pulmonary vascular resistance compared to its effect on systemic vascular resistance and often had no effect on pulmonary pressures.[15] Hence, there is no specificity for the pulmonary circulation, and the systemic effects have limited its use in PAH, for which it fails to improve pulmonary haemodynamics significantly.[14] These studies seemed to contradict the serotonin hypothesis. However, the red herring in these studies was the assumption that it is the 5-HT_{2A} receptor that mediates the pulmonary effects of serotonin in humans. Evidence now suggests that it is a 5-HT_{1} receptor that mediates responses to serotonin in the human pulmonary artery. In 1993, MacIntyre et al. conducted an assessment of the vasoactive effects of the anti-migraine drug sumatriptan (a 5-$HT_{1B/D}$ agonist) on the systemic and pulmonary circulations and the coronary artery vasculature. They discovered that sumatriptan had a profound effect on pulmonary pressures and resistance.[16] Further study of isolated human small and large pulmonary arteries identified the 5-HT_{1B} receptor as that mediating serotonin-induced vasoconstriction.[17,18] An increased expression of the 5-HT_{1B} receptor has subsequently been demonstrated in a small cohort of PAH patients,[19] and many factors present in PAH (increased vascular tone, decreased nitric oxide synthase) can potentiate responses to 5-HT_{1B} agonists in a synergistic fashion.[20] Subsequent studies in experimental animals have shown that, in mice and rats, respectively, if the 5-HT_{1B} receptor is knocked out or the animal is treated with a 5-HT_{1B} antagonist, there is a reduction in the development of hypoxia-induced PAH.[21] In chronic over-circulation-induced PAH in growing piglets, there is also an increased expression of the 5-HT_{1B} receptor.[22] It has also recently been shown that the 5-HT_{1B} receptor can mediate proliferation in human pulmonary arterial smooth muscle cells (PASMCs).[23]

Curiously, Raynaud's phenomenon and PAH have been associated, for example, in systemic lupus erythematosus.[24] The 5-HT_{1B} receptor mediates constriction in digital arteries, and the 5-HT_{1B} gene has been suggested to be involved in the

susceptibility to primary Raynaud's phenomenon.[25,26] In addition, the use of anti-migraine 5-HT_{1B} agonists has been associated with Raynaud's phenomenon and lupus.[27] This is another curious indirect potential association of 5-HT_{1B} receptors with PAH that is worthy of consideration and perhaps future research.

5-HT_2 receptors may influence pulmonary arterial vasoconstriction and proliferation, at least in animal models. For example, in rat PASMCs, the 5-HT_{2A} receptor inhibits native K–V and $hK_v1.5$ currents, and this may contribute to vasoconstriction.[28] In rat pulmonary arterial fibroblasts, the 5-HT_{2A} receptor also mediates hypoxia-associated 5-HT proliferation.[29] The development of hypoxia-induced PAH in mice is ablated in $5HT_{2B}$ receptor knockout mice,[19] and this receptor may control serotonin plasma levels in mice.[30] However, paradoxically, loss of serotonin $5HT_{2B}$ receptor function may predispose to fenfluramine-associated PAH in humans.[31]

2.2 The Serotonin Transporter

The SERT belongs to the Na^+/Cl^- family of transporters, which also includes the noradrenaline, dopamine, γ-aminobutyric acid (GABA) and glycine transporters. These operate by using Na^+ influx down a concentration gradient as the driving force for the transport of proteins into the cell. Serotonin, Na^+, and Cl^- bind simultaneously to a SERT-binding site on the exterior of the cell, triggering a conformational change that transports these molecules to the cytoplasmic surface of the membrane. Serotonin, Na^+ and Cl^- then dissociate from the SERT, and K^+ binds to the same binding site to drive a conformational change in the SERT back to its original form.[32] The SERT can also act in reverse, transporting serotonin out of the cell. SERT substrates and indirect serotonergic agonists are taken into the cytoplasm via the SERT in exchange for serotonin, which is pumped out of the cell. Once inside the cytoplasm, SERT substrates can also disrupt the storage of serotonin in vesicles and thus increase cytoplasmic concentrations of serotonin available for release[12] (Fig. 20.1).

The SERT is particularly highly expressed in the lung and placenta. A single gene encodes the SERT, which is located on chromosome 17q11.2. The SERT expression and function is controlled by a repetitive element of varying length in the promoter region of the gene. Alleles are commonly composed of either 14 (short) or 16 (long) repeated elements. The long (L) allele induces a twofold to threefold higher rate of SERT gene transcription than the short (S) allele and is associated with increased SERT messenger RNA (mRNA) expression, protein expression and uptake activity.

The 'LL' variant of the SERT was found to be more prevalent in a small group of IPAH patients than controls, an observation that focused attention on the role of the SERT in the pathobiology of PAH.[33] The LL genotype has also been associated with exaggerated PAH in patients with chronic obstructive lung disease,[34] with an increased risk of developing PAH at high altitudes[35] and with an increased pulmonary arterial pressure in patients suffering from heart failure.[36] Studies in larger cohorts of PAH patients, however, found no variation in the prevalence of the LL

allele of the SERT gene between controls and patients with IPAH or FPAH.[37,38] Patients with FPAH who have the LL variant of the SERT may, however, present earlier than those without.[38]

Experimentally, there is evidence for a role for SERT in the development of PAH. Mice deficient for the SERT are less susceptible to hypoxia-induced PAH and develop less hypoxia-induced pulmonary vascular remodelling and right ventricular hypertrophy than wild-type mice.[39] Mice ubiquitously over-expressing the SERT ($SERT^+$ mice) exhibit elevated pulmonary pressures and exaggerated hypoxia-induced PAH, which is associated with elevated hypoxia-induced pulmonary vascular remodelling and right ventricular hypertrophy.[40] Mice that selectively over-express the SERT in smooth muscle cells also exhibit a similar phenotype.[41] Fawn-hooded rats, which have an inherited platelet storage defect and increased expression of the SERT, have increased susceptibility to PAH secondary to hypoxia.[42,43] The SERT inhibitors citalopram and fluoxetine both protect against PAH secondary to hypoxia in mice,[44] and fluoxetine also protects against monocrotaline-induced PAH in rats.[45] Monocrotaline-induced PAH in rats is associated with an up-regulation of the SERT, and the protective effect of statins on monocrotaline-induced PAH is associated with a down-regulation of the SERT.[46]

2.2.1 SERT and Pulmonary Vascular Remodelling and Vasoconstriction

Serum- and serotonin-induced proliferation is elevated in PASMCs derived from IPAH and secondary PAH patients. These cells also have increased expression of the SERT compared to those from controls, and the proliferative effects of serotonin are abolished by SERT inhibitors.[47] The SERT has also been shown to be involved in proliferation of human, bovine and rodent PASMCs and pulmonary arterial fibroblasts.[23,29,48,49] There is also evidence that SERT activity can co-operate with the $5\text{-}HT_{1B}$ receptor in the modulation of both vasoconstriction and proliferation.[23,42] In fawn-hooded rat pulmonary resistance arteries (where the SERT is over-expressed), SERT inhibitors potentiate the contractile responses to serotonin. Consistent with this, in pulmonary resistance arteries from $SERT^+$ mice, citalopram increases serotonin-induced contraction.[42,50] This may be due to increased extracellular concentrations of serotonin available to activate serotonin receptors. We have therefore hypothesised that dual inhibition of the SERT and the $5\text{-}HT_{1B}$ receptor is required to maximally inhibit serotonin-induced pulmonary vascular contraction. Indeed, whilst the $5\text{-}HT_{1B}$ receptor antagonist SB224289 and the SERT inhibitor fluoxetine both inhibit serotonin-induced contractile response in pulmonary resistance arteries from normoxic and chronically hypoxic Sprague–Dawley rats, there is synergy between the effects of these when given simultaneously. Moreover, the combined $5\text{-}HT_{1B}$ receptor/SERT antagonist LY393558 is the most potent inhibitor of serotonin-induced constriction,[42] and is more effective than SERT inhibition alone at preventing and reversing PAH secondary to both hypoxia and SERT over-expression[68]. Therapeutically, therefore, we propose that the optimum therapeutic strategy would be to inhibit both the serotonin transporter and the $5\text{-}HT_{1B}$ receptor.

2.3 Serotonin Synthesis

Tryptophan hydroxylase catalyses the rate-limiting step in the synthesis of serotonin from tryptophan. By studying *Tph1–/–* mice, Walther et al. demonstrated that there are two isoforms of Tph, now classified as Tph1 and Tph2.[51] Tph2 is present exclusively in the brain but not the periphery. The classical Tph gene, now termed Tph1, is mainly expressed in the gut and mediates the generation of serotonin in the periphery.[51] It has been shown that expression of the *Tph1* gene is increased in lungs and pulmonary endothelial cells from patients with IPAH.[52] Hypoxia-induced PAH is ablated in *Tph1*$^{-/-}$ mice devoid of peripheral serotonin synthesis.[53] Dfen-induced PAH is also ablated in *Tph1*$^{-/-}$ mice.[50] We have also shown that in normoxic mice there is little evidence for expression of Tph1 in pulmonary arterial endothelial cells. However, after 2 weeks of hypoxia, there is substantial Tph1 expression, suggesting that hypoxia induces Tph1 expression and de novo synthesis of serotonin (Fig. 20.2). This is consistent with the observation that serotonin is overproduced in lungs and endothelial cells from patients with IPAH.[52] Thus, endothelium-derived serotonin may act on underlying PASMCs in a paracrine fashion (Fig. 20.3).

2.4 Serotonin-Induced Signalling in Pulmonary Arteries

Signal transduction initiated by 5-HT involves SERT-dependent generation of reactive oxygen species (ROS) and activation of the extra-cellular signal regulated kinase (ERK) pathway in PASMCs and fibroblasts from many species, including human.[23,48,54] The down-stream transcriptional factor GATA-4 mediates 5-HT-induced growth of PASMCs.[55] The mechanism by which serotonin internalisation via SERT activates

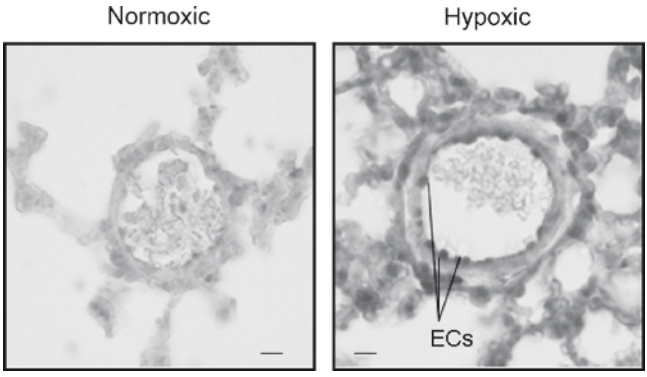

Fig. 20.2 Tryptophan hydroxylase 1 (Tph1) expression in small pulmonary arteries in control mice (normoxic) and in mice following 2 weeks of chronic hypoxia (hypoxic). There is no evidence for Tph1 in normoxic mouse pulmonary artery but dense Tph1 staining is evident in the endothelial cells (ECs) of remodelled pulmonary arteries removed from hypoxic mice. Scale bar = 10 μm

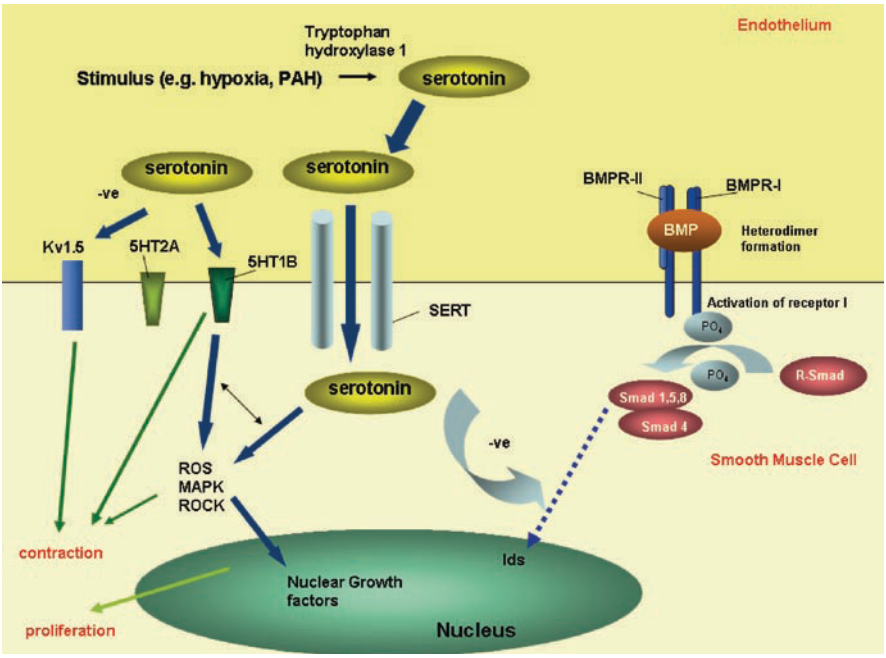

Fig. 20.3 The serotonin hypothesis of IPAH and FPAH. See text for details. Serotonin synthesis is increased in pulmonary arterial endothelial cells and acts in a paracrine fashion on underlying PASMCs. Serotonin can enter the PASMCs via the serotonin transporter (SERT), and signal transduction is initiated by SERT-dependent generation of reactive oxygen species (ROS), Rho kinase (ROCK) and mitogen-activated protein kinases (MAPKs). This may contribute to contraction or, via nuclear translocation of pERK1/2, increase expression of nuclear growth factors such as GATA 4, leading to proliferation. Serotonin may also stimulate the 5-HT receptors, and in the human this is likely to be the 5-HT_{1B} receptor. This contributes to ROS, ROCK and MAPK activation and may operate with SERT activation in a co-operative fashion to facilitate contraction and proliferation. Serotonin may also inhibit K_v1.5 channels, which would contribute to vasoconstriction. Signalling by wild-type BMPR-II involves heterodimerization with the transmembrane serine/threonine kinases type I BMPR-IA and -1B receptors at the cell membrane. On ligand binding, the constitutively active BMPR-II phosphorylates the type I receptor. Activated type I receptors phosphorylate the cytoplasmic signalling proteins known as receptor-mediated Smads (R-Smads) -1, -5 and -8. These complex with Smad4 and translocate to the nucleus, where they activate downstream target genes such as the inhibitors of DNA binding 3 (Ids), which inhibit proliferation. Serotonin may antagonise the anti-proliferative BMPR-II/Smad1, -5, -8 pathway, inhibit Id3 activation and facilitate proliferation

ROS may be species specific. In bovine PASMCs, serotonin induces ROS through activation of NADPH (nicotinamide adenine dinucleotide phosphate) oxidase.[56,57] However, studies on human PASMCs provide evidence that ROS is produced via the breakdown of serotonin by monoamine oxidase.[23]

The small G protein RhoA and its downstream effector Rho kinase (ROCK) play a central role in multiple cellular functions, including proliferation and migration, smooth muscle contraction and cytoskeletal rearrangement. Activation of the RhoA/ROCK pathway by internalised serotonin may play a role in SERT-mediated

proliferation. We have shown that in mice, SERT over-expression increases ROCK-dependent pulmonary remodelling, and SERT over-expression is associated with elevated ROCK1/2 levels. ROCK inhibition also ablates the increased pulmonary arterial remodelling and hypertension observed in SERT+ mice.[58] In platelets and aortic smooth muscle cells, internalised serotonin is transamidated to small guanosine triphosphatases (GTPases) such as Rho A by transglutaminases, rendering these GTPases constitutively active.[59,60] Interestingly, serotonin was found to be transamidated to RhoA in pulmonary but not systemic arteries from rats exposed to hypoxia, and this effect was blocked by the SERT inhibitor fluoxetine.[59] This may provide a further mechanism by which serotonin can activate ROCK.

Both ROS and ROCK are involved in the activation of ERK1/2, although the precise mechanism for this may be dependent on cell type or species. Studies in human PASMCs showed phosphorylation of ERK1/2 to occur via activation of the 5-HT_{1B} receptor, with ROS responsible for nuclear translocation of phosphorylated ERK1/2.[23] In bovine PASMCs, ROS mediates phosphorylation of ERK1/2, while activation of ROCK via the 5-HT_{1B} receptor mediates nuclear translocation of phosphorylated ERK1/2.[49] In Chinese hamster lung fibroblasts, ROCK has been shown to function downstream of the SERT in allowing efficient 5-HT_{1B} receptor-stimulated mitogen-activated protein kinase (MEK) phosphorylation of ERK1/2.[58] Nuclear translocation of phosphorylated ERK1/2 results in DNA binding of transcription factors such as GATA-4, cyclin-D1, early growth response factor 1 (Egr-1) and Ets like-1 (Elk-1) and thus increased expression of proteins that are involved in cellular proliferation.[49] One such protein is S100A4/Mts1, a calcium-binding protein that is involved in proliferation of human PASMCs.[23]

Hypoxia is a stimulus for the proliferation pulmonary arterial fibroblasts in some species, and we demonstrated that this involves the SERT, the 5-HT_{2A} receptor and the p38 mitogen-activated protein (MAP) kinase pathway. Pulmonary arterial fibroblasts derived from SERT+ mice, unlike those derived from wild-type mice, proliferate in response to hypoxia in the absence of any growth factors in the media. This effect is not inhibited by citalopram but rather by antagonism of the 5-HT_{2A} receptor.[50] Hypoxia-induced proliferation of human, bovine, rat and SERT+ mouse pulmonary arterial fibroblasts is associated with an increase in phosphorylated p38 MAP kinase and can be ablated by inhibition of p38 MAP kinase.[29,50,61,62]

3 Dexfenfluramine-Induced PAH

As described, the original ‘serotonin hypothesis of PAH’ was derived from the observation that appetite suppressants such as aminorex and Dfen were associated with an increased risk of developing PAH. However, the serotonin hypothesis of anorexigen-induced PAH has been controversial. Dfen has been reported to have non-serotonergic and direct effects on pulmonary arteries, including inhibition of potassium channels, increased intracellular calcium, vasoconstriction (albeit with very low potency), and mitogenic effects.[8–11] In addition, the Dfen metabolite nor-Dfen

is an agonist at 5-HT_{2A} and 5-HT_{2B} receptors.[12] Moreover, Dfen can actually protect against both hypoxia- and monocrotaline-induced PAH.[50,63,64] However, our recent observation that mice deficient in Tph1 are protected against increased pulmonary pressures and increased pulmonary vascular remodelling in response to Dfen lends weight to the serotonin hypothesis.[50] With respect to the protective effects of Dfen on hypoxia-induced PAH, we have provided evidence that this may be due to the inhibition of p38 MAP kinase. Dfen inhibited hypoxia-induced increases in right ventricular pressure and pulmonary vascular remodelling in $SERT^+$ mice. In pulmonary arterial fibroblasts derived from $SERT^+$ mice, Dfen inhibited both hypoxia-induced proliferation and hypoxia-induced phosphorylation of p38 MAP kinase.[50] As p38 MAP kinase is essential for hypoxia-induced proliferation of pulmonary arterial fibroblasts from various species, including humans, inhibition of p38 MAP kinase may provide the mechanism by which Dfen protects against hypoxia-induced pulmonary vascular remodelling and therefore hypoxia-induced PAH.

4 SERT and BMPR-II

Haploinsufficiency of the BMPR-II receptor is common in patients with FPAH. In the lung, the BMPR-II receptor is expressed on pulmonary vascular endothelial, smooth muscle and fibroblast cells. BMPR-II receptors can inhibit proliferation of pulmonary vascular cells via signalling through the Smad1, -5, -8 pathway. Smad1, -5 and -8 must dimerize with Smad4 to enter the nucleus and regulate transcription of target genes such as the inhibitor of DNA binding 3.[65] Consistent with this, BMP4, a ligand for the BMPR-II receptor, has a reduced ability to suppress proliferation in PASMCs from patients with BMPR-II mutations, and these cells are also deficient in Smad signalling.[66] However, disease penetrance in BMPR-II mutation carriers is low, and it is likely that an additional risk factor is required to mediate development of PAH. Serotonin infusion in vivo has been shown to uncover a PAH phenotype in $BMPR\text{-}II^{+/-}$ mice, and this was associated with an inhibition of phosphorylation of Smad1/5.[67] Expression of the inhibitor of DNA binding 3 mRNA in response to BMP2 was lower in $BMPR\text{-}II^{+/-}$ mice than their wild-type controls, and this was inhibited by serotonin.[67] Therefore, it would appear that serotonin can antagonise the anti-proliferative BMPR-II/Smad 1/5 pathway. This suggests that serotonin may be the 'second hit' influence required to uncover a PAH phenotype in face of BMPR-II haploinsufficiency.

5 The Serotonin Hypothesis Revisited

The influence of serotonin, as discussed here, is summarised in Fig. 20.3. The original serotonin hypothesis was based on a role for serotonin in anorexigen-induced PAH. Clearly, there is now substantial evidence that serotonin influences pulmonary vas-

cular remodelling processes and pulmonary arterial vasoconstriction at many levels, and serotonin may play a facilitating role in different forms of PAH. This may be via intracellular cross talk, with key pathways believed to play a pivotal role in PAH, including the BMPR-II system, K_v channels, ROCK, ROS, MAP kinases and transcription factors. As SERT/5-HT_{1B}-mediated proliferation and vasoconstriction are pulmonary specific, these offer new pulmonary-specific therapeutic targets for PAH. In addition, targeting Tph1 specifically may be a novel therapeutic approach.

References

1. Simonneau G, Galiè N, Rubin LJ et al (2004) Clinical classification of pulmonary hypertension. J Am Coll Cardiol 43:5S–12S
2. Rothman RB, Ayestas MA, Dersch CM, Baumann MH (1999) Aminorex, fenfluramine, and chlorphentermine are serotonin transporter substrates - implications for primary pulmonary hypertension. Circulation 100:869–875
3. Ulus IH, Maher TJ, Wurtman RJ (2000) Characterization of phentermine and related compounds as monoamine oxidase (MAO) inhibitors. Biochem Pharmacol 59:1611–1621
4. Hervé P, Launay JM, Scrobohaci ML et al (1995) Increased plasma serotonin in primary pulmonary hypertension. Am J Med 99:249–254
5. Herve P, Drouet L, Dosquet C et al (1990) Primary pulmonary hypertension in a patient with a familial platelet storage pool disease: role of serotonin. Am J Med 89:117–120
6. Martin F, Artigas F (1992) Simultaneous effects of para-chloroamphetamine, D-fenfluramine, and reserpine on free and stored 5-hydroxytryptamine in brain and blood. J Neurochem 59:1138–1144
7. Zolkowska D, Baumann MH, Rothman RB (2008) Chronic fenfluramine administration increases plasma serotonin (5-hydroxytryptamine) to nontoxic levels. J Pharmacol Exp Ther 324:791–797
8. Lee SL, Wang WW, Fanburg BL (2001) Dexfenfluramine as a mitogen signal via the formation of superoxide anion. FASEB J 15:1324–1325
9. Patnaude LA, Undem BJ, O'Rourke ST (2000) Dexfenfluramine-induced contraction of human and rat isolated pulmonary arteries. Eur J Pharmacol 401:229–234
10. Reeve HL, Archer SL, Soper M, Weir EK (1999) Dexfenfluramine increases pulmonary smooth muscle intracellular Ca^{2+} independent of membrane potential. Am J Physiol Lung Cell Mol Physiol 277:L662–L666
11. Weir EK, Reeve HL, Huang JMC, Michelakis E, Nelson DP, Hampl V et al (1996) Anorexic agents aminorex, fenfluramine, and dexfenfluramine inhibit potassium current in rat pulmonary vascular smooth muscle and cause pulmonary vasoconstriction. Circulation 94:2216–2220
12. Rothman RB, Baumann MH (2002) Therapeutic and adverse actions of serotonin transporter substrates. Pharmacol Ther 95:73–88
13. Alexander SPH, Mathie A, Peters JA (2005) Guide to receptors and channels, 1st edition (2005 revision). Br J Pharmacol 144:S1–S128
14. Frishman WH, Huberfeld S, Okin S, Wang YH, Kumar A, Shareef B (1995) Serotonin and serotonin antagonism in cardiovascular and non-cardiovascular disease. J Clin Pharmacol 35:541–572
15. McGoon MD, Vlietstra RE (1987) Acute hemodynamic response to the S2-serotonergic receptor antagonist, ketanserin, in patients with primary pulmonary hypertension. Int J Cardiol 14:303–309
16. MacIntyre PD, Bhargava B, Hogg KJ, Gemmill JD, Hillis WS (1993) Effect of subcutaneous sumatriptan, a selective 5Ht1 agonist, on the systemic, pulmonary, and coronary circulation. Circulation 87:401–405

17. MacLean MR, Clayton RA, Templeton AGB, Morecroft I (1996) Evidence for 5-HT1-like receptor-mediated vasoconstriction in human pulmonary artery. Br J Pharmacol 119:277–282
18. Morecroft I, Heeley RP, Prentice HM, Kirk A (1999) MacLean MR. 5-hydroxytryptamine receptors mediating contraction in human small muscular pulmonary arteries: importance of the 5-HT_{1B} receptor. Br J Pharmacol 128:730–734
19. Launay JM, Herve P, Peoc'h K et al (2002) Function of the serotonin 5-hydroxytryptamine 2B receptor in pulmonary hypertension. Nat Med 8:1129–1135
20. MacLean MR (1999) Pulmonary hypertension, anorexigens and 5-HT: pharmacological synergism in action? Trends Pharmacol Sci 20:490–495
21. Keegan A, Morecroft I, Smillie D, Hicks MN, MacLean MR (2001) Contribution of the 5-HT_{1B} receptor to hypoxia-induced pulmonary hypertension – converging evidence using 5-HT_{1B}-receptor knockout mice and the 5-$HT_{1B/1D}$-receptor antagonist GR127935. Circ Res 89:1231–1239
22. Rondelet B, Van Beneden R, Kerbaul F et al (2003) Expression of the serotonin 1b receptor in experimental pulmonary hypertension. Eur Respir J 22:408–412
23. Lawrie A, Spiekerkoetter E, Martinez EC et al (2005) Interdependent serotonin transporter and receptor pathways regulate S100A4/Mts1, a gene associated with pulmonary vascular disease. Circ Res 97:227–235
24. Kasparian A, Floros A, Gialafos E et al (2007) Raynaud's phenomenon is correlated with elevated systolic pulmonary arterial pressure in patients with systemic lupus erythematosus. Lupus 16:505–508
25. Bailey SR, Elliott J (1998) Evidence for different 5-$HT_{1B/1D}$ receptors mediating vasoconstriction of equine digital arteries and veins. Eur J Pharmacol 355:175–187
26. Susol E, MacGregor AJ, Barrett JH et al (2000) A two-stage, genome-wide screen for susceptibility loci in primary Raynaud's phenomenon. Arthritis Rheum 43:1641–1646
27. Bernatsky S, Pineau CA, Lee JL, Clarke AE (2006) Headache, Raynaud's syndrome and serotonin receptor agonists in systemic lupus erythematosus. Lupus 15:671–674
28. Cogolludo A, Moreno L, Lodi F et al (2006) Serotonin inhibits voltage-gated K^+ currents in pulmonary artery smooth muscle cells – role of 5-HT_{2A} receptors, caveolin-1, and K_V1.5 channel internalization. Circ Res 98:931–938
29. Welsh DJ, Harnett M, Maclean M, Peacock AJ (2004) Proliferation and signaling in fibroblasts – role of 5-hydroxytryptamine$_{2A}$ receptor and transporter. Am J Respir Crit Care Med 170:252–259
30. Callebert J, Esteve JM, Hervé P et al (2006) Evidence for a control of plasma serotonin levels by 5-hydroxytryptamine$_{2B}$ receptors in mice. J Pharmacol Exp Ther 317:724–731
31. Blanpain C, Le Poul E, Parma J, Knoop C, Detheux M, Parmentier M et al (2003) Serotonin 5-HT_{2B} receptor loss of function mutation in a patient with fenfluramine-associated primary pulmonary hypertension. Cardiovasc Res 60:518–528
32. Torres GE, Gainetdinov RR, Caron MG (2003) Plasma membrane monoamine transporters: structure, regulation and function. Nat Rev Neurosci 4:13–25
33. Eddahibi S, Humbert M, Fadel E et al (2001) Serotonin transporter overexpression is responsible for pulmonary artery smooth muscle hyperplasia in primary pulmonary hypertension. J Clin Invest 108:1141–1150
34. Eddahibi S, Chaouat A, Morrell N et al (2003) Polymorphism of the serotonin transporter gene and pulmonary hypertension in chronic obstructive pulmonary disease. Circulation 108:1839–1844
35. Long L, Aldashev AA, Hensiek A et al (2002) Preliminary identification of genetic loci associated with high altitude pulmonary hypertension by association mapping. Thorax 57:S110
36. Olson TP, Snyder EM, Frantz RP, Turner ST, Johnson BD (2007) Repeat length polymorphism of the serotonin transporter gene influences pulmonary artery pressure in heart failure. Am Heart J 153:426–432
37. Machado RD, Koehler R, Glissmeyer E et al (2006) Genetic association of the serotonin transporter in pulmonary arterial hypertension. Am J Respir Crit Care Med 173:793–797

38. Willers ED, Newman JH, Loyd JE et al (2006) Serotonin transporter polymorphisms in familial and idiopathic pulmonary arterial hypertension. Am J Respir Crit Care Med 173:798–802
39. Eddahibi S, Hanoun N, Lanfumey L et al (2000) Attenuated hypoxic pulmonary hypertension in mice lacking the 5-hydroxytryptamine transporter gene. J Clin Invest 105:1555–1562
40. MacLean MR, Deuchar GA, Hicks MN et al (2004) Overexpression of the 5-hydroxytryptamine transporter gene – effect on pulmonary hemodynamics and hypoxia-induced pulmonary hypertension. Circulation 109:2150–2155
41. Guignabert C, Izikki M, Tu LI et al (2006) Transgenic mice overexpressing the 5-hydroxytryptamine transporter gene in smooth muscle develop pulmonary hypertension. Circ Res 98:1323–1330
42. Morecroft I, Loughlin L, Nilsen M et al (2005) Functional interactions between 5-hydroxytryptamine receptors and the serotonin transporter in pulmonary arteries. J Pharmacol Exp Ther 313:539–548
43. Sato K, Webb S, Tucker A et al (1992) Factors influencing the idiopathic development of pulmonary hHypertension in the fawn hooded rat. Am Rev Respir Dis 145:793–797
44. Marcos E, Adnot S, Pham MH et al (2003) Serotonin transporter inhibitors protect against hypoxic pulmonary hypertension. Am J Respir Crit Care Med 168:487–493
45. Guignabert C, Raffestin B, Benferhat R et al (2005) Serotonin transporter inhibition prevents and reverses monocrotaline-induced pulmonary hypertension in rats. Circulation 111:2812–2819
46. Laudi S, Trump S, Schmitz V et al (2007) Serotonin transporter protein in pulmonary hypertensive rats treated with atorvastatin. Am J Physiol Lung Cell Mol Physiol 293:L630–L6L8
47. Marcos E, Fadel E, Sanchez O et al (2004) Serotonin-induced smooth muscle hyperplasia in various forms of human pulmonary hypertension. Circ Res 94:1263–1270
48. Lee SL, Wang WW, Lanzillo JJ, Fanburg BL (1994) Serotonin produces both hyperplasia and hypertrophy of bovine pulmonary artery smooth muscle cells in culture. Am J Physiol 266:L46–L52
49. Liu YL, Suzuki YJ, Day RM, Fanburg BL (2004) Rho kinase-induced nuclear translocation of ERK1/ERK2 in smooth muscle cell mitogenesis caused by serotonin. Circ Res 95:579–586
50. Dempsie Y, Morecroft I, Welsh DJ et al (2008) Converging evidence in support of the serotonin hypothesis of dexfenfluramine-induced pulmonary hypertension with novel transgenic mice. Circulation 117:2928–2937
51. Walther DJ, Bader M (2003) A unique central tryptophan hydroxylase isoform. Biochem Pharmacol 66:1673–1680
52. Eddahibi S, Guignabert C, Barlier-Mur AM et al (2006) Cross talk between endothelial and smooth muscle cells in pulmonary hypertension – critical role for serotonin-induced smooth muscle hyperplasia. Circulation 113:1857–1864
53. Morecroft I, Dempsie Y, Bader M et al (2007) Effect of tryptophan hydroxylase 1 deficiency on the development of hypoxia-induced pulmonary hypertension. Hypertension 49:232–236
54. Lee SL, Wang WW, Finlay GA, Fanburg BL (1999) Serotonin stimulates mitogen-activated protein kinase activity through the formation of superoxide anion. Am J Physiol Lung Cell Mol Physiol 277:L282–L291
55. Suzuki YJ, Day RM, Tan CC et al (2003) Activation of GATA-4 by serotonin in pulmonary artery smooth muscle cells. J Biol Chem 278:17525–17531
56. Lee SL, Wang WW, Fanburg BL (1998) Superoxide as an intermediate signal for serotonin-induced mitogenesis. Free Radic Biol Med 24:855–858
57. Liu JQ, Folz RJ (2004) Extracellular superoxide enhances 5-HT-induced murine pulmonary artery vasoconstriction. Am J Physiol Lung Cell Mol Physiol 287:L111–L118
58. Mair KM, MacLean MR, Morecroft I, Dempsie Y, Palmer TM (2008) Novel interactions between the 5-HT transporter, 5-HT_{1B} receptors and Rho kinase in vivo and in pulmonary fibroblasts. Br J Pharmacol 155:606–616

59. Guilluy C, Rolli-Derkinderen M, Tharaux PL, Melino G, Pacaud P, Loirand G (2007) Transglutaminase-dependent RhoA activation and depletion by serotonin in vascular smooth muscle cells. J Biol Chem 282:2918–2928
60. Walther DJ, Peter JU, Winter S et al (2003) Serotonylation of small GTPases is a signal transduction pathway that triggers platelet alpha-granule release. Cell 115:851–862
61. Das M, Bouchey DM, Moore MJ, Hopkins DC, Nemenoff RA, Stenmark KR (2001) Hypoxia-induced proliferative response of vascular adventitial fibroblasts is dependent on G protein-mediated activation of mitogen-activated protein kinases. J Biol Chem 276: 15631–15640
62. Mortimer HJ, Peacock AJ, Kirk A, Welsh DJ P38 MAP (2007) kinase: essential role in hypoxia-mediated human pulmonary artery fibroblast proliferation. Pulm Pharmacol Ther 20:718–725
63. Mitani Y, Mutlu A, Russell JC, Brindley DN, DeAlmeida J, Rabinovitch M (2002) Dexfenfluramine protects against pulmonary hypertension in rats. J Appl Physiol 93:1770–1778
64. Rochefort GY, Lemaire MC, Eder V et al (2006) Dexfenfluramine does not worsen but moderates progression of chronic hypoxia-induced pulmonary hypertension. Eur J Pharmacol 550:149–154
65. Massagué J (2003) Integration of Smad and MAPK pathways: a link and a linker revisited. Genes Dev 17:2993–2997
66. Yang XD, Long L, Southwood M et al (2005) Dysfunctional Smad signaling contributes to abnormal smooth muscle cell proliferation in familial pulmonary arterial hypertension. Circ Res 96:1053–1063
67. Long L, MacLean MR, Jeffery TK et al (2006) Serotonin increases susceptibility to pulmonary hypertension in BMPR2-deficient mice. Circ Res 98:818–827
68. Morecroft I, Pang L, Baranowska M et al (2009) In vivo effects of a combined 5-HT1B receptor/SERT antagonist in experimental pulmonary hypertension. Cardiovasc Res In press.

Impaired Vascular Endothelial Growth Factor Signaling in the Pathogenesis of Neonatal Pulmonary Vascular Disease

Steven H. Abman

Abstract Of diverse growth factors that contribute to normal lung development, vascular endothelial growth factor (VEGF) plays an especially prominent role in the normal growth and development of the pulmonary circulation in the fetus and newborn. Strong experimental and clinical data support the role of impaired VEGF signaling in the pathogenesis of two major clinical disorders of the developing lung circulation: persistent pulmonary hypertension of the newborn (PPHN) and bronchopulmonary dysplasia (BPD). These disorders are each characterized by impaired vascular growth, structure and reactivity, which are at least partly due to endothelial cell dysfunction. This chapter will briefly discuss VEGF signaling during normal lung development and how disruption of VEGF signaling contribute to the pathogenesis of neonatal pulmonary vascular disease in these settings.

Keywords Persistent pulmonary hypertension of the newborn • bronchopulmonary dysplasia • endothelial cells • lung development • pulmonary circulation • nitric oxide

1 Introduction

Rapid adaptation of the fetal cardiopulmonary system at birth is required for the lung to assume its essential postnatal role of gas exchange. Perhaps the most dramatic event at birth involves the lung circulation, which must undergo a marked fall in pulmonary vascular resistance (PVR) to allow for about an eightfold increase in pulmonary blood flow.[1] The fall in PVR at birth is due to increased oxygen tension, ventilation, and shear stress, which cause vasodilation through enhanced release of nitric oxide (NO) and prostacyclin and decreased production of vasoconstrictors, such as endothelin 1.[2–5] Failure to achieve or sustain this drop in PVR at birth leads to profound hypoxemia and constitutes the syndrome known as *persistent pulmonary*

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hypertension of the newborn (PPHN). Successful transition of the pulmonary circulation at birth requires the precise orchestration of diverse growth factors and signaling pathways to ensure normal functional and structural maturation of the lung circulation. Mechanisms that disrupt this process prior to birth and contribute to the pathogenesis of PPHN are incompletely understood.

In addition to its key role at birth, normal pulmonary vascular development remains essential throughout postnatal life.[6] The ability of the lung to achieve normal gas exchange requires ongoing growth and maintenance of an intricate system of airways and vessels, including the establishment of a thin yet vast blood–gas interface. In animal models, disruption of angiogenesis during lung development not only increases the risk for pulmonary hypertension but also impairs alveolarization.[7] Clinically, there has been growing recognition of the importance of understanding basic mechanisms of lung vascular growth in the context of premature birth and the risk for chronic lung disease, known as *bronchopulmonary dysplasia* (BPD). BPD is characterized by arrested alveolar and vascular growth, and infants with BPD are at high risk for pulmonary hypertension.[8,9] Recent studies suggested that impaired lung angiogenesis plays a critical role in the pathogenesis of BPD; however, little is known about basic mechanisms of pulmonary vascular injury in the immature lung and the impact of this injury on subsequent lung vascular growth and function. Thus, not only do abnormalities of the developing lung circulation contribute to the development of pulmonary hypertension in term and preterm infants, but also disruption of vascular growth during the perinatal period can cause long-standing aberrations of lung architecture.

Of the diverse growth factors and signaling pathways that contribute to normal lung development, vascular endothelial growth factor (VEGF) plays an especially prominent role in growth and development of the pulmonary circulation. Strong experimental and clinical data support the role of impaired VEGF signaling in the pathogenesis of two major disorders of the developing lung circulation: PPHN and BPD. This chapter briefly discusses VEGF signaling during normal lung development and how disruption of VEGF signaling may contribute to the pathogenesis of neonatal pulmonary vascular disease in the settings of PPHN and premature infants with BPD.

2 VEGF Signaling During Lung Development

Although diverse growth and transcription factors modulate blood vessel formation during development, VEGF (VEGF-A) is one of the most potent and critical regulators of lung vascular growth, development, and maintenance throughout embryonic, fetal, and postnatal life.[9–13] Beginning in the embryonic period, VEGF is strongly expressed in the developing respiratory epithelium. VEGF acts through two distinct tyrosine kinase receptors, VEGFR-1 (VEGF receptor 1) and VEGFR-2 (VEGF receptor 2), which are each critical for embryonic vascular development. VEGFR-2 (Flk-1) is present within the lung mesenchyme during the embryonic stage and is an early marker for endothelial and endothelial progenitor cells.

Studies of genetic mouse models have provided unequivocal evidence for the critical roles of VEGF and VEGFRs during vascular development. Targeted disruption of the VEGF gene causes severe defects in the formation of blood vessels, and loss of a single VEGF allele results in early embryonic death due to a marked reduction in endothelial cells prior to embryonic day 9.5.[14,15] Targeted inactivation of VEGFRs is lethal due to defective vascular development, including the absence of vasculature in mice lacking the VEGFR-2 gene and increased endothelial cell number but a lack of normal tubular networks in VEGFR-1 null mice.[16,17] Complex interactions between VEGFR-1 and VEGFR-2 activities modulate the net effects of VEGF on postnatal angiogenesis.[18]

Lung epithelium and mesenchyme express VEGF ligand and receptor, respectively, from the embryonic stage of lung development and throughout fetal life. However, VEGF is also strongly expressed in isolated fetal pulmonary artery endothelial cells, suggesting autocrine functions for VEGF during development as well.[19] Because loss of VEGF causes early embryonic death, few studies have examined the roles of VEGF signaling during distinct stages of lung development. Inhibition of VEGF signaling during the pseudoglandular stage markedly disrupts vessel formation and leads to the loss of lung architecture in rat fetal lung explants.[20] In an experimental model of lung hypoplasia, nitrofen treatment impaired lung growth and markedly downregulated VEGF and VEGFR-2 expression in fetal rat lung explants.[21] Genetic disruption of VEGF in respiratory epithelium inhibited vascular and saccular growth in fetal mice, further demonstrating the critical role for VEGF signaling in development of the distal lung.[22]

VEGF-A exists as three prominent isoforms: VEGF 120, 164, and 188. Each isoform has different properties, including varying affinity for the heparin sulfate component of the extracellular matrix and different avidities for VEGFR-1 and VEGFR-2 binding. Each isoform is present in alveolar type II cells in the developing mouse lung, with expression peaking during the period of accelerated vascular growth during the canalicular stage of lung development.[23] VEGF 120 is highly diffusible due to the lack of heparin sulfate binding and probably serves a key early role in vascular formation through its effects on endothelial differentiation and proliferation. The importance of the VEGF 164 and 188 isoforms was demonstrated in mice engineered to express only the VEGF 120 isoform. Rodents exclusively expressing the VEGF 120 isoform had fewer air–blood barriers and decreased airspace-to-parenchyma ratios compared to wild-type littermates.[24] Thus, as development proceeds, the pattern of VEGF isoform expression becomes more restrictive, and VEGF isoforms may have different roles during lung development.

VEGF has other family members, including VEGF-B, -C, and -D. These VEGFs have different affinities for specific VEGFRs, with VEGF-C and -D demonstrating an ability to bind to VEGFR-3 that is probably crucial for development of the lymphatic vascular system. Studies of VEGF-D expression during mouse lung development suggested a pattern that is distinct from VEGF-A, suggesting strong mesenchymal expression of VEGF-D, perhaps by fibroblasts, that may potentially influence endothelial growth and function during lung development.[25] Regulation of the expression of pro- and antiangiogenic isoforms of VEGF by growth and splice factors has

been demonstrated, further illustrating the complexity of VEGF regulation.[26] Thus, the temporal and spatial patterns of expression of VEGF and its receptors during development are crucial for normal growth and patterning of the lung circulation, but more information is need to define their distinct and interactive roles.

Multiple mechanisms regulate VEGF expression during development, including low oxygen tension, hypoxia-inducible factor 1α, sonic hedgehog, fibroblast growth factor 9, and others. NO has been shown not only to mediate many downstream effects of VEGF during lung development and with postnatal angiogenesis[27] but also to upregulate VEGF expression ("reciprocal regulation"). Disruption of VEGF-NO interactions may be especially important in the pathogenesis of pulmonary vascular disease and lung growth, including PPHN, BPD, alveolar–capillary dysplasia, primary lung hypoplasia, and others (as discussed further in this chapter[28,29]). For example, NO corrects lung vascular and airspace growth after VEGFR inhibition in fetal rat lungs in vitro and neonatal rat lungs in vivo.[21,30]

Interactions with other growth factors can modulate the effects of VEGF on lung vascular development. For example, the angiopoietins (Angs) are growth factors that act on vascular endothelium through its receptors Tie 1 and Tie 2 (tyrosine kinase with immunoglobulin and epidermal growth factor-[EGF]-like domains).[31] Ang-Tie signaling is critical for normal vascular development, as Ang $1^{-/-}$ or Tie $2^{-/-}$ mice are embryonic lethal due to the failure of vascular integrity.[32,33] Ang 1 is produced by lung mesenchyme and smooth muscle, whereas Tie 2, its receptor, is largely restricted to endothelial expression. Ang 1 binding to Tie 2 causes receptor tyrosine phosphorylation and downstream signals for endothelial cell survival through PI3K/Akt signaling.[34] As noted with VEGF, angiogenic actions of Ang (angiopoietins) 1 may require endothelium-derived NO. Ang 1 promotes interactions between endothelial cells, extracellular matrix, and pericytes that are required for vessel maturation. Ang 1–VEGF interactions are critical for normal vascular maturation, but their interactive effects are complex and dependent on multiple factors. In hyperoxia-induced lung injury in neonatal rats, combined gene therapy of VEGF and Ang 1 stimulated lung growth and vascular maturation more effectively than VEGF gene therapy alone.[35]

In addition, hepatocyte growth factor (HGF) and c-met, its receptor, mediates many of the effects of VEGF-induced cross talk between epithelium and vasculature during lung development.[22] Thus, VEGF plays a central role in lung vascular development, with key effects on endothelial growth, differentiations, survival, and vascular formation.

3 Altered VEGF Signaling in the Pathogenesis of PPHN

PPHN is a clinical syndrome that is characterized by the failure of PVR to fall at birth (Fig. 21.1). Although the pathogenesis of PPHN is uncertain, abnormal pulmonary vasoreactivity (or *maladaptation*) is a central clinical feature of PPHN. Clinical studies have further emphasized the presence of pulmonary vascular remodeling (*maldevelopment*) in PPHN, even in newborns dying during their first days of life. These observations suggest the importance of chronic intrauterine events in the pathogenesis of PPHN. This hypothesis is supported by experimental

studies demonstrating that adverse stimuli, such as chronic hypertension in utero, can alter pulmonary vascular reactivity and structure, causing an inability of the pulmonary vasculature to dilate at birth. Disorders with severe PPHN are also characterized by decreased arterial density (*underdevelopment*), especially in the setting of lung hypoplasia. Work has shown that sustained intrauterine pulmonary hypertension reduces vascular growth, which is further associated with decreased alveolarization and lung weight.[36] Mechanisms that link pulmonary hypertension, decreased arterial growth, and reduced alveolarization in severe PPHN, especially in congenital diaphragmatic hernia or lung hypoplasia, are poorly understood but may relate to altered VEGF–NO signaling (Table 21.1). Advances in the treatment

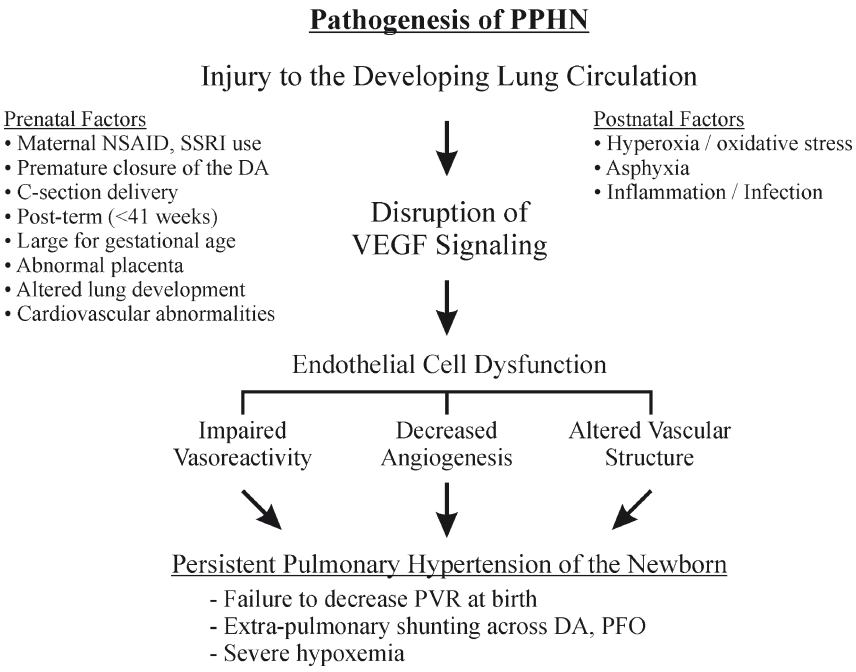

Fig. 21.1 Schematic illustration regarding impaired VEGF signaling in the pathogenesis of PPHN

Table 21.1 Impaired VEGF Signaling in PPHN

1. Experimental PPHN	
• Lung VEGF expression decreased in experimental PPHN	40
• Inhibition of VEGF impairs pulmonary vasoreactivity, increases smooth muscle hyperplasia, and causes RVH in fetal sheep	40
• Prolonged rhVEGF treatment restores endothelial function and vasoreactivity and attenuates pulmonary hypertension in experimental PPHN	41
2. Clinical PPHN	
• Decreased serum VEGF levels in human infants with PPHN	43

PPHN persistent pulmonary hypertension of the newborn; *VEGF* vascular endothelial growth factor; *RVH* right ventricular hypertrophy

of PPHN, such as inhaled NO, have improved outcomes, but treatment of severe PPHN, especially when associated with lung hypoplasia, continues to remain poorly responsive to current interventions and has high mortality.

Several experimental models have been used to explore the pathogenesis of PPHN. Such models include exposure to acute or chronic hypoxia after birth, chronic hypoxia in utero, placement of meconium into the airways of neonatal animals, sepsis, and others. Each model has key strengths for examining potential mechanisms relevant to PPHN but often lacks the capacity to sufficiently explore prenatal events that lead to the pulmonary vascular changes prior to birth that are characteristic of human PPHN. Adverse intrauterine stimuli during late gestation, such as abnormal hemodynamics, changes in substrate or hormone delivery to the lung, hypoxia, inflammation, or others may potentially alter lung vascular function and structure, contributing to abnormalities of postnatal adaptation.

Several investigators have examined the effects of chronic intrauterine stresses, such as hypoxia or hypertension, in animal models to mimic the clinical problem of PPHN. Since prenatal closure of the ductus arteriosus (DA) is a known cause of PPHN, an experimental model of surgical DA constriction in fetal lambs was developed as a model of PPHN.[37,38] Pulmonary hypertension induced by DA constriction in fetal lambs alters lung vascular reactivity and structure, causing the failure of postnatal adaptation at delivery, as observed in human PPHN. Over days, pulmonary artery pressure and PVR progressively increase, but flow remains low, and PaO_2 is unchanged.[37] Marked right ventricular hypertrophy and structural remodeling of small pulmonary arteries develop within 8–12 days of hypertension. After delivery, these lambs have persistent elevation of PVR despite mechanical ventilation with high oxygen concentrations. Studies with this model showed that chronic hypertension without high flow can alter fetal lung vascular structure and function.

This model is further characterized by marked endothelial cell dysfunction and altered smooth muscle cell reactivity and growth, including findings of impaired NO production and activity due to downregulation of lung endothelial NO synthase messenger RNA (mRNA) and protein expression and decreased NO bioavailablity.[39] Abnormalities of NO production and responsiveness contribute to altered structure and function of the developing lung circulation, leading to failure of postnatal cardiorespiratory adaptation.

Since VEGF is critical for maintenance of endothelial growth, function, and survival, we hypothesized that disruption of VEGF signaling may contribute to endothelial dysfunction in experimental PPHN. To determine whether impaired VEGF signaling contributes to endothelial dysfunction and related vascular abnormalities of PPHN, we performed a series of experiments in fetal sheep. In comparison with age-matched controls, lung VEGF protein expression was reduced by 75% of control levels in fetal sheep exposed to chronic DA compression.[40] Lung VEGFR-2 and eNOS-endothelial nitric oxide synthase (eNOS) expression were also markedly decreased in PPHN sheep. Tissue studies suggested that VEGF immunoreactivity was reduced in the vessel wall of small pulmonary arteries in the distal lung of PPHN sheep.

To determine the role of endogenous VEGF activity in the developing lung, we studied the effects of chronic VEGF inhibition by treating normal late-gestation

fetal sheep with daily intrapulmonary infusions of a highly selective VEGF 165 aptamer.[40] We found that chronic VEGF inhibition impaired endothelium-dependent vasodilation, decreased lung eNOS protein expression, caused progressive elevation of pulmonary artery pressure and PVR, increased arterial smooth muscle cell hyperplasia, and reduced arterial density.[40] Thus, selective VEGF inhibition mimicked the pulmonary vascular findings in this experimental model of PPHN.

Based on these findings, we further sought to determine whether treatment with VEGF could preserve endothelial function and reduce the severity of pulmonary hypertension in this model of experimental PPHN. In this model, daily intrapulmonary infusions of (recombinant human VEGF) rhVEGF$_{165}$ protein prevented the loss of acetylcholine-induced vasodilation, sustained eNOS expression, maintained normal vascular wall structure, and reduced right ventricular hypertrophy.[41] Thus, VEGF$_{165}$ preserved endothelial cell function and prevented the development of PPHN.

Gien et al. performed in vitro studies of fetal pulmonary artery endothelial cells (PAECs) from sheep with experimental PPHN.[19] In comparison with control cells, PAECs from PPHN sheep maintained an abnormal phenotype in vitro, as characterized by decreased growth and tube formation and marked reduction of VEGF, VEGFR-2, and eNOS protein content from cell lysates. Treatment with either exogenous VEGF or NO enhanced PAEC growth and tube formation, suggesting that impaired VEGF–NO signaling contributes to endothelial dysfunction in this model of PPHN.

Overall, these findings suggest that VEGF expression is decreased, and that impaired VEGF signaling contributes to endothelial dysfunction and abnormalities of pulmonary vascular reactivity, wall thickness, and angiogenesis in PPHN. Further insights into the role of VEGF and mechanisms of action may lead to novel treatment strategies for refractory PPHN, especially in the setting of severe lung hypoplasia.

4 Disruption of VEGF Signaling in the Pathogenesis of BPD

BPD is the chronic lung disease of infancy that follows mechanical ventilation and oxygen therapy for acute respiratory distress after birth in premature newborns.[8] BPD is defined by the presence of persistent respiratory signs and symptoms, the need for supplemental oxygen, and an abnormal chest radiograph at 36 weeks corrected age. There is a growing recognition that infants with chronic lung disease after premature birth have a different clinical course and pathology than was traditionally observed in infants dying with BPD during this “presurfactant era.” Lung histology of infants with BPD now shows less fibrosis and a pattern of enlarged distal airspaces and reduced growth of the capillary bed. Thus, the “new BPD” of the postsurfactant period represents inhibition of lung development with altered lung structure, growth, and function of the distal airspaces and vasculature. This marked reduction in alveolar–capillary surface area contributes to impaired gas exchange with an increased risk for exercise intolerance, pulmonary hypertension, and severe deterioration with respiratory infections.

In addition to its effects on the airway, lung injury impairs growth, structure, and function of the developing pulmonary circulation. Endothelial cells are particularly susceptible to oxidant injury from hyperoxia and inflammation. The media of small pulmonary arteries also undergoes striking changes, which include smooth muscle cell proliferation and incorporation of fibroblasts or myofibroblasts into the vessel wall. Structural changes in the lung vasculature contribute to high PVR due to narrowing of the vessel diameter and decreased vascular compliance. Decreased angiogenesis reduces vascular surface area, causing further elevations of PVR, especially in response to high cardiac output with exercise or stress or increased flow due to shunt lesions. Thus, in addition to pulmonary hypertension, it is now clear that pulmonary vascular disease in BPD also includes reduced vascular density due to impaired angiogenesis, which contributes to physiologic abnormalities of abnormal gas exchange as well as the pathogenesis of BPD[8,42–44] (Fig. 21.2).

Experimental data support the hypothesis that impaired angiogenesis decreases alveolarization, and that strategies that preserve and enhance endothelial cell survival, growth, and function may provide new therapeutic approaches for the prevention of BPD. Of multiple growth factors and signaling systems that have been shown to play important roles in normal lung vascular growth, VEGF plays an especially prominent role. Several studies have examined how premature delivery and changes in oxygen tension, inflammatory cytokines, and other signals can decrease VEGF expression and signaling, thereby altering lung structure (Table 21.2). Experimentally, hyperoxia, which impairs alveolar and vascular growth and inhibits alveolarization in neonatal rats, also downregulates lung VEGF and VEGFR expression, and pharmacologic inhibition of VEGFRs inhibits lung vascular and alveolar growth in newborn rats.[7,45,46] Furthermore, lung VEGF expression is impaired in primate and

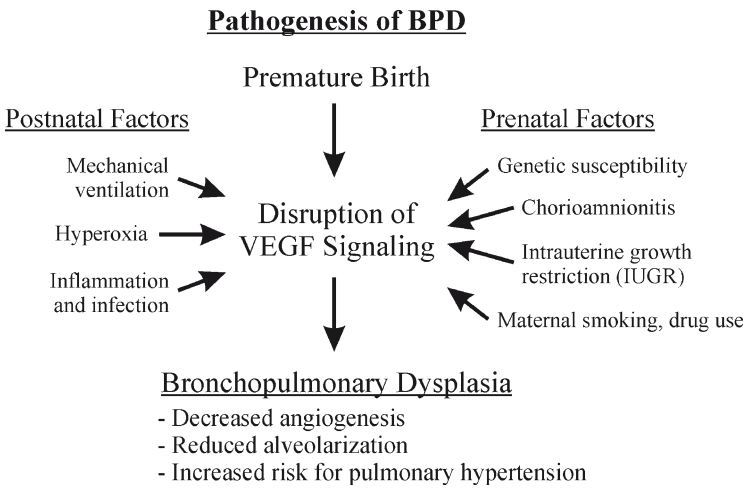

Fig. 21.2 Schematic illustration regarding mechanisms that impair VEGF signaling and its role in the pathogenesis of BPD as based on experimental and clinical studies (see text)

Table 21.2 Impaired VEGF signaling in BPD

1. Experimental BPD	Reference
• Decreased VEGF and VEGFR expression in models of BPD (e.g., neonatal hyperoxia, prolonged ventilation, and antenatal endotoxin)	43
• VEGFR inhibition reduces vascular growth, impairs alveolarization, and causes pulmonary hypertension in newborn rats	7, 46
• Early and late rhVEGF protein treatment restores vascular growth and lung structure in neonatal hyperoxia	52, 53
• VEGF gene therapy improves lung structure in neonatal hyperoxia	35
2. Clinical BPD	
• Decreased lung VEGF and VEGFR expression in human infants dying with BPD	42
• Decreased VEGF levels in preterm infants who develop BPD	43

BPD bronchopulmonary dysplasia; *VEGF* vascular endothelial growth factor; *VEGFR* VEGF receptor; *rhVEGF* recombinant human VEGF

ovine models of BPD induced by mechanical ventilation after premature birth, further supporting the hypothesis that impaired VEGF signaling contributes to the pathogenesis of BPD.[47] As initially suggested in animal models, impaired lung VEGF expression was also demonstrated in human BPD. Bhatt and coworkers first demonstrated decreased VEGF and VEGFR-1 mRNA and protein in the lungs of premature infants who died with BPD.[42] Decreased VEGF levels were reported in tracheal fluid samples from premature neonates who subsequently developed BPD when compared with patients without chronic lung disease.[43]

To determine whether angiogenesis is necessary for alveolarization, the effects of antiangiogenesis drugs thalidomide and fumagillin on lung growth were studied in neonatal rat pups.[7] In comparison with vehicle-treated controls, postnatal treatment with angiogenesis inhibitors reduced lung vascular density, alveolar number, and lung weight. Similar effects were found after treatment of newborn rats with SU5416, a VEGFR inhibitor.[7,46] In these studies, a single injection of SU5416 immediately after birth caused progressive pulmonary hypertension and markedly reduced lung vascular density and alveolar growth in infant rats. Early endothelial cell apoptosis without inflammation preceded changes in vascular and alveolar structure.[48] These findings suggest that angiogenesis is necessary for alveolarization during lung development, and that disruption of lung vascular growth impedes alveolar growth after premature birth.

Mechanisms through which impaired VEGF signaling inhibits vascular growth and alveolarization are uncertain but may in part be mediated by altered NO production. Past in vitro and in vivo studies have shown that VEGF stimulates endothelial eNOS expression in endothelial cells from the systemic circulation. In addition to its effects on vascular tone, NO can alter angiogenesis, but data are conflicting on its effects. NO mediates the angiogenic effects of VEGF on fetal pulmonary artery endothelial cells,[19] which is likely through activation of VEGFR-2 and stimulation of the Akt–PI3K pathway. Studies with the eNOS$^{-/-}$ fetal mouse model suggested that NO plays a critical role in vascular and alveolar

growth in utero, and that eNOS$^{-/-}$ newborns are more susceptible to hypoxia-induced inhibition of alveolarization than wild-type mice.[28,29,49] Importantly, lung eNOS expression is decreased in primate and ovine models of BPD, which may contribute to lung structural abnormalities in these settings.[50,51] VEGFR inhibition with SU5416 in neonatal rats was shown to decrease lung eNOS expression and NO production throughout infancy, and that prolonged treatment with inhaled NO prevented endothelial apoptosis, blocked the development of pulmonary hypertension, improved vascular growth, and enhanced alveolarization.[30,48]

To test the therapeutic potential for angiogenic growth factor modulation in experimental lung disease characterized by alveolar damage, we studied whether the effects of recombinant human VEGF 165 (rhVEGF) treatment of newborn rats during or after exposure to hyperoxia enhances vessel growth and improves alveolarization.[52,53] Similarly, postnatal intratracheal adenovirus-mediated VEGF gene therapy improves survival, promotes lung capillary formation, preserves alveolar development, and regenerates new alveoli in this same model of irreversible lung injury.[25] In these studies, VEGF stimulated sprouting of immature and leaky capillaries, leading to lung edema. Indeed, despite its central role in vascular formation, VEGF works in concert with other factors, notably Angs. Ang 1 is required to stabilize the vessel wall by maximizing interactions between endothelial cells and their surrounding support cells and matrix. Accordingly, combined lung VEGF and Ang 1 gene transfer preserves alveolarization and enhances angiogenesis with more mature capillaries that are less permeable, reducing the vascular leakage seen in VEGF-induced capillaries.[25]

Previous studies have shown that inhaled NO attenuates hyperoxia-induced acute lung injury, which may enhance subsequent vascular and lung growth. These studies suggest that decreased VEGF signaling downregulates lung eNOS expression, and that impaired NO production may contribute to abnormal lung growth during development.[30] Importantly, a randomized single-center study has shown that inhaled NO treatment reduced the combined endpoint of BPD and death in human premature newborns with moderate respiratory distress syndrome (RDS).[54] Multicenter clinical trials suggested that early treatment with inhaled NO may attenuate the risk for BPD in premature infants with birth weights above 1,000 g[55] and in older infants who require mechanical ventilation beyond the first week of life.[56] However, mechanisms through which inhaled NO improved outcomes in these clinical studies are uncertain.

5 Summary

In summary, lung vascular growth and development involve a dynamic process that includes critical changes throughout development, beginning in the embryonic period and continuing throughout gestation and during postnatal life. Production of proangiogenic growth factors, especially VEGF and its receptors, maintains pulmonary vascular growth and structure in normal and disease states

due to enhanced endothelial cell differentiation, survival, and function. Future work is needed to better define basic mechanisms of lung vascular growth and development, which will likely lead to novel therapeutic approaches to diseases associated with neonatal pulmonary vascular diseases, especially in the settings of severe PPHN and BPD.

References

1. Dawes G, Mott JC, Widdicombe JG (1953) Changes in the lungs of the newborn lamb. J Physiol 121:141–162
2. Abman SH, Chatfield BA, Hall SL, McMurtry IF (1990) Role of endothelium-derived relaxing factor during transition of pulmonary circulation at birth. Am J Physiol 259:H1921–H1927
3. Cornfield DN, Reeve HL, Tolarova S, Weir EK, Archer S (1996) Oxygen causes fetal pulmonary vasodilation through activation of a calcium-dependent potassium channel. Proc Natl Acad Sci U S A 93:8089–8094
4. Velvis H, Moore P, Heymann MA (1991) Prostaglandin inhibition prevents the fall in pulmonary vascular resistance as the result of rhythmic distension of the lungs in fetal lambs. Pediatr Res 30:62–67
5. Ivy DD, Kinsella JP, Abman SH (1994) Physiologic characterization of endothelin A and B receptor activity in the ovine fetal lung. J Clin Invest 93:2141–2148
6. deMello DE, Reid LM (2002) Prenatal and postnatal development of the pulmonary circulation. In: Haddad CG, Abman SH, Chernick VC (eds) Basic mechanisms of pediatric respiratory disease. Decker, Hamilton, ON, pp 77–101
7. Jakkula M, Le Cras TD, Gebb S et al (2000) Inhibition of angiogenesis decreases alveolarization in the developing rat lung. Am J Physiol Lung Cell Mol Physiol 279:L600–L6L7
8. Coalson JJ (2000) Pathology of chronic lung disease of early infancy. In: Bland RD, Coalson JJ (eds) Chronic lung disease of early infancy. Dekker, New York, pp 85–124
9. Abman SH (2000) Pulmonary hypertension in chronic lung disease of infancy. Pathogenesis, pathophysiology and treatment. In: Bland RD, Coalson JJ (eds) Chronic lung disease of infancy. Dekker, New York, pp 619–668
10. Ferrara N, Gerber HP, LeCouter J (2003) The biology of VEGF and its receptors. Nat Med 9:669–672
11. Flamme I, Breier G, Risau W (1995) VEGF and VEGF receptor 2 are expressed during vasculogenesis and vascular differentiation in the quail embryo. Dev Biol 169:699–712
12. Millauer B, Wizigmann-Voos S, Schnurch H et al (1993) High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. Cell 72:835–846
13. Akeson AL, Greenberg JM, Cameron JE et al (2003) Temporal and spatial regulation of VEGF-A controls vascular patterning in the embryonic lung. Dev Dyn 264:443–455
14. Carmeliet P, Ferreira V, Breier G et al (1996) Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 380:435–439
15. Ferrara N, Carver-Moore K, Chen H et al (1992) Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 380:439–442
16. Fong G, Rossant H, Gertsenstein M, Breitman ML (1995) Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature 376:66–70
17. Shalaby F, Rossant J, Yamaguchi TP et al (1995) Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature 376:62–66
18. Nishi J, Minamino T, Miyauchi H et al (2008) Vascular endothelial growth factor receptor-1 regulates postnatal angiogenesis through inhibition of the excessive activation of Akt. Circ Res 103:261–268

19. Gien J, Seedorf G, Balasubramaniam V, Markham N, Abman SH (2007) Chronic intrauterine pulmonary hypertension impairs endothelial cell growth and angiogenesis in vitro. Am J Respir Crit Care Med 176:1146–1153
20. van Tuyl M, Liu J, Wang J, Kuliszewski M, Tibboel D, Post M (2005) Role of oxygen and vascular development in epithelial branching morphogenesis of the developing mouse lung. Am J Physiol Lung Cell Mol Physiol 288:L167–L178
21. Muehlethaler V, Kunig A, Seedorf G, Balasubramaniam V, Abman SH (2008) Impaired VEGF and NO signaling after nitrofen exposure in rat fetal lung explants. Am J Physiol Lung Cell Mol Physiol 294:L110–L120
22. Yamamoto H, Yun EJ, Gerber HP, Ferrara N, Whitsett JA, Vu TH (2007) Epithelial – vascular cross talk mediated by VEGF A and HGF signaling directs primary septae formation during distal lung morphogenesis. Dev Biol 308:44–53
23. Ng Y, Rohan R, Sunday ME, deMello DE, D'Amore PA (2001) Differential expression of VEGF isoforms in mouse during development and in the adult. Dev Dyn 220:112–121
24. Galumbos C, Ng YS, Ali A et al (2002) Defective pulmonary development in the absence of heparin-binding VEGF isoforms. Am J Respir Cell Mol Biol 27:194–203
25. Greenberg JM, Thompson FY, Brooks SK et al (2002) Mesenchymal expression of VEGF D and A defines vascular patterning in developing lung. Dev Dyn 224:144–153
26. Nowak DG, Woolard J, Amin EM et al (2008) Expression of pro- and anti-angiogenic isoforms of VEGF is differentially regulated by splicing and growth factors. J Cell Sci 121:3487–3495
27. Ziche M, Morbidelli L, Choudhuri R et al (1997) NO synthase lies downstream from vascular endothelial growth factor – induced but not basic fibroblast growth factor-induced angiogenesis. J Clin Invest 99:2625–2634
28. Balasubramaniam V, Tang JR, Maxey A, Plopper CG, Abman SH (2003) Mild hypoxia impairs alveolarization in the endothelial nitric oxide synthase-deficient mouse. Am J Physiol Lung Cell Mol Physiol 284:L964–L971
29. Han RN, Babei S, Robb M et al (2004) Defective lung vascular development and fatal respiratory distress in eNOS deficient mice: a model of alveolar capillary dysplasia. Circ Res 94:1115–1123
30. Tang JR, Markham NE, Lin YJ et al (2004) Inhaled NO attenuates pulmonary hypertension and improves lung growth in infant rats after neonatal treatment with a VEGF receptor inhibitor. Am J Physiol Lung Cell Mol Physiol 287:L344–L351
31. Loughna S, Sato TN (2001) Angiopoietin and Tie signaling pathways in vascular development. Matrix Biol 20:319–325
32. Dumont DJ, Gradwohl G, Fong GH et al (1994) Dominant negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis in the embryo. Genes Dev 8:1897–1909
33. Sato TN, Tozawa Y, Deutsch U et al (1995) Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. Am J Respir Cell Mol Biol 22:157–165
34. Kim I, Kim JH, So Moon, Kwak NJ, Kim NG, Koh GY (2000) Angiopoietin-2 at high concentrations can enhance endothelial cell survival through the PI3K/Akt signal transduction pathway. Oncogene 19:4549–4552
35. Thébaud B, Ladha F, Michelakis ED et al (2005) VEGF gene therapy increases survival, promotes lung angiogenesis, and prevents alveolar damage in hyperoxia-induced lung injury: evidence that angiogenesis participates in alveolarization. Circulation 112:2477–2486
36. Grover TR, Parker TA, Balasubramaniam V, Markham NE, Abman SH (2005) Pulmonary hypertension impairs alveolarization and lung growth in the ovine fetus. Am J Physiol Lung Cell Mol Physiol 288:L648–L654
37. Abman SH, Shanley PF, Accurso FJ (1989) Failure of postnatal adaptation of the pulmonary circulation after chronic intrauterine pulmonary hypertension in fetal lambs. J Clin Invest 83:1849–1858
38. Morin FC (1989) Ligating the ductus arteriosus before birth causes persistent pulmonary hypertension of the newborn lamb. Pediatr Res 25:245–250

39. Villamor E, Le Cras TD, Horan M, Halbower AC, Tuder R, Abman SH (1997) Chronic intrauterine pulmonary hypertension impairs endothelial nitric oxide synthase in the ovine fetus. Am J Physiol 272:L1013–L1020
40. Grover TR, Parker TA, Zenge JP, Markham NE, Abman SH (2003) Intrauterine pulmonary hypertension decreases lung VEGF expression and VEGF inhibition causes pulmonary hypertension in the ovine fetus. Am J Physiol Lung Cell Mol Physiol 284:L508–L517
41. Grover TR, Parker TA, Markham NE (2005) Abman SH. rhVEGF treatment improves pulmonary vasoreactivity and structure in an experimental model of pulmonary hypertension in fetal sheep. Am J Physiol Lung Cell Mol Physiol 289:L529–L535
42. Bhatt AJ, Pryhuber GS, Huyck H, Watkins RH, Metlay LA, Maniscalco WM (2001) Disrupted pulmonary vasculature and decreased VEGF, flt-1, and Tie 2 in human infants dying with BPD. Am J Resp Crit Care Med 164:1971–1980
43. Lassus P, Turanlahti M, Heikkilä P et al (2001) Pulmonary vascular endothelial growth factor and Flt-1 in fetuses, in acute and chronic lung disease, and in persistent pulmonary hypertension of the newborn. Am J Respir Crit Care Med 164:1981–1987
44. Abman SH (2001) BPD: a vascular hypothesis. Am J Respir Crit Care Med 164:1755–1756
45. Maniscalco WM, Watkins RH, D'Angio CT, Ryan RM (1997) Hyperoxic injury decreases alveolar epithelial cell expression of vascular endothelial growth factor (VEGF) in neonatal rabbit lung. Am J Respir Cell Mol Biol 16:557–567
46. Le Cras TD, Markham NE, Tuder RM, Voelkel NF, Abman SH (2002) Treatment of newborn rats with a VEGF receptor inhibitor causes pulmonary hypertension and abnormal lung structure. Am J Physiol Lung Cell Mol Physiol 283:L555–L562
47. Maniscalco WM, Watkins RH, Pryhuber GS, Bhatt A, Shea C, Huyck H (2002) Angiogenic factors and the alveolar vasculature: development and alterations by injury in very premature baboons. Am J Physiol Lung Cell Mol Physiol 282:L811–L823
48. Tang JR, Seedorf G Balasubramaniam V, Maxey A, Markham N, Abman SH (2007) Early inhaled NO treatment decreases apoptosis of endothelial cells in neonatal rat lungs after VEGF inhibition. Am J Physiol Lung Cell Mol Physiol 293:L1271–L1280
49. Young SL, Evans K, Eu JP (2002) Nitric oxide modulates branching morphogenesis in fetal rat lung explants. Am J Physiol Lung Cell Mol Physiol 282:L379–L385
50. Afshar S, Gibson LL, Yuhanna IS et al (2003) Pulmonary NO synthase expression is attenuated in a fetal baboon model of chronic lung disease. Am J Physiol Lung Cell Mol Physiol 284:L749–L758
51. MacRitchie AN, Albertine KH, Sun J et al (2001) Reduced endothelial nitric oxide synthase in lungs of chronically ventilated preterm lambs. Am J Physiol Lung Cell Mol Physiol 281:L1011–L1020
52. Kunig AM, Balasubramaniam V, Markham NE, Seedorf G, Gien J, Abman SH (2006) Recombinant human VEGF treatment transiently increases lung edema but enhances lung structure after neonatal hyperoxia. Am J Physiol Lung Cell Mol Physiol 291:L1068–L1078
53. Kunig AM, Balasubramaniam V, Markham NE et al (2005) Recombinant human VEGF treatment enhances alveolarization after hyperoxic lung injury in neonatal rats. Am J Physiol Lung Cell Mol Physiol 289:L529–L535
54. Schreiber MD, Gin-Mestan K, Marks JD, Huo D, Lee G, Srisuparp P (2003) Inhaled NO in premature infants with respiratory distress syndrome. N Engl J Med 349:2099–2107
55. Kinsella JP, Cutter GR, Walsh WF et al (2006) Early inhaled nitric oxide therapy in premature newborns with respiratory failure. N Engl J Med 355:354–364
56. Ballard RA, Truog WE, Cnaan A et al (2006) Inhaled nitric oxide in preterm infants undergoing mechanical ventilation. N Engl J Med 355:343–353

Part V
Receptors and Transporters: Role in Cell Function and Hypoxic Pulmonary Vasoconstriction

Mitochondrial Regulation of Oxygen Sensing

Navdeep S. Chandel

Abstract Hypoxia promotes physiological processes such as energy metabolism, angiogenesis, cell proliferation, and cell viability through the transcription factor Hypoxia Inducible Factor (HIF). Hypoxia also diminishes the activity of ATP consuming processes to promote cell survival. The mechanism(s) by which hypoxia activates HIF and diminishes ATP demand are a subject of intensive research. Here we outline the model in which mitochondrial complex III regulate the activity of HIF and diminish ATP utilization processes through the increased production of ROS during hypoxia.

Keywords Mitochondria • HIF • ROS • Na/K ATPase

1 Introduction

Oxygen is necessary for cellular processes, most notably the production of adenosine triphosphate (ATP) by mitochondrial oxidative phosphorylation in higher organisms. As mammalian cells encounter lower oxygen levels (hypoxia, 2–20 torr or 0.3–3% O_2), they have mechanisms to prevent depletion of oxygen to anoxic levels (0–2 torr or 0–0.3% O_2). Cells that reside under hypoxia do not undergo cell death. However, cells that encounter anoxia for a sustained period will undergo cell death.[1] There are two mechanisms that cells invoke to during hypoxia to prevent depletion of oxygen to anoxic levels. First, cells can increase their oxygen supply through the transcriptional upregulation of vascular endothelial growth factor (VEGF).[2] The increase in VEGF stimulates angiogenesis, which would help prevent depletion of oxygen (Fig. 22.1). The second mechanism is to decrease the cellular demand for oxygen. This is mediated in part by the downregulation of Na/K adenosine triphos-

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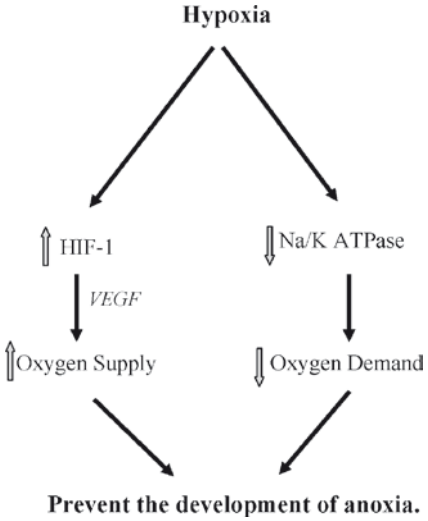

Fig. 22.1 Cellular oxygen supply and demand during hypoxia. We propose that HIF-1 activates VEGF to stimulate angiogenesis to increase oxygen supply during hypoxia. Simultaneously, hypoxia decreases the activity of Na/K ATPase to diminish cellular oxygen demand. Collectively, the increase in VEGF and the decrease in Na/K ATPase prevent the depletion of oxygen under hypoxic conditions to anoxic levels

phatase (ATPase) activity.[3] The decrease in oxygen demand during hypoxia diminishes the rate at which the cells reach anoxia. It is important to note that these events occur at oxygen levels at which cellular bioenergetics are not compromised. Cells become energetically comprised under anoxic conditions. This is because the K_m of the cytochrome *c* oxidase is less than 1 μM.[4] Cytochrome *c* oxidase is the main enzyme in the electron transport chain that transfers electrons and binds to oxygen. Thus, availability of oxygen regulates oxidative phosphorylation through cytochrome *c* oxidase. This ensures that mitochondria have the ability to generate ATP at maximal levels throughout the physiological range of oxygen tensions (1–20% O_2) that cells encounter.[5] Since cells can regulate oxygen supply and demand under hypoxia, it implies that cells must have oxygen-sensing mechanisms during hypoxia independent of cell's bioenergetic status. This review focuses on the current models and controversies surrounding cellular oxygen sensing as it pertains to the regulation of oxygen supply and demand during hypoxia.

2 Mitochondria Regulate Oxygen Supply Through an Increase in Hypoxia-Inducible Factor 1

The transcription factor hypoxia-inducible factor 1 (HIF-1) regulates the increase in oxygen supply observed during hypoxia. HIF-1 is a transcriptional activator that is required for the upregulation of VEGF gene during hypoxia.[6] The increase in

VEGF during hypoxia provides a mechanism to stimulate angiogenesis, thus preventing depletion of local oxygen. HIF-1 is a heterodimer of two basic helix loop-helix Per-Arnt-Sim (PAS) proteins containing HIF-1α and the aryl hydrocarbon nuclear translocator (ARNT or HIF-1β).[7] The ARNT protein is constitutively expressed, and its level is not significantly affected by oxygen. In contrast, HIF-1α protein is stabilized within minutes of exposure to hypoxia. The molecular regulation of HIF-1 by oxygen is controlled exclusively by the α subunit. The α subunit contains four distinct domains.[8] The N-terminus contains the basic helix-loop-helix (bHLH) and PAS domains that mediate the dimerization to ARNT and DNA binding of the heterodimer to a consensus sequence (5′-CGTGC-3′) in promoters or enhancers of a variety of genes. The middle of the protein contains an oxygen-dependent domain (ODD, residues 401–603), which controls the protein stability of the α subunit as a function of the oxygen tension (Fig. 22.2). This domain contains a

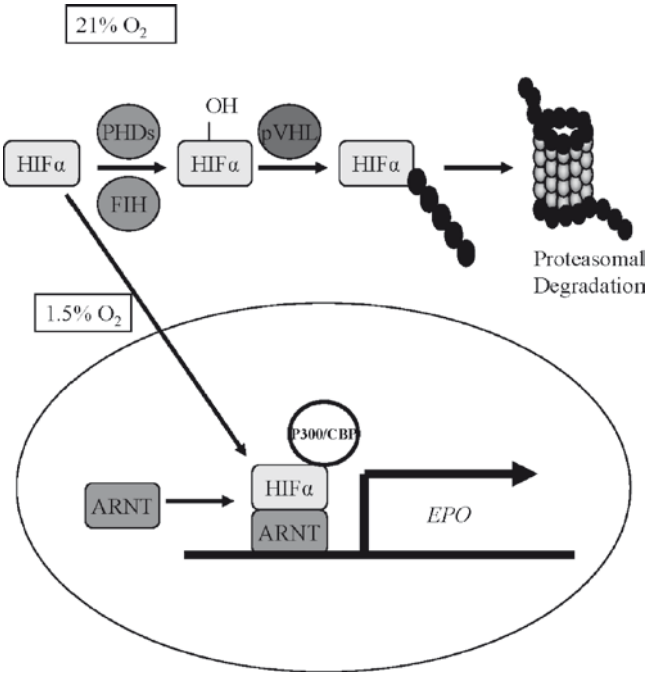

Fig. 22.2 Hypoxic regulation of HIF-1α. HIF-1α is hydroxylated at two different proline residues and an aspargine residue under normoxia. The proline residues reside in the oxygen-dependent domain (ODD, residues 401–603) of HIF-1α. The hydroxylation of proline residues occurs by a family of prolyl hydroxylases (PHDs 1–3). The hydroxylation of proline residues serves as a recognition motif for pVHL. The binding of pVHL targets the HIF-1α protein for ubiquitin-mediated degradation. HIF-1α contains two transactivation domains, referred to as TAD-N (531–575) and TAD-C (786–826). The asparagine residue resides in TAD-C. The hydroxylation of asparagine prevents the binding of transcriptional coactivators such as p300/CBP. Under hypoxia, the hydroxylation of proline and asparagine is diminished, which allows for the protein to be stabilized and to bind to HIF-1β as well as p300/CBP to allow HIF-1-dependent gene transcription

transactivation domain (531–575) referred to as TAD-N. The C-terminus contains another TAD (786–826) referred to as TAD-C. The TADs interact with the coactivators' p300 and creb binding protein (CBP), which are required for activating transcription of target genes.

During normoxia (21% O_2), HIF-1α is polyubiquitinated and targeted for degradation by an E3 ubiquitin ligase complex that contains the von Hippel-Lindau tumor-suppressor protein (pVHL), elongin B, elongin C, Cul2, and Rbx.[9] The binding of pVHL to HIF-1α is dependent on the hydroxylation of proline residues 402 and 564 within the ODD of HIF-1α.[10–12] This constitutes the pVHL substrate recognition unit. The enzymatic hydroxylation reaction is inherently oxygen dependent since the oxygen atom of the hydroxyl group is derived from molecular oxygen. In addition, prolyl hydroxylation requires 2-oxoglutarate and iron as cofactors. 2-Oxoglutarate is required because the hydroxylation reaction is coupled to the decarboxylation of 2-oxoglutarate to succinate, which accepts the remaining oxygen atom. In mammalian cells, HIF prolyl hydroxylation is carried out by a distinct family of prolyl hydroxylases (PHDs 1–3).[13]

Oxygen tension also regulates the interaction of HIF-1α with transcriptional coactivators p300 and CBP (Fig. 22.2). Asparagine hydroxylation of residue 803 in HIF-1α by the enzyme FIH-1 (factor-inhibiting HIF-1) blocks the binding of p300 and CBP to HIF-1α, thus inhibiting HIF-1-mediated gene transcription.[14–16] Under hypoxic conditions, the rate of asparagine and proline hydroxylation decreases. pVHL cannot bind to HIF-1α in the absence of proline hydroxylation, thus resulting in a decreased rate of HIF-1α degradation. In the absence of asparagine hydroxylation, p300 and CBP can bind to HIF-1α, allowing transcriptional activation of HIF-1 target genes.

The fact that cells are able to increase glycolytic capacity at oxygen tensions in the range at which mitochondrial oxidative phosphorylation is not limited by oxygen suggests that cells have oxygen-sensing mechanisms to regulate oxygen supply independent of ATP. In search of cellular oxygen-sensing mechanisms, investigators focused on understanding the observation that iron chelators, such as desferrioxamine (DFO), are able to stabilize HIF-1α protein levels and activate HIF-1-dependent gene targets.[17] This initially led to the proposal that a rapidly turning over heme protein that interacts with O_2 might be a sensor. However, studies using heme synthesis inhibitors failed to show any effect on the hypoxic activation of HIF-1.[18] The discovery that hydroxylation of proline residues by PHDs regulate the stabilization of HIF-1α protein has provided a mechanistic explanation for the action of iron chelators. Since iron is required as a cofactor for the hydroxylation of proline residues within HIF-1α, iron chelators are likely to suppress this hydroxylation reaction and allow HIF-1α protein levels to be stabilized. The hydroxylation reaction also requires oxygen as a substrate, thus making the hydroxylation step an oxygen-dependent process.

It is clear that the PHDs are the most proximal molecules regulating the stabilization of the HIF-1α protein. We propose that there are likely to be signaling molecules that are required to regulate PHD activity during hypoxia. For example, we have shown that a functional electron transport chain is required for the hypoxic stabilization of the HIF-1α protein.[19,20] This hypothesis is supported by the observation that cells depleted of their mitochondrial DNA (mtDNA; ρ° cells) are not able to

stabilize the HIF-1α protein or activate HIF-1-dependent gene transcription under hypoxia. The ρ° cells are unable to carry out functional electron transport because they lack critical subunits of complexes I, III, and IV.[21] Since neither mitochondrial ATP levels nor the mitochondrial membrane potential change during hypoxia, we have focused on the generation of reactive oxygen species (ROS) by the electron transport chain as possible signaling molecules linking the electron transport chain to the stabilization of HIF-1α protein. The mitochondrial electron transport chain can generate superoxide at complex I, II, or III.[22] ROS do not appear to be generated by complex IV. The best-studied site of ROS generation under physiological conditions is during the ubiquinone (Q) cycle within complex III (Fig. 22.3). The Q cycle involves the transfer of two electrons to ubiquinone from complex I and complex II, resulting in the reduction of ubiquinone to ubiquinol (QH2).[23] The subsequent oxidation of ubiquinol to ubiquinone requires the donation of two electrons (Fig. 22.3). The first electron transfer is to the Reiske iron–sulfur center protein (Fe–S). This electron is then transferred first to cytochrome *c1* and then to cytochrome *c*. This one electron transfer converts ubiquinol to ubisemiquinone (Q^{-}). The second electron residing in ubisemiquinone is transferred to the two heme groups of cytochrome *b*, referred to as heme b_L and heme b_H. The two hemes have

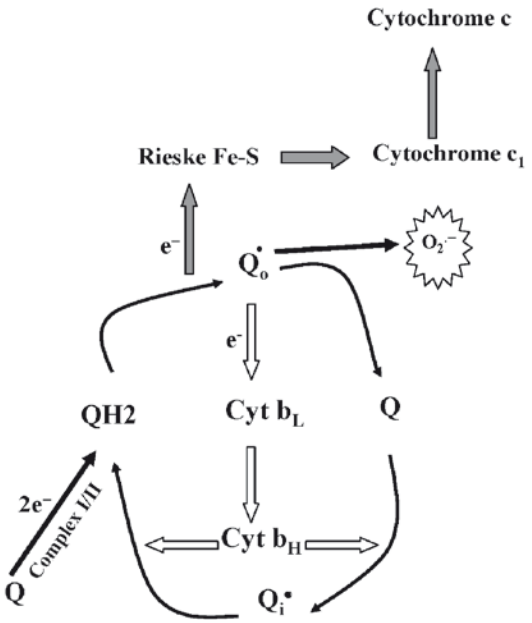

Fig. 22.3 The ubiquinone (Q) cycle. The Q cycle involves the transfer of two electrons to ubiquinone from complex I and complex II, resulting in the reduction of ubiquinone to ubiquinol (QH2). Ubiquinol transfers one electron to Reiske iron–sulfur protein (Fe–S), resulting in the formation of ubisemiquinone (Q^{-}). This electron is sequentially transferred to cytochromes *c*1, cytochrome *c*, and cytochrome *c* oxidase. The second electron residing in ubisemiquinone (Q^{-}) is transfered to the two heme groups of cytochrome *b*, referred to as heme b_L and heme b_H

different electron affinities because they are located in different polypeptide environments. Heme b_L is located closer to the intermembrane space and has a lower affinity for an electron compared with heme b_H, which is located closer to the matrix side. Ubisemiquinone transfers its electron to b_L to form ubiquinone. Heme b_L in turn donates an electron to heme b_H, which subsequently reduces ubiquinone to ubisemiquinone. The Q cycle at this stage is only half complete since only one of the electrons from ubiquinol has been transferred to cytochrome *c*. Ubiquinol is a two-electron carrier, whereas cytochrome *c* is a one-electron carrier. The Q cycle has to go through two rounds to efficiently donate two electrons from ubiquinol to cytochrome *c*. During the second round of the Q cycle, ubiquinol donates one electron to Fe-S, followed by cytochromes *c1* and *c*. Again, this step generates ubisemiquinone, which sequentially reduces heme b_L and heme b_H. However, in this second cycle heme b_H reduces ubisemiquinone left from the first cycle. This completes the Q cycle. Thus, two rounds of the cycle result in the oxidation of two ubiquinol molecules to form ubiquinone, of which one of the ubiquinone molecules is reduced to regenerate one ubiquinol.

The unstable radical ubisemiquinone (Q^{-}) can donate electrons to oxygen to generate superoxide within the Q cycle. Ubisemiquinone generated after ubiquinol oxidation can potentially produce superoxide and release it in the intermembrane space and eventually into the cytosol through voltage-dependent anion channels.[24] Much of the information regarding superoxide generation within the Q cycle comes from the use of mitochondrial inhibitors.[25] The generation of superoxide by ubisemiquinione can be prevented by stigmatellin, which inhibits the oxidation of ubiquinol to ubisemiquinone by preventing electron flux to the iron–sulfur protein. In fact, any strategy to prevent electron flux to the iron–sulfur protein, cytochrome *c1*, or cytochrome *c* will prevent the oxidation of ubiquinol to ubisemiquinone, thereby diminishing the generation of superoxide.[25] Antimycin increases the generation of superoxide within the Q cycle by preventing the oxidation of Heme b_H (Fig. 22.3). This increases the steady-state concentration of ubisemiquinone (Q^{-}), thereby increasing the production of superoxide.[26] Inhibitors of cytochrome *c* oxidase such as cyanide or azide can also modulate the production of superoxide by the Q cycle. Acute exposures to these inhibitors will prevent the reduced cytochrome *c* from being oxidized by cytochrome *c* oxidase. The pool of cytochrome *c*, similar to the pool of ubiquinol, is present in excess compared to the other components of the respiratory chain.[27,28] Thus, the pool of cytochrome *c* can continue to accept electrons from ubiquinol via the iron–sulfur protein and cytochrome *c1* even in the presence of cytochrome *c* oxidase inhibitors until the pool of cytochrome *c* becomes fully reduced. This will ensure the generation of superoxide in the presence of cytochrome *c* oxidase inhibitors. However, once the pool of cytochrome *c* becomes fully reduced, no electron flux will occur from ubiquinol oxidation to cytochrome *c*. This will prevent ubiquinol from being oxidized and forming ubisemiquinone and prevent the production of superoxide.

Hypoxia paradoxically increases the generation of ROS from complex III. ROS generation has been primarily detected by the oxidation of 2′,7′-dichlorodihydrofluorescin (DCFH) dye.[19] Oxidation of the dye within the cytosol of the cell yields

the fluorescent compound 2′,7′-dichlorofluorescein (DCF). Cells do not show any substantial increase in the oxidation of DCFH between 21% and 5% O_2. Cells do display a graded increase in the oxidation of DCFH between 5 and 1% O_2. The increase in DCFH oxidation can be prevented by antioxidants that specifically scavenge hydrogen peroxide but not superoxide or nitric oxide. This indicates that this dye is primarily being oxidized by the production of hydrogen peroxide under hypoxia. It is interesting to note that this graded increase in ROS observed during hypoxia is similar in kinetics to the graded increase in HIF-1α protein. The ρ° cells do not display an increase in the oxidation of DCFH during hypoxia.[19] Furthermore, rotenone or stigmatellin prevents the increase in oxidation of DCFH and stabilization of HIF-1α protein during hypoxia.[20] Rotenone inhibits complex I, while stigmatellin blocks electron transfer to the Rieske iron–sulfur center within complex III. Antimycin A, which inhibits the oxidation of heme b_H within complex III, does not decrease the oxidation of DCFH or the accumulation of the HIF-1α protein during hypoxia. Based on the Q cycle, this indicates that hypoxia increases the generation of ROS at the ubisemiquinone site that is proximal to the intermembrane space (Fig. 22.3). Thus, mitochondrial inhibitors such as rotenone or stigmatellin that prevent the formation of ubisemiquinone proximal to intermembrane space diminish ROS generation and HIF-1α protein accumulation during hypoxia. By contrast, mitochondrial inhibitors such as antimycin that prevent the oxidation of ubisemiquinone proximal to the intermembrane space do not diminish ROS generation or the stabilization of HIF-1α protein during hypoxia.

These results are supported genetically by the observation that loss of the Rieske iron–sulfur protein or cytochrome *c* prevents stabilization of HIF-1α protein during hypoxia.[29–31] We further corroborated our findings by utilizing cells deficient in cytochrome *b*, which will maintain a Q_o site of ROS generation. Cytochrome *b* gene is encoded by mtDNA, and current small-interfering RNA (siRNA) techniques do not target mtDNA transcripts. Thus, we utilized cytochrome *b* mutant cybrids, generated by reconstituting 143B ρ° cells with wild-type mtDNA or that contained a 4-bp deletion in the cytochrome *b* gene found in a patient suffering from parkinsonism.[32] Loss of cytochrome *b* renders these cells incapable of oxygen consumption and unable to generate ROS at the Qi site specifically. However, these cells were capable of upregulating hypoxic ROS and stabilizing HIF-1α protein.[33] The administration of the mitochondrial antioxidant mitoquinone (MITOQ) prevented the HIF-1α protein stabilization. We also utilized RNA interference (RNAi) to knock down Rieske iron–sulfur protein to abolish ROS generation at the Q_o site in mutant cytochrome *b* cybrids. This prevented hypoxic ROS generation and HIF-1α protein stabilization, implicating the Q_o cite of complex III as the key site in hypoxic ROS generation and HIF-1α protein stabilization. These cells, however, retained the ability to stabilize HIF-1α protein after direct PHD inhibition by dimethyloxallyl glycine (DMOG), showing an otherwise-intact HIF signaling pathway. Furthermore, we established a link between hypoxic ROS generation and hydroxylation of HIF-1α protein. Neutralizing the ROS with antioxidants allowed HIF-1α protein to remain hydroxylated (and therefore primed for degradation) under hypoxic conditions.[33] On the contrary, increasing ROS levels under normoxia by

overexpressing glucose oxidase prevented normoxic HIF-1α protein hydroxylation. Importantly, these data demonstrated that the ROS generated by the mitochondria under hypoxia prevent hydroxylation of HIF-1α protein (Fig. 22.4).

How might the generation of mitochondrial ROS link to the stabilization of the HIF-1α protein? To date, the best evidence is the requirement of small guanosine triphosphatases (GTPases) such as Rac or Rho as regulators of HIF-1α protein stabilization downstream of mitochondrial electron transport chain. For example, Semenza and colleagues demonstrated that hypoxia stimulates Rac1 activity, and that Rac1 is required for the hypoxic stabilization of HIF-1α protein in human embryonic kidney 293 (HEK-293) cells.[34] Both the hypoxic activation of Rac1 and the stabilization of HIF-1α protein were abolished by the complex I inhibitor rotenone. These results indicate that Rac1 is downstream of mitochondrial signaling. Béliveau and colleagues demonstrated that hypoxia increases ROS production and activates Rho proteins.[35] Inhibitors of ROS production prevent the activation of Rho. HIF-1α protein accumulation during hypoxia is reduced in cells incubated with an inhibitor of RhoA, the toxin C3. These results indicate that mitochondrial-derived ROS elicit an activation of small GTPases to stabilize HIF-1α. Presently, the molecular basis by which ROS activate GTPases remains unknown.

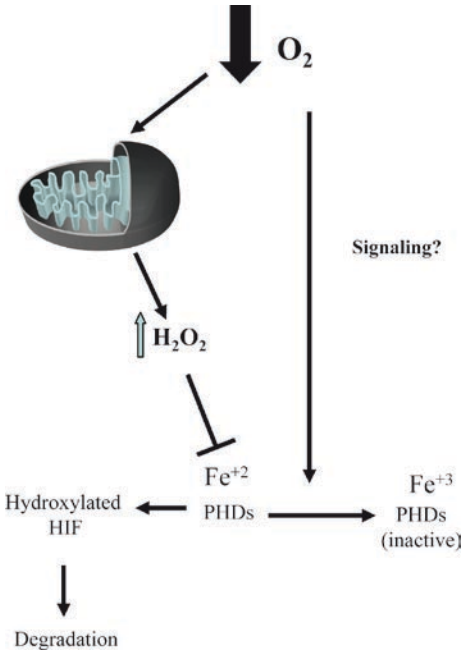

Fig. 22.4 Mitochondrial regulation of PHDs. Hypoxia could both directly decrease the PHDs activity and cause an increase in mitochondrial ROS generation, resulting in diminished PHD activity. Alternatively, hypoxia could invoke signaling pathways that make the PHDs catalytically inactive

3 Mitochondria Regulate Oxygen Demand Through a Decrease in Na/K ATPase Activity

Oxygen consumption of cells is primarily a reflection of the oxygen demands of cellular ATPases. Cells exposed to hypoxia acutely (seconds) do not display a decrease in oxygen consumption.[36] However, as cells are exposed to hypoxia for longer periods (minutes to hours) they display a reversible suppression of oxygen consumption. Cells display this decrease in metabolism under hypoxia in which ATP levels are not compromised and at oxygen levels well above the K_m for cytochrome *c* oxidase. The decrease in oxygen consumption under hypoxia prevents the depletion of oxygen to anoxic levels. During hypoxia, cells that fail to decrease oxygen consumption are likely to become anoxic faster than cells that can suppress their rate of oxygen consumption. The decrease in oxygen consumption during hypoxia dictates a suppression of cellular ATPases. The Na/K ATPase activity alone can account for 20–70% of the oxygen expenditure of mammalian cells.[37] Na/K ATPase is a transmembrane protein found in higher eukaryotes that transports Na^+ and K^+ across the plasma membrane to maintain ionic gradients. Na/K ATPase is a heterodimer composed of α and β subunits.[38] The α subunit is a transmembrane protein that cleaves high-energy phosphate bonds and exchanges intracellular Na^+ for extracellular K^+ coupled to the hydrolysis of ATP. The smaller β subunit is a glycosylated transmembrane molecule that controls the heterodimer assembly and insertion into the plasma membrane. Multiple investigators have reported that hypoxia reversibly suppresses Na/K ATPase activity.[39–41] Classically, the Na/K ATPase activity is thought to be primarily regulated by changes in catalytic activity brought about by changes in affinity for its major substrates. However, reports have demonstrated that the Na/K ATPase activity is regulated by phosphorylation, which results in either endocytosis or exocytosis of this molecule from the plasma membrane to internal compartments.[42] Indeed, exposure to hypoxia for as little as 15 min decreases Na/K ATPase activity. The hypoxia-induced decrease of the Na/K ATPase activity is due to endocytosis of the α subunit from the plasma membrane. Hypoxia does not affect the total Na/K ATPase protein abundance in cell lysates during this short interval (<1 h).

The decrease in the Na/K ATPase activity and the endocytosis of the α subunit from the plasma membrane is also mediated by an increase in mitochondrial ROS.[41] The ρ° cells do not display a decrease in the Na/K ATPase activity or the endocytosis of the α subunit. Rotenone, but not antimycin, prevents the decrease in Na/K ATPase activity during hypoxia. Catalase also prevents the endocytosis of the α subunit, whereas exogenous hydrogen peroxide activates the endocytosis of the α subunit. Thus, ROS are required and sufficient for the decrease in Na/K ATPase activity during hypoxia.

How might the generation of mitochondrial ROS mediate the endocytosis of the α subunit? One possible mechanism is through the activation of protein kinase C (PKC) by ROS. Physiological levels of ROS can activate PKC. Exposure of hydrogen peroxide can result in the tyrosine phosphorylation and activation of various

PKC isoforms.[43] PKC phosphorylates the serine-18 residue within the α subunit of the Na/K ATPase, which signals and targets the α subunit for endocytosis.[44] Hypoxia-induced phosphorylation and endocytosis of the Na/K ATPase is dependent on PKC.[41] A mutation in the serine-18 residue of the α subunit prevents the hypoxia-induced endocytosis of Na/K ATPase. This mechanism of regulated endocytosis is specific as there is no decrease in the levels of the glucose transporter GLUT-1. Finally, other key proteins that require ATP may be downregulated, but this requires further studies.

4 Controversies in Oxygen Sensing

We propose a model in which hypoxia induces the increased generation of ROS within complex III, resulting in activation of signaling cascades (e.g., GTPase, PKC) that result in the decrease in Na/K ATPase activity and the activation of HIF-1 (Fig. 22.5). Despite progress in identifying mitochondria as a potential component of an oxygen-sensing mechanism, some concerns have been raised with respect to this model. These include (1) whether prolyl hydroxylases alone are the

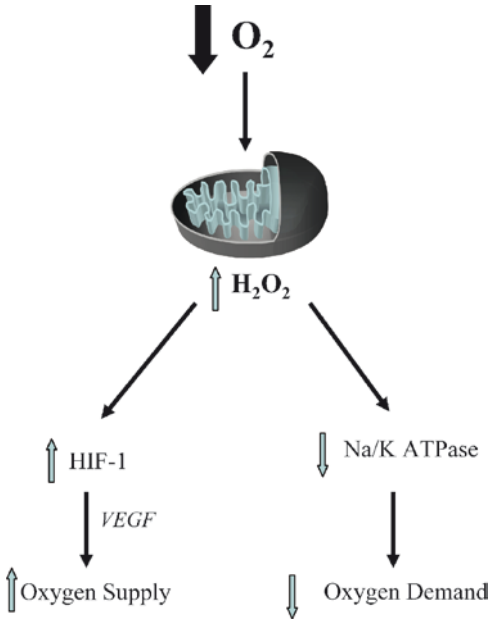

Fig. 22.5 Mitochondrial complex III regulates oxygen sensing. We propose that complex III within the mitochondrial electron transport chain generates ROS during hypoxia to (1) increase HIF-1-dependent gene transcription to stimulate oxygen supply and (2) decrease the activity of Na/K ATPase to diminish cellular oxygen demand

oxygen sensors; (2) whether hypoxia increases the production of ROS; (3) whether the mitochondrial electron transport chain plays a significant role in oxygen sensing. Next, we explore the basis of these controversies.

4.1 Do Prolyl Hydroxylases Serve as Oxygen Sensors?

The discovery of prolyl hydroxylases as the regulators of the stabilization of HIF-1α protein levels raised the possibility that the prolyl hydroxylases might serve as oxygen sensors.[45] These hydroxylases utilize oxygen as a substrate for the hydroxylation reaction and would make ideal candidates as oxygen sensors. Furthermore, iron is also required for the hydroxylation reaction. Thus, prolyl hydroxylases could explain the effects of hypoxia and iron chelators on HIF-1α protein stabilization. It is clear that in the complete absence of oxygen the prolyl hydroxylases serve as oxygen sensors since they cannot carry out the hydroxylation of the HIF-1α protein. However, it is less clear that they serve as oxygen sensors in the hypoxic region (1–3% O_2). To fulfill this role, the hydroxylases would have to have a K_m in the hypoxic region. Recombinant prolyl hydroxylases have a K_m of ambient air (20.9% O_2) in vitro, indicating that the prolyl hydroxylases are decreasing their enzymatic activity throughout the physiological range of PO_2.[46] Therefore, if the prolyl hydroxylases were in fact the sensors, one would predict a continuous increase in the accumulation of HIF-1α protein as oxygen levels fall from 21% O_2 to 0% O_2. In fact, the HIF-1α protein begins to accumulate around 5% O_2, and its concentration increases as the oxygen levels approach anoxia.[47] It would be important to decipher the K_m of prolyl hydroxylases in cultured cells.

Another implication of prolyl hydroxylases as the oxygen sensors is that there would be no need for the activation of signaling pathways during hypoxia for the stabilization of HIF-1α protein. The first hint that hypoxic signaling is likely to be different from iron chelation came from the observation that diphenylene iodonium (DPI), an inhibitor of a wide range of flavoproteins, including complex I, prevents stabilization of HIF-1α protein and HIF-1 target genes at oxygen levels of 1% but not in the presence of iron chelators under normoxia.[48] Subsequently, the literature is abundant with examples of the requirement of signaling molecules for stabilization of the HIF-1α protein during hypoxia. These include but are not limited to the requirement of diacylglycerol kinase, small GTPases, ROS, and Phosphainositide 3-Kinase is PI3K an AKT is protein Kinase B (PI3K/AKT). We interpret these findings as suggesting that the prolyl hydroxylases might be modified by signaling molecules within cells to allow the K_m of the enzyme to change from 21% O_2 (in vitro) to a K_m in the hypoxic region. A K_m of PHDs in the hypoxic region would theoretically fit with the experimental observation of the levels of HIF-1α protein as a function of lower oxygen. A major step in understanding HIF-1α protein stabilization during hypoxia would be to elucidate the oxygen dependence of the proline hydroxylation reaction of HIF-1α protein in cultured cells.

4.2 Does Hypoxia Increase the Production of ROS?

The most confounding observation about mitochondrial ROS as a component of an oxygen-sensing model is that hypoxia increases the production of mitochondrial ROS. At first, this observation seems paradoxical; however, the levels of oxygen are sufficient to generate nanomolar concentrations of ROS under hypoxia. Multiple investigators have confirmed our initial observations demonstrating the oxidation of DCFH during hypoxia in cell culture models and in whole-animal models.[49,50] However, there have been reports in the literature demonstrating a decrease in ROS levels utilizing other dyes that measure oxidative stress, such as dihydrorhodamine 123 or horseradish peroxidase (HRP)-enhanced luminol chemiluminescence.[51] All of these dyes have limitations in their measurements of intracellular oxidative stress.[52]

Poyton and colleagues utilized oxidative protein carbonylation to assess whether mitochondria increase oxidative stress during low oxygen concentrations.[53] They observed that yeast cells increased protein carbonylation, and the mitochondrial respiratory chain was responsible for this carbonylation. Gillespie and colleagues demonstrated that hypoxia causes oxidative lesions in nuclear DNA in mammalian cells exposed to hypoxia.[54] Perhaps the best analytical approach that permits the direct detection of free radicals is electron spin resonance (ESR). Presently, there is only a single study that examined radical formation by ESR using the spin trap 5-diethyoxyphospharyl-5 methyl-1-pyrroline N-oxide (DEPMPO) during hypoxia.[55] This study demonstrated that porcine pulmonary arteries increased production of hydroxyl and alkyl radicals, and that this increase was inhibited by superoxide dismutase. It is likely that hypoxia increases ROS production; however, the molecular basis by which lower oxygen concentration increases mitochondrial ROS production remains unknown.

4.3 Does the Mitochondrial Electron Transport Chain Play a Significant Role in Oxygen Sensing?

The major evidence that the mitochondrial electron transport chain regulates stabilization of the HIF-1α protein is the observation that ρ° cells and mitochondrial inhibitors prevent the formation of ubisemiquinone within the Q cycle of complex III, preventing hypoxic stabilization of the HIF-1α protein. Multiple studies have demonstrated that ρ° cells do not stabilize the HIF-1α protein during hypoxia (1–3% O_2), and a variety of complex I inhibitors, including rotenone, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and DPI prevent hypoxic stabilization of the HIF-1α protein.[20,56] However, there are studies that indicated some ρ° cells are still able to stabilize HIF-1α protein levels.[57–59]

What is the basis of this inconsistency? Two potential reasons might explain this discrepancy. First, in some studies the response of ρ° cells was examined at oxygen concentrations close to 0% O_2 (anoxia), while others were in the range of 1–3% O_2. Oxygen is required as a substrate for the hydroxylation of the HIF-1α protein, and in the absence of oxygen this reaction would not occur. HIF-1α protein will not be hydroxylated and targeted for ubiquitin-mediated degradation under anoxia due to

substrate limitation. We have demonstrated that ρ° cells do not stabilize HIF-1α protein levels under hypoxia but are still able to stabilize HIF-1α protein levels under anoxia.[1] This is consistent with the idea that when cells are subjected to conditions close to 0% O_2, the proline hydroxylation reaction would become substrate limited, eliminating the requirement for any signaling molecules. A second reason for the failure of some ρ° cells to prevent hypoxic stabilization of HIF-1α protein might be that ρ° cells are still able to generate ROS from mitochondria. Mitochondrial DNA encodes subunits for complex I, III, and IV of the electron transport chain but not complex II. The ρ° cells have a functional complex II and have been shown to generate ROS through this complex.[60] Furthermore, in some cell types complex II has been shown to regulate the hypoxic increase in ROS production.[61] Thus, in some cells complex II, in addition to complex III, might contribute to significant ROS generation for the stabilization of the HIF-1α protein. We predict that only in the complete absence of mitochondrial ROS generation during hypoxia should cells then be unable to stabilize the HIF-1α protein.

Moncada and colleagues suggested an alternative explanation for the discrepancy in results obtained with respiratory inhibitors of ρ° cells.[62] They demonstrated that inhibiting respiration at any complex within the electron transport chain is sufficient to prevent stabilization of the HIF-1α protein at 1% O_2. They suggested that cells consuming oxygen would likely be experiencing much lower intracellular oxygen levels compared with ρ° cells or cells incubated with respiratory inhibitors. In the presence of respiratory inhibitors, cells exposed to 1% O_2 will have intracellular oxygen levels closer to 1% O_2 and will allow the prolyl hydroxylases to effectively hydroxylate the HIF-1α protein. In this model, the mitochondria were regulating the availability of intracellular oxygen to prolyl hydroxylases. Based on their model, the prolyl hydroxylases would decrease their rate of hydroxylation of the HIF-1α protein significantly only when oxygen tensions fall below 1% O_2. This would make the hydroxylases regulating HIF-1α protein stabilization in a very narrow range of oxygen levels (0–1%) and suggests that the HIF-1α protein will not be stabilized if the intracellular oxygen levels are 1% O_2 or greater. These data are not compatible with previous observations that the increase in HIF-1α protein levels begins around 5% O_2 and continues to increase exponentially as the oxygen levels approach 0% O_2.[47] Furthermore, respiratory-deficient cells such as cytochrome *b* null cells that can still increase ROS are able to stabilize HIF-1α protein during hypoxia.[33]

5 Conclusions and Perspectives

Much remains to be learned about how cells sense low oxygen. Currently, there is confusion in defining the oxygen levels that investigators utilize to study the biology of low oxygen. Clearly, the biology of cells exposed to oxygen levels close to 0% O_2 (anoxia) is different from cells exposed to 1–3% O_2 (hypoxia). There might be completely different oxygen-sensing mechanisms under hypoxia as compared to anoxia. Since cells exposed to hypoxia display multiple responses from the increase in gene transcription to the decrease in the Na/K ATPase activity, a fundamental

question arises whether multiple O_2 sensors exist in the same cell or whether a single O_2 sensor regulates the diverse responses to hypoxia. Currently, there are multiple oxygen-sensing models to explain the diverse biological effects of hypoxia. The advent of genetic tools such as RNAi is likely to yield new insights into which putative oxygen sensors are required in different mammalian cell types for divergent biological responses to hypoxia or anoxia.

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References

1. Schroedl C, McClintock DS, Budinger GRS, Chandel NS (2002) Hypoxic but not anoxic stabilization of HIF-1α requires mitochondrial reactive oxygen species. Am J Physiol Lung Cell Mol Physiol 283:L922–L931
2. Semenza GL (1999) Regulation of mammalian O_2 homeostasis by hypoxia-inducible factor 1. Annu Rev Cell Dev Biol 15:551–578
3. Planès C, Friedlander G, Loiseau A, Amiel C, Clerici C (1996) Inhibition of Na,K-ATPase activity after prolonged hypoxia in an alveolar epithelial cell line. Am J Physiol 271:L71–L78
4. Chandel NS, Budinger GR, Schumacker PT (1996) Molecular oxygen modulates cytochrome c oxidase function. J Biol Chem 271:18672–18677
5. Aw TY, Jones DP (1982) Secondary bioenergetic hypoxia. Inhibition of sulfation and glucuronidation reactions in isolated hepatocytes at low O_2 concentration. J Biol Chem 257:8997–9004
6. Forsythe JA, Jiang BH, Iyer NV et al (1996) Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol 16:4604–4613
7. Wang GL, Jiang BH, Rue EA, Semenza GL (1995) Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. Proc Natl Acad Sci U S A 92:5510–5514
8. Jiang BH, Zheng JZ, Leung SW, Roe R, Semenza GL (1997) Transactivation and inhibitory domains of hypoxia-inducible factor 1α modulation of transcriptional activity by oxygen tension. J Biol Chem 272:19253–19260
9. Maxwell PH, Wiesener MS, Chang GW et al (1999) The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature 399:271–275
10. Jaakkola P, Mole DR, Tian YM et al (2001) Targeting of HIF-α to the von Hippel–Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. Science 292:468–472
11. Masson N, Willam C, Maxwell PH, Pugh CW, Ratcliffe PJ (2001) Independent function of two destruction domains in hypoxia-inducible factor-α chains activated by prolyl hydroxylation. EMBO J 20:5197–5206
12. Ivan M, Kondo K, Yang H et al (2001) HIFα targeted for VHL-mediated destruction by proline hydroxylation: implications for O_2 sensing. Science 292:464–468
13. Epstein AC, Gleadle JM, McNeill LA et al (2001) C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell 107:43–54
14. Lando D, Peet DJ, Gorman JJ, Whelan DA, Whitelaw ML, Bruick R (2002) FIH-1 is an asparaginyl hydroxylase that regulates the transcriptional activity of hypoxia inducible factor. Genes Dev 16:1466–1471
15. Lando D, Peet DJ, Whelan DA, Gorman JJ, Whitelaw ML (2002) Asparagine hydroxylation of the HIF transactivation domain: a hypoxic switch. Science 295:858–861
16. Mahon PC, Hirota K, Semenza GL (2001) FIH-1: a novel protein that interacts with HIF-1α and VHL to mediate repression of HIF-1 transcriptional activity. Genes Dev 15:2675–2686

17. Bunn HF, Poyton RO (1996) Oxygen sensing and molecular adaptation to hypoxia. Physiol Rev 76:839–885
18. Srinivas V, Zhu X, Salceda S, Nakamura R, Caro J (1998) Hypoxia-inducible factor 1α (HIF-1α) is a non-heme iron protein. Implications for oxygen sensing. J Biol Chem 273:18019–18022
19. Chandel NS, Maltepe E, Goldwasser E, Mathieu CE, Simon MC, Schumacker PT (1998) Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. Proc Natl Acad Sci U S A 95:5015–5019
20. Chandel NS, McClintock DS, Feliciano SE et al (2000) Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1α during hypoxia: a mechanism of O_2 sensing. J Biol Chem 275:25130–25138
21. King MP, Attardi G (1988) Injection of mitochondria into human cells leads to a rapid replacement of the endogenous mitochondrial DNA. Cell 52:811–819
22. Turrens JF (2003) Mitochondrial formation of reactive oxygen species. J Physiol 552:335–344
23. Hunte C, Palsdottir H, Trumpower BL (2003) Protonmotive pathways and mechanisms in the cytochrome bc1 complex. FEBS Lett 545:39–46
24. Han D, Antunes F, Canali R, Rettori D, Cadenas E (2003) Voltage-dependent anion channels control the release of the superoxide anion from mitochondria to cytosol. J Biol Chem 278:5557–5563
25. Turrens JF, Alexandre A, Lehninger AL (1985) Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. Arch Biochem Biophys 237:408–414
26. Boveris A, Cadenas E, Stoppani AO (1976) Role of ubiquinone in the mitochondrial generation of hydrogen peroxide. Biochem J 156:435–444
27. Gupte S, Wu ES, Hoechli L et al (1984) Relationship between lateral diffusion, collision frequency, and electron transfer of mitochondrial inner membrane oxidation-reduction components. Proc Natl Acad Sci U S A 81:2606–2610
28. Kroger A, Klingenberg M (1973) Further evidence for the pool function of ubiquinone as derived from the inhibition of the electron transport by antimycin. Eur J Biochem 39:313–323
29. Mansfield KD, Guzy RD, Pan Y et al (2005) Mitochondrial dysfunction resulting from loss of cytochrome c impairs cellular oxygen sensing and hypoxic HIF-alpha activation. Cell Metab 1:393–399
30. Guzy RD, Hoyos B, Robin E et al (2005) Mitochondrial complex III is required for hypoxia-induced ROS production and cellular oxygen sensing. Cell Metab 1:401–408
31. Brunelle JK, Bell EL, Quesada NM et al (2005) Oxygen sensing requires mitochondrial ROS but not oxidative phosphorylation. Cell Metab 1:409–414
32. Rana M, de Coo I, Diaz F, Smeets H, Moraes CT (2000) An out-of-frame cytochrome b gene deletion from a patient with parkinsonism is associated with impaired complex III assembly and an increase in free radical production. Ann Neurol 48:774–781
33. Bell EL, Klimova TA, Eisenbart J et al (2007) The Qo site of the mitochondrial complex III is required for the transduction of hypoxic signaling via reactive oxygen species production. J Cell Biol 177:1029–1036
34. Hirota K, Semenza GL (2001) Rac1 activity is required for the activation of hypoxia-inducible factor 1. J Biol Chem 276:21166–21172
35. Turcotte S, Desrosiers RR, Beliveau R (2003) HIF-1α mRNA and protein upregulation involves Rho GTPase expression during hypoxia in renal cell carcinoma. J Cell Sci 116:2247–2260
36. Chandel NS, Budinger GR, Choe SH, Schumacker PT (1997) Cellular respiration during hypoxia. Role of cytochrome oxidase as the oxygen sensor in hepatocytes. J Biol Chem 272:18808–18816
37. Milligan LP, McBride BW (1985) Energy costs of ion pumping by animal tissues. J Nutr 115:1374–1382
38. Kaplan JH (2002) Biochemistry of Na,K-ATPase. Annu Rev Biochem 71:511–535
39. Carpenter TC, Schomberg S, Nichols C, Stenmark KR, Weil JV (2003) Hypoxia reversibly inhibits epithelial sodium transport but does not inhibit lung ENaC or Na-K-ATPase expression. Am J Physiol Lung Cell Mol Physiol 284:L77–L83
40. Mairbaurl H, Wodopia R, Eckes S, Schulz S, Bartsch P (1997) Impairment of cation transport in A549 cells and rat alveolar epithelial cells by hypoxia. Am J Physiol Lung Cell Mol Physiol 273:L797–L806

41. Dada LA, Chandel NS, Ridge KM, Pedemonte C, Bertorello AM, Sznajder JI (2003) Hypoxia-induced endocytosis of Na,K-ATPase in alveolar epithelial cells is mediated by mitochondrial reactive oxygen species and PKC-ς. J Clin Invest 111:1057–1064
42. Chibalin AV, Ogimoto G, Pedemonte CH et al (1999) Dopamine-induced endocytosis of Na^+,K^+-ATPase is initiated by phosphorylation of Ser-18 in the rat α subunit and is responsible for the decreased activity in epithelial cells. J Biol Chem 274:1920–1927
43. Konishi H, Tanaka M, Takemura Y et al (1997) Activation of protein kinase C by tyrosine phosphorylation in response to H_2O_2. Proc Natl Acad Sci U S A 94:11233–11237
44. Feschenko MS, Sweadner KJ (1997) Phosphorylation of Na,K-ATPase by protein kinase C at Ser18 occurs in intact cells but does not result in direct inhibition of ATP hydrolysis. J Biol Chem 272:17726–17733
45. Bruick RK (2003) Oxygen sensing in the hypoxic response pathway: regulation of the hypoxia-inducible transcription factor. Genes Dev 17:2614–2623
46. Hirsila M, Koivunen P, Gunzler V, Kivirikko KI, Myllyharju J (2003) Characterization of the human prolyl 4-hydroxylases that modify the hypoxia-inducible factor. J Biol Chem 278:30772–30780
47. Jiang BH, Semenza GL, Bauer C, Marti HH (1996) Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O_2 tension. Am J Physiol 271:C1172-C1180
48. Gleadle JM, Ebert BL, Ratcliffe PJ (1995) Diphenylene iodonium inhibits the induction of erythropoietin and other mammalian genes by hypoxia. Implications for the mechanism of oxygen sensing. Eur J Biochem 234:92–99
49. Killilea DW, Hester R, Balczon R, Babal P, Gillespie MN (2000) Free radical production in hypoxic pulmonary artery smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 279:L408-L412
50. Wood JG, Johnson JS, Mattioli LF, Gonzalez NC (1999) Systemic hypoxia promotes leukocyte-endothelial adherence via reactive oxidant generation. J Appl Physiol 87:1734–1740
51. Fandrey J, Frede S, Jelkmann W (1994) Role of hydrogen peroxide in hypoxia-induced erythropoietin production. Biochem J 303:507–510
52. Tarpey MM, Fridovich I (2001) Methods of detection of vascular reactive species: nitric oxide, superoxide, hydrogen peroxide, and peroxynitrite. Circ Res 89:224–236
53. Dirmeier R, O'Brien KM, Engle M, Dodd A, Spears E, Poyton RO (2002) Exposure of yeast cells to anoxia induces transient oxidative stress. Implications for the induction of hypoxic genes. J Biol Chem 277:34773–34784
54. Grishko V, Solomon M, Breit JF et al (2001) Hypoxia promotes oxidative base modifications in the pulmonary artery endothelial cell VEGF gene. FASEB J 15:1267–1269
55. Liu Q, Kuppusamy P, Sham JSK, Shimoda LA, Zweier JL, Sylvester JT (2001) Increased production of reactive oxygen species (ROS) by pulmonary arterial smooth muscle is required for hypoxic pulmonary vasoconstriction (HPV). Am J Respir Crit Care Med 163:A395
56. Agani FH, Pichiule P, Chavez JC, LaManna JC (2000) The role of mitochondria in the regulation of hypoxia-inducible factor 1 expression during hypoxia. J Biol Chem 275:35863–35867
57. Enomoto N, Koshikawa N, Gassmann M, Hayashi J, Takenaga K (2002) Hypoxic induction of hypoxia-inducible factor-1α and oxygen-regulated gene expression in mitochondrial DNA-depleted HeLa cells. Biochem Biophys Res Commun 297:346–352
58. Srinivas V, Leshchinsky I, Sang N, King MP, Minchenko A, Caro J (2001) Oxygen sensing and HIF-1 activation does not require an active mitochondrial respiratory chain electron-transfer pathway. J Biol Chem 276:21995–21998
59. Vaux EC, Metzen E, Yeates KM, Ratcliffe PJ (2001) Regulation of hypoxia-inducible factor is preserved in the absence of a functioning mitochondrial respiratory chain. Blood 98:296–302
60. Miranda S, Foncea R, Guerrero J, Leighton F (1999) Oxidative stress and upregulation of mitochondrial biogenesis genes in mitochondrial DNA-depleted HeLa cells. Biochem Biophys Res Commun 258:44–49
61. Paddenberg R, Ishaq B, Goldenberg A et al (2003) Essential role of complex II of the respiratory chain in hypoxia-induced ROS generation in the pulmonary vasculature. Am J Physiol Lung Cell Mol Physiol 284:L710-L719
62. Hagen T, Taylor CT, Lam F, Moncada S (2003) Redistribution of intracellular oxygen in hypoxia by nitric oxide: effect on HIF1α.Science 302:1975–1978

Reactive Oxygen Species and RhoA Signaling in Vascular Smooth Muscle: Role in Chronic Hypoxia-Induced Pulmonary Hypertension

Thomas C. Resta, Brad R. S. Broughton, and Nikki L. Jernigan

Abstract Increases in myofilament Ca^{2+} sensitivity resulting from stimulation of RhoA and Rho kinase represent a primary mechanism of vasoconstriction and associated pulmonary hypertension resulting from chronic hypoxia (CH). This chapter summarizes recent advances in the understanding of RhoA/Rho kinase signaling mechanisms in pulmonary vascular smooth muscle (VSM) that increase the sensitivity of the contractile apparatus to Ca^{2+} and contribute to vasoconstriction in this setting. Such advances include the discovery of myogenic tone in small pulmonary arteries from CH rats that contributes to vasoconstriction through a mechanism inherent to the VSM, dependent on Rho kinase-induced Ca^{2+} sensitization but independent of L-type voltage-gated Ca^{2+} channels. Additional studies have revealed an important contribution of superoxide anion (O_2^-)-induced RhoA activation to both receptor-mediated and membrane depolarization-induced myofilament Ca^{2+} sensitization in hypertensive pulmonary arteries. Xanthine oxidase and NADPH oxidase isoforms are potential sources of O_2^- that mediate RhoA-dependent vasoconstriction and associated pulmonary hypertension.

Keywords Myofilament Ca^{2+} sensitization • Rho kinase • myogenic tone • membrane depolarization • endothelin-1 • superoxide anion

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1 Chronic Hypoxia Augments RhoA/Rho Kinase-Mediated Ca^{2+} Sensitization in Pulmonary Vascular Smooth Muscle: A New Paradigm of Pulmonary Hypertension

Chronic hypoxia (CH) associated with obstructive lung diseases, sleep apnea, and high-altitude exposure leads to increased pulmonary vascular resistance and pulmonary hypertension. The resulting increase in afterload on the right heart often leads to clinical manifestations of cor pulmonale and peripheral edema. The pulmonary vasoconstrictor component of CH-induced pulmonary hypertension is widely thought to be mediated by generalized hypoxic pulmonary vasoconstriction resulting from global airway hypoxia. However, hypoxic vasoreactivity is largely blunted following long-term hypoxic exposure,[1] suggesting that additional mechanisms provide a major contribution to the development of CH-induced pulmonary hypertension. Indeed, the pulmonary hypertension in conscious rats that persists on acute return to a normoxic environment is substantial[2] and until recently has been considered to be mediated largely by fixed increases in pulmonary vascular resistance resulting from arterial remodeling and increases in blood viscosity associated with polycythemia. However, polycythemia alone does not induce pulmonary hypertension,[3] and morphometric studies of the hypertensive pulmonary circulation in rats have revealed that proliferation and hypertrophy of pulmonary vascular smooth muscle (VSM) resulting from CH do not significantly reduce the luminal diameter of small pulmonary arteries[4] and thus would not be expected to increase pulmonary vascular resistance. Furthermore, findings that basal tone is elevated in pulmonary arterial rings isolated from CH rats studied under normoxic conditions,[5–7] that CH augments pulmonary vasoconstrictor reactivity to receptor-mediated agonists, and that vasodilators dramatically lower pulmonary vascular resistance in CH rats returned to a normoxic environment[5,8] provide intriguing evidence that the vasoconstrictor response to CH is multifaceted (Fig. 23.1).

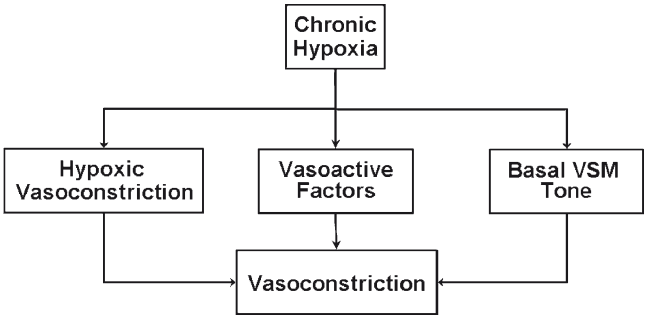

Fig. 23.1 Vasoconstrictor mechanisms in addition to acute hypoxic vasoconstriction, including enhanced arterial reactivity to endogenous vasoactive factors and increased basal vascular smooth muscle (VSM) tone, may play a primary role in CH-mediated increases in pulmonary vascular resistance and resultant hypertension

Consistent with these findings are recent studies that have identified a novel effect of CH to enhance both basal arterial tone and agonist-dependent vasoconstriction through a RhoA–Rho kinase (ROCK)-mediated Ca^{2+} sensitization pathway in pulmonary VSM,[5,6,9–12] a response that may contribute to the pathogenesis of pulmonary hypertension[13–15] (a more in-depth analysis of the contribution of ROCK-dependent vasoconstriction to pulmonary hypertension is provided in Chap. 13). The objective of the present chapter is to summarize RhoA signaling mechanisms in VSM and how these are altered in CH-induced pulmonary hypertension to mediate elevated basal tone and vasoconstriction to receptor-mediated agonists and depolarizing stimuli.

2 RhoA/ROCK Signaling: A Central Component of VSM Ca^{2+} Sensitization

Phosphorylation of the 20 kDa regulatory myosin light chain (MLC) is a key determinant of VSM contraction, allowing increased myosin adenosine triphosphatase (ATPase) activity and subsequent cross-bridge cycling. The phosphorylation state of MLC is determined largely by the balance of activities of Ca^{2+}/calmodulin-activated myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) (Fig. 23.2). It is well established that MLCP activity is highly regulated to

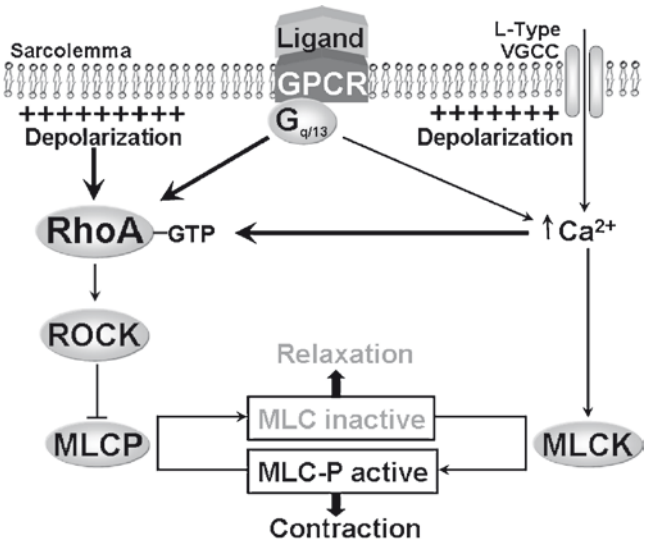

Fig. 23.2 Stimuli for RhoA activation include membrane depolarization, which can activate RhoA through both Ca^{2+}-independent and Ca^{2+}-dependent mechanisms, and stimulation of $G_{q/13}$-coupled receptors (GPCRs). *VGCC* voltage-gated Ca^{2+} channel, *ROCK* Rho kinase, *MLCP* myosin light chain phosphatase, *MLCK* myosin light chain kinase, *MLC* myosin light chain

mediate changes in the sensitivity of the contractile apparatus to Ca^{2+} and thus VSM tone.[16] For example, stimulation of many G protein-coupled receptors leads to inactivation of MLCP, allowing accumulation of phosphorylated MLC and greater contraction for a given concentration of cytosolic free Ca^{2+} ($[Ca^{2+}]_i$). This phenomenon of MLC phosphorylation occurring independently of changes in VSM $[Ca^{2+}]_i$ is referred to as *myofilament Ca^{2+} sensitization*. Activation of the RhoA/ROCK pathway and consequent inhibition of MLCP are central to receptor-mediated Ca^{2+} sensitization of systemic VSM, although roles for protein kinase C (PKC)-dependent inhibition of MLCP and MLCP-independent mechanisms have also been implicated.

RhoA is a small guanosine triphosphate (GTP)-binding protein that is activated in response to stimulation of many G protein-coupled receptors[13] (Fig. 23.2). Activation of RhoA requires Rho-specific guanine nucleotide exchange factors (RhoGEFs) that catalyze the exchange of guanosine diphosphate (GDP) for GTP on cytoplasmic RhoA. The activated RhoA–GTP complex then undergoes translocation to the sarcolemma and stimulates ROCK. RhoA activity is further regulated by Rho guanosine triphosphatase (GTPase)-activating proteins, which inactivate RhoA by dephosphorylating GTP to GDP, and guanine dissociation inhibitors (GDIs), which inhibit membrane translocation of RhoA from the cytosol.

RhoA-induced stimulation of ROCK leads to decreased MLCP catalytic activity and consequent myofilament Ca^{2+} sensitization[13,16] (Fig. 23.2). MLCP is a heterotrimer consisting of a myosin-binding subunit MYPT1, a catalytic subunit PP1c, and a small noncatalytic subunit of unknown function. Phosphorylation of MYPT1 by ROCK at Thr^{696} and Thr^{853} induces dissociation of MYPT1 from PP1c, thereby inhibiting catalytic activity. ROCK can also inhibit MLCP indirectly through phosphorylation of CPI-17, a smooth muscle-specific phosphoprotein that inhibits the catalytic subunit of MLCP. Further evidence suggests that ROCK directly phosphorylates MLC at Ser^{19}, the same amino acid that is phosphorylated by MLCK, although the functional significance of this response is not well defined. Two isoforms of ROCK have been identified in the pulmonary circulation: ROCK1 (or ROKβ) and ROCK2 (or ROKα).[4,9,17,18] The currently available ROCK inhibitors, including Y-27632, fasudil hydrochloride (HA-1077), and HA-1152P, exhibit relatively high selectivity for ROCK and equally inhibit ROCK1 and ROCK2 by competing with ATP binding.[19]

In addition to activation of RhoA signaling by G protein-coupled receptors, ROCK-mediated VSM Ca^{2+} sensitization can also be elicited by depolarizing stimuli.[20–23] Although membrane potential depolarization-induced VSM contraction appears to be mediated in part via Ca^{2+}-dependent stimulation of RhoA/ROCK,[20–23] a role for depolarization to stimulate this pathway independent of changes in $[Ca^{2+}]_i$ has been suggested in renal tubule epithelial cells[24] (Fig. 23.2). Preliminary evidence supports a similar effect of membrane depolarization to induce pulmonary VSM Ca^{2+} sensitization independent of changes in VSM $[Ca^{2+}]_i$ in small pulmonary arteries from rats.[25,26] Whereas this effect of depolarization is independent of ROCK in normotensive pulmonary arteries, a contribution of ROCK is evident in pulmonary VSM from CH rats.

3 Role of RhoA/ROCK in the Development of CH-Induced Pulmonary Hypertension

Studies of chronic ROCK inhibition in both mice and rats have revealed a role for this enzyme in the development of pulmonary hypertension. For example, Hyvelin et al.[4] found that chronic ROCK inhibition with Y-27632 attenuated the right ventricular hypertrophy associated with pulmonary hypertension in CH rats. Studies by Fagan et al.[27] demonstrated a similar inhibitory effect of Y-27632 on right ventricular systolic pressure, right ventricular hypertrophy, and pulmonary arterial neomuscularization in CH mice. Considering the emerging role for RhoA/ROCK as a mediator of systemic vascular remodeling,[28] it is possible that ROCK directly contributes to the arterial remodeling component of pulmonary hypertension. However, an alternative possibility is that ROCK contributes to arterial remodeling secondary to direct vasoconstrictor actions that induce pulmonary hypertension. Indeed, a role for ROCK-mediated vasoconstriction in CH-induced pulmonary hypertension in rats has been provided by Nagaoka et al.,[5] who found that intravenous administration of the ROCK inhibitor Y-27632 dose-dependently reduced both mean pulmonary arterial pressure and total pulmonary resistance in conscious, chronically instrumented CH rats that had been acutely returned to normoxia. Interestingly, the highest dose of Y-27632 normalized total pulmonary resistance between control and CH rats. Similar acute reductions in mean pulmonary arterial pressure were observed in response to inhaled Y-27632 and fasudil in CH rats[8] as well as to intravenous administration of ROCK inhibitors to anesthetized CH rats[4] and neonatal rats with pulmonary hypertension.[29] These findings suggest that vasoconstrictor mechanisms involving ROCK are of greater importance in mediating CH-induced pulmonary hypertension in rats than fixed components of hypertension (i.e., arterial remodeling and polycythemia).

Whether ROCK-induced pulmonary hypertension represents a mechanism intrinsic to the VSM or rather involves alterations in endothelial control of vascular tone is not fully understood. Consistent with known inhibitory influences of ROCK on endothelial nitric oxide synthase (eNOS) messenger RNA (mRNA) stability,[30] ROCK inhibition is associated with increased eNOS expression in lungs from pulmonary hypertensive mice[27,31] and in pulmonary arterial endothelial cells from fetal sheep.[32] Further evidence supports a role for RhoA or ROCK to stimulate Ca^{2+} influx in smooth muscle through voltage-gated or nonselective cation channels.[33–37] Nevertheless, studies have implicated a major contribution of ROCK-dependent VSM Ca^{2+} sensitization to enhanced vasoconstrictor sensitivity in hypertensive pulmonary arteries as detailed in sections 4–7 below.

4 Contribution of RhoA/ROCK Signaling to Increased Basal Pulmonary Arterial Tone Following CH

Although greater Ca^{2+} entry through L-type channels or store-operated cation channels in pulmonary VSM may contribute to increased basal pulmonary arterial tone following CH,[38,39] studies have begun to examine an alternative hypothesis that

RhoA/ROCK-mediated Ca^{2+} sensitization is central to this vasoconstrictor mechanism. Consistent with this hypothesis are observations that ROCK inhibitors reverse nitric oxide synthase (NOS) inhibition-induced vasoconstriction in isolated lungs from CH rats,[5] whereas inhibitors of MLCK, L-type Ca^{2+} channels, tyrosine kinases, and PKC caused little or no reduction of basal tone.[5] A similar increase in ROCK-dependent basal tone has been observed in endothelium-intact arterial rings[5] and pressurized, small pulmonary arteries from CH rats.[10]

Increases in basal tone in hypertensive pulmonary arteries have also been demonstrated in endothelium-denuded preparations,[6,11] suggesting this vasoconstrictor mechanism is intrinsic to the VSM. However, discrepancies exist with respect to the contribution of ROCK to this response. For example, Weigand et al.[6] found that elevated basal tone in endothelium-denuded, isolated pulmonary arterial rings from CH rats was unaltered by ROCK inhibitors but was significantly reduced by the MLCK inhibitor ML-9. In contrast, studies from our laboratory using isolated, pressurized pulmonary arteries support an important contribution of ROCK to this response.[11] Our initial observations of elevated basal VSM tone following CH came from studies demonstrating that the nitric oxide (NO) donor (Z)-1-[N-[3-aminopropyl]-N-[4-(3-aminopropylammonio)butyl]-amino]diazen-1-ium-1,2-diolate (spermine NONOate) elicited vasodilation in isolated, endothelium-denuded pulmonary arteries from CH rats but not controls under resting conditions. Furthermore, this NO-mediated reduction in basal tone in CH arteries occurred without a change in VSM $[Ca^{2+}]_i$, thus implicating a Ca^{2+} sensitization mechanism in mediating increased VSM tone following CH.

Considering our earlier observations that CH mediates a shift in NO signaling to mechanisms involving protein kinase G (PKG)-dependent inhibition of RhoA/ROCK-induced VSM Ca^{2+} sensitization,[9] we examined the hypothesis that elevated basal tone in CH arteries is dependent on VSM ROCK activity. Consistent with this possibility is evidence that CH increases basal intrapulmonary arterial RhoA activity and ROCK2 expression.[9,10] Furthermore, the selective ROCK inhibitor HA-1077 produced a concentration-dependent reduction in basal tone only in arteries from CH rats without altering VSM $[Ca^{2+}]_i$, whereas the broad-spectrum PKC inhibitor GF 109203X was without effect on baseline inner diameter or $[Ca^{2+}]_i$ in arteries from either control or CH rats.[11] These data therefore support a critical role for the RhoA/ROCK pathway in mediating elevated basal pulmonary arterial tone following CH.

5 CH Induces ROCK-Dependent Myogenic Tone in Small Pulmonary Arteries

The *myogenic response*, defined as vasoconstriction in response to increasing transmural pressure, is a mechanism intrinsic to the VSM that contributes to resting vascular tone and autoregulation of blood flow in the systemic circulation. Although the mechanism by which VSM stretch mediates contraction appears to

involve depolarization-induced Ca^{2+} influx through L-type voltage-dependent Ca^{2+} channels,[40] a contribution of ROCK-mediated VSM Ca^{2+} sensitization to myogenic behavior has also been demonstrated in systemic vascular preparations.[41] Pressure-mediated vasoconstriction also occurs in lungs from late-gestation fetal lambs and newborn guinea pigs[42–45]; however, myogenic tone in the adult normotensive pulmonary circulation is minimal or nonexistent.[42,43,46] Furthermore, the degree of VSM stretch is limited in the adult normoxic vasculature since arterial pressures are low. In contrast, upon the development of pulmonary hypertension secondary to CH exposure, it is possible that pressure may reach a threshold for myogenic reactivity not achieved in the normotensive circulation.

Because of the similarities between the pulmonary vasculature of the fetus and the pulmonary circulation of the adult exposed to CH (i.e., thickened medial layer), studies have examined the hypothesis that elevated basal pulmonary arterial tone following CH is due to the development of myogenic reactivity.[11,47] Consistent with this hypothesis, Broughton et al.[11] demonstrated myogenic behavior independent of changes in VSM $[Ca^{2+}]_i$ in isolated, pressurized small pulmonary arteries from CH rats. In contrast, no such myogenic tone was observed in pulmonary arteries from normotensive animals. This study further identified an exclusive contribution of ROCK-induced VSM Ca^{2+} sensitization to this myogenic tone in CH arteries, consistent with elevated basal arterial RhoA activity following CH.[4,9,10] However, in contrast to effects of ROCK inhibitors, this myogenicity in hypertensive pulmonary arteries was unaltered by PKC inhibition or by blockade of T-type or L-type Ca^{2+} channels.[11] These data stand in marked contrast to numerous studies of the systemic circulation and fetal/neonatal pulmonary circulation demonstrating a central role for Ca^{2+} influx through L-channels in myogenic reactivity[41] and emphasize the divergent mechanisms that mediate pressure-induced tone in the adult systemic vs. pulmonary vasculatures. Interestingly, myogenic tone in CH vessels increased as a function of decreasing arterial diameter,[11] which may explain previous observations that slightly larger arteries from these animals do not demonstrate pressure-induced vasoconstriction.[47] In summary, these findings support a unique effect of CH to promote myogenic reactivity in the adult pulmonary circulation and further implicate a major role for a ROCK-dependent VSM Ca^{2+} sensitization mechanism in mediating this response.

6 Contribution of RhoA/ROCK Signaling to Enhanced Agonist-Induced Pulmonary Vasoreactivity Following CH

A substantial body of evidence supports a major role for endogenous vasoconstrictors, including endothelin 1 (ET-1),[48–50] serotonin (5-HT),[48,51] and thromboxanes[52] in the development of CH-induced pulmonary hypertension. Whereas the vasoconstrictor component of CH-induced pulmonary hypertension may result from increased synthesis or bioavailability of these mediators, studies from our laboratory

and others have demonstrated an effect of CH to increase pulmonary vasoreactivity to these agonists.[5,9,10,53,54] Consistent with the hypothesis that CH augments agonist-induced pulmonary VSM tone via RhoA/ROCK-dependent VSM Ca^{2+} sensitization, ROCK has been implicated in contributing to the VSM contractile response to each of these agonists.[6,12,55–57] In direct support of this hypothesis are studies indicating that the ROCK inhibitor Y-27632 markedly attenuated ET-1-induced contraction of pulmonary arterial rings from CH rats.[6,12]

Controversy exists, however, regarding the effect of CH on agonist-induced RhoA/ROCK signaling in pulmonary VSM. For example, a study by Sauzeau et al.[17] demonstrated decreased agonist-mediated contraction in conduit pulmonary arteries from CH rats vs. controls as a result of RhoA downregulation and abolishment of RhoA-mediated Ca^{2+} sensitization. These discrepancies in the literature may reflect segmental differences in RhoA/ROCK signaling between conduit and small pulmonary arteries or differences in the duration of hypoxic exposure. Studies have begun to clarify these discrepancies using pressurized small pulmonary arteries to evaluate VSM Ca^{2+} sensitization signaling mechanisms.

To directly assess a role for VSM Ca^{2+} sensitization in mediating augmented agonist-dependent vasoconstriction following CH, we examined vasoconstrictor responses to receptor-mediated agonists in endothelium-denuded small pulmonary arteries in which VSM $[Ca^{2+}]_i$ had been clamped with the Ca^{2+} ionophore ionomycin. Consistent with our hypothesis, both UTP[9] (uridine triphosphate) and ET-1-mediated vasoconstrictor responses[10] were greater in Ca^{2+}-permeabilized arteries from CH rats vs. controls. Furthermore, the ROCK inhibitor Y-27632 markedly inhibited reactivity in CH vessels, effectively normalizing constriction to that of control arteries. In contrast, neither PKC inhibition nor tyrosine kinase inhibition altered reactivity in arteries from either group.[9,10] Furthermore, VSM Ca^{2+} sensitization to the PKC agonist phorbol 12-myristate 13 acetate (PMA) was not different between control and CH arteries,[9,11] indicating that CH selectively augments ROCK-induced Ca^{2+} sensitization. Supporting a role for ROCK-dependent inhibition of MLCP in mediating ET-1-induced Ca^{2+}-sensitization in CH arteries, we found that ROCK inhibition prevented Thr^{696} phosphorylation of the MYPT1 subunit of MLCP in response to ET-1.[10] Collectively, these data support an effect of CH to increase agonist-induced ROCK-dependent VSM Ca^{2+} sensitization through phosphorylation of MYPT1, whereas PKC-dependent Ca^{2+} sensitization is unaltered by CH.

Although increased basal VSM Ca^{2+} sensitivity following CH may be mediated in part by increased pulmonary arterial ROCK expression,[4,9,10] an additional mechanism involving elevated basal RhoA activity may contribute to this response. Indeed, whereas hypoxic acclimation does not alter RhoA expression in intrapulmonary arteries,[9,10] CH is associated with enhanced basal as well as agonist-stimulated RhoA activity. [9,10]. The mechanism by which CH mediates this response is the focus of current research efforts and involves ROS-dependent stimulation of RhoA as discussed in sections 9 and 10 below.

7 CH Promotes Membrane Depolarization-Induced VSM Ca^{2+} Sensitization Through a ROCK Signaling Mechanism

In addition to effects of CH to augment basal and agonist-induced vasoconstriction, reactivity to depolarizing concentrations of KCl has been demonstrated to be increased following hypoxic acclimation,[5,12,58] although this is not a universal finding.[7,47] Interestingly, KCl-dependent increases in pulmonary VSM $[Ca^{2+}]_i$ are markedly blunted following CH,[47] thus implicating a myofilament Ca^{2+} sensitization mechanism in mediating depolarization-dependent vasoconstriction in hypertensive arteries. However, a contribution of VSM ROCK to this response has not been consistently observed. Whereas vasoreactivity to KCl is attenuated by inhibitors of ROCK in isolated perfused lungs[5,27,58] and endothelium-intact arteries from CH rats,[5,6,58] no such inhibition was observed in endothelium-denuded vessels from CH rats.[6,12] These findings support a contribution of the endothelium to depolarization-induced VSM Ca^{2+} sensitization following CH. Interestingly, Homma et al.[58] found that the augmented KCl-induced vasoconstriction in lungs from pulmonary hypertensive rats was reduced by either endothelin or serotonin receptor antagonism, implicating a role for stimulation of ET_A and $5\text{-}HT_{1B/1D}$ receptors by endogenous ET-1 and 5-HT in enhanced KCl-dependent Ca^{2+} sensitization following hypoxic acclimation.

It is further possible that increased depolarization-dependent pulmonary VSM Ca^{2+} sensitization following CH is explained by a mechanism inherent to the VSM. Indeed, whereas RhoA stimulation in VSM is well established to occur secondary to activation of G protein-coupled receptors,[13,14,19] RhoA-mediated VSM Ca^{2+} sensitization can also be elicited by depolarizing stimuli.[20–23] Although membrane depolarization-induced VSM contraction appears to be mediated in part via Ca^{2+}-dependent stimulation of RhoA/ROCK,[20–23] a role for depolarization to stimulate this pathway via a Ca^{2+}-independent mechanism has been demonstrated in renal tubule epithelial cells.[24]

In preliminary studies, we tested the hypothesis that a similar mechanism of RhoA activation exists in VSM, and that such a mechanism contributes to enhanced depolarization-induced pulmonary vasoconstriction following exposure to CH. In direct support of membrane depolarization-dependent Ca^{2+} sensitization in pulmonary VSM, we found that a depolarizing stimulus of KCl elicited constriction in arteries in which VSM $[Ca^{2+}]_i$ had been clamped with the calcium ionophore ionomycin.[25,26] In addition, K^+-induced Ca^{2+} sensitization was greater in CH vs. control arteries, indicating that CH augments membrane depolarization-induced VSM Ca^{2+} sensitization through a unique Ca^{2+}-independent mechanism. ROCK inhibition with HA-1077 attenuated these responses to high extracellular K^+ only in arteries from CH rats, thus providing intriguing support for a contribution of ROCK to membrane depolarization-dependent Ca^{2+} sensitization in CH arteries.

Consistent with these findings, a depolarizing concentration of KCl (60 m*M*) increased RhoA activity (GTP-bound RhoA levels as assessed by pull-down assay) in intrapulmonary arteries from CH rats but not controls (unpublished observations). Furthermore, blockade of L channels or PKC inhibition was without effect

on depolarization-induced constriction in Ca^{2+}-permeabilized arteries from either normoxic or CH rats. Interestingly, substantial vasoconstriction remained following ROCK inhibition in both groups, suggesting that additional Ca^{2+} sensitization pathways contribute to depolarization-dependent constriction in the pulmonary circulation. Collectively, these observations suggest that both endothelium-dependent and -independent mechanisms account for the effect of CH to increase depolarization-induced Ca^{2+} sensitization in pulmonary VSM through enhanced ROCK signaling. Considering that isolated pulmonary arteries exhibit pressure-dependent depolarization that is augmented following CH,[47] such a mechanism of depolarization-induced RhoA activation could contribute to the ROCK-dependent myogenicity observed in these vessels.

8 Role of ROS in the Development of PH

Endogenous ROS are physiologically important intracellular second-messenger molecules known to be involved in immune responses and various cell signaling pathways that regulate gene expression, cell proliferation and motility, kinase activation, phosphatase inhibition, regulation of ion channels, Ca^{2+} signaling, and myofilament Ca^{2+} sensitization.[59] Consequently, ROS are integrally involved in the regulation of both VSM cell phenotype and contractility. In pulmonary VSM, endogenous ROS facilitate the proliferation of pulmonary VSM cells in culture[60] and contribute to hypoxic pulmonary vasoconstriction[61] and 5-HT-mediated contraction[62] of small pulmonary arteries. However, excessive ROS production has also been implicated in aging and the progression of various disease states, including pulmonary hypertension.

Although ROS generation has been reported to be reduced in isolated lungs from CH rats,[63] a critical role for ROS in the development of CH pulmonary hypertension is provided by evidence that chronic ROS inhibition with the superoxide dismutase (SOD) mimetic tempol inhibits right ventricular hypertrophy and arterial remodeling in CH rats[64] as well as ROS production and vascular remodeling in CH mice.[65] Sources of ROS in the vasculature include NADPH oxidase (NOX) isoforms, mitochondria, xanthine oxidase, uncoupled NOS, cytochrome P450, and cyclooxygenase (COX). However, NOX[66–70] and xanthine oxidase[71,72] have been the major sources of ROS implicated in the development of pulmonary hypertension.

The prototypical NOX isoform, NOX2 (also known as $gp91^{phox}$), was first identified in neutrophils and macrophages.[59,73] This enzyme functions to produce superoxide anion (O_2^-) and is critically involved in host defense. However, since the discovery of NOX2, other isoforms of NOX have been identified. Of the four primary NOX isoforms expressed in VSM (NOX1, NOX2, NOX4, and NOX5), NOX2 has been most studied and may be the major isoform involved in generation of O_2^- in resistance vessels.[59,73,74] The NOX2 enzyme complex consists of two membrane-bound subunits ($gp91^{phox}$ and $p22^{phox}$); three cytosolic subunits ($p47^{phox}$, $p67^{phox}$ and $p40^{phox}$) and the small G proteins Rac1 and Rac2. Activation of NOX2 involves a complex series of protein–protein interactions, involving translocation of the cytosolic subunits to the

membrane, which requires the association of GTP-bound Rac with $p67^{phox}$. Association of the cytosolic subunits with the $gp91^{phox}/p22^{phox}$ complex increases the catalytic activity of $gp91^{phox}$, thus allowing binding of nicotinamide adenine dinucleotide phosphate (NADPH) and generation of O_2^-. Like NOX2, NOX1 is constitutively bound to $p22^{phox}$ and is activated by Rac and migration of $p47^{phox}$ and $p67^{phox}$ homologs (NOX organizer 1 and NOX activator 1, respectively) to the membrane. NOX4 and NOX5 are also expressed in VSM, although these isoforms have no well-defined regulatory subunits and little is known regarding their role in vascular ROS signaling.[59,73,74] NOX5 is unique among NOX isoforms in being activated by binding of Ca^{2+} to the intracellular N-terminus and has gained attention in contributing to human aortic VSM cell proliferation[75] and oxidant stress in human coronary artery disease.[76] Interestingly, NOX isoforms vary in their subcellular localization. For example, whereas NOX4 colocalizes with the cytoskeletal components vinculin and α-actin in cultured rat aortic smooth muscle cells,[77,78] NOX1 is expressed primarily at the sarcolemma in association with caveolin 1.[77] Although the significance of such compartmentalized expression to NOX signaling is not well characterized, the subcellular localization of NOX isoforms may be functionally important in receptor signaling, oxygen sensing, mechanotransduction, and regulation of gene expression.

Several studies support a contribution of ROS produced by NOX in the development of CH-induced pulmonary hypertension in both adult and neonatal animals. A role for NOX2 in mediating CH-induced pulmonary hypertension in mice was provided by Liu et al.,[66] who found that CH-mediated increases in ROS generation and pulmonary hypertension were markedly reduced in mice deficient in $gp91^{phox}$. In agreement with these findings, Grobe et al.[67] measured elevated Rac1 and $p47^{phox}$ expression in lungs from pulmonary hypertensive lambs. This same group further reported that the NOX inhibitors apocynin and diphenylene iodonium reduced pulmonary ROS levels in these same animals. NOX4 gene and protein expression is also increased by both chronic sustained hypoxia[79] and chronic intermittent hypoxia[80] in murine pulmonary arteries and lung tissue and is similarly upregulated in lung tissue from patients with idiopathic pulmonary hypertension.[80] However, the contribution of NOX4 to pulmonary hypertension is unknown. Furthermore, while these studies suggest that ROS generated by NOX enzymes are involved in the pulmonary hypertensive response to CH, the intracellular signaling mechanisms involved are not well defined. Interestingly, studies from our laboratory and others have begun to define a role for ROS in mediating enhanced pulmonary vasoconstrictor sensitivity following CH (discussed next), a response that may contribute to the etiology of pulmonary hypertension.

9 Contribution of ROS to Enhanced Vasoconstrictor Reactivity After CH

Studies have also implicated ROS in mediating enhanced sensitivity to vasoconstrictor stimuli in CH hypertensive pulmonary arteries. A role for NOX-derived ROS in this response was provided by Liu et al.,[66] who found that CH-induced increases in contractility of small pulmonary arteries to both 5-HT and the thromboxane analog

U-46619 are attenuated in gp91^{phox} (NOX2) knockout mice compared to wild-type controls. Furthermore, Fike et al.[70] reported that ROS contribute to enhanced vasoconstrictor reactivity of small pulmonary arteries in piglets with CH-induced pulmonary hypertension. The involvement of NOX in this response was supported by an effect of apocynin not only to attenuate vasoconstriction to acetylcholine (ACh) in hypertensive arteries but also to inhibit NOX-stimulated ROS generation in small pulmonary arteries from CH piglets as assessed by lucigenin-enhanced chemiluminescence. ROS generated by xanthine oxidase also contribute to pulmonary hypertension and endothelial dysfunction in intrapulmonary arteries from CH neonatal rats.[72] Consequently, endogenous ROS derived from various enzymatic sources appear to contribute to CH-induced increases in vasoconstrictor sensitivity and associated pulmonary hypertension. These observations are further supported by studies from our laboratory, which have begun to address the mechanistic relationship among receptor stimulation, membrane depolarization, ROS, and RhoA-induced myofilament Ca^{2+} sensitization in the hypertensive pulmonary circulation.

10 CH Augments ET-1- and Membrane Depolarization-Induced Pulmonary VSM Ca^{2+} Sensitization Through ROS-Dependent Stimulation of RhoA/ROCK Signaling

Based on evidence that exogenous reactive oxygen species (ROS) mediate ROCK-dependent VSM contraction[81] and that membrane depolarization increases ROS production in endothelial cells, macula densa, and isolated mouse and rat lungs,[82–84] we hypothesized that both receptor-mediated and depolarization-induced ROS generation mediates enhanced RhoA-dependent Ca^{2+} sensitization in pulmonary VSM following CH. This hypothesis was tested by assessing effects of the O_2^- scavenger tiron[9,10] on receptor-mediated and depolarization-induced vasoconstriction, VSM $[Ca^{2+}]_i$, and ROS generation in Ca^{2+}-permeabilized, pressurized, small pulmonary arteries from both normoxic and CH rats. We also evaluated the contribution of ROS to ET-1- and depolarization-induced RhoA activity in intrapulmonary arteries from each group. In agreement with this hypothesis, we found that, similar to effects of ROCK inhibition, tiron largely inhibited both KCl[25] and ET-1-induced vasoconstriction[10] in CH arteries and normalized vasoconstrictor responses between groups. In more recent experiments, we found that a tiron/HA-1077 cocktail did not provide greater attenuation of KCl-dependent reactivity than scavenging of O_2^- alone (unpublished observations). This lack of an additive effect of ROCK inhibition and O_2^- scavenging provides additional support for our hypothesis that O_2^- signals in series with RhoA/ROCK to increase myofilament Ca^{2+} sensitivity in CH arteries.

To determine whether enhanced basal and agonist-dependent VSM Ca^{2+} sensitivity in CH arteries is associated with increased O_2^- generation, we measured dihydroethydine (DHE) oxidation by fluorescence microscopy as an index of O_2^- production in isolated, pressurized arteries. Interestingly, we found that basal DHE fluorescence was greater in arteries from CH rats compared to controls.[10,25]

Furthermore, addition of either KCl or ET-1 increased DHE fluorescence in arteries from CH but not control animals. As expected, tiron abolished the CH-induced elevation in basal ROS as well as both depolarization- and ET-1-stimulated ROS production without altering DHE fluorescence in control arteries. In contrast, ROCK inhibition with HA-1077 was without effect on either CH-dependent increases in basal ROS or KCl-induced ROS generation. These data argue against a potential role for ROCK to enhance basal and depolarization-induced Ca^{2+} sensitization through stimulated ROS production and are consistent with our hypothesis that RhoA is activated by ROS in CH pulmonary arteries.

In further agreement with this hypothesis is the correlation between elevated basal ROS generation[10,25] and RhoA activity[9,10] in arteries from pulmonary hypertensive rats. In addition, both ET-1[10] and KCl (unpublished observations) were found to increase GTP-bound RhoA levels in arteries from CH but not control animals. Consistent with a role for ROS in mediating enhanced ET-1-mediated Ca^{2+} sensitization in CH arteries, tiron abolished both the CH-dependent elevation in basal and ET-1-induced RhoA activity in small pulmonary arteries but was without effect in control arteries. These results provide direct evidence for ROS-dependent RhoA activation, and together with data from Ca^{2+} -permeabilized arteries and DHE experiments, demonstrate a novel link among receptor stimulation, membrane depolarization, ROS generation, and RhoA/ROCK signaling that mediates enhanced myofilament Ca^{2+} sensitivity in the hypertensive pulmonary circulation (Fig. 23.3). These findings may also establish a mechanistic basis for previous studies that have established a crucial role for ROS in the development of PH.[64,71]

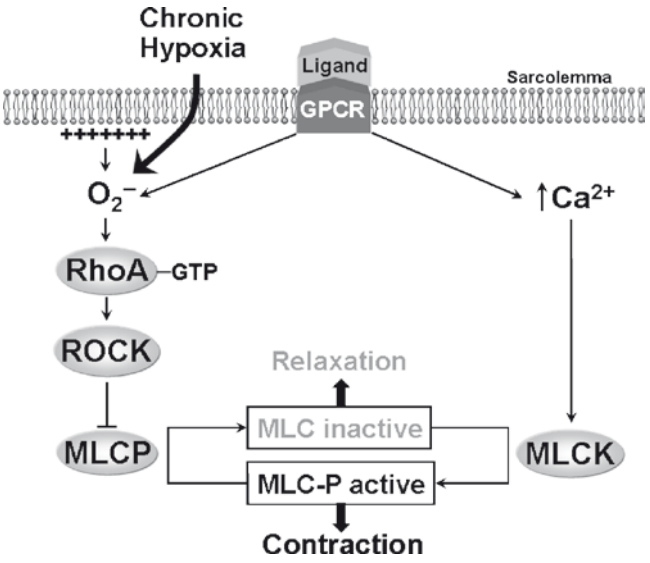

Fig. 23.3 CH augments both depolarization- and GPCR-induced Ca^{2+}-sensitization through O_2^--dependent activation of RhoA/ROCK signaling in pulmonary VSM

11 Summary and Future Directions

Pulmonary hypertension resulting from long-term hypoxic exposure has been historically considered to be mediated by increases in pulmonary vascular resistance resulting from hypoxic pulmonary vasoconstriction, arterial remodeling, and polycythemia. However, findings suggest that vasoconstrictor influences in addition to acute hypoxic vasoconstriction, including elevated basal VSM tone and enhanced vasoconstrictor reactivity, play prominent roles in mediating CH-induced pulmonary hypertension. Furthermore, increases in myofilament Ca^{2+} sensitivity resulting from stimulation of RhoA and ROCK signaling represent a primary mechanism of vasoconstriction and associated pulmonary hypertension resulting from CH. This chapter summarized recent developments in the understanding of RhoA/ROCK signaling mechanisms in pulmonary VSM that increase the sensitivity of the contractile apparatus to $[Ca^{2+}]_i$ and contribute to vasoconstriction in this setting. Such developments include the discovery of myogenic tone in small pulmonary arteries from adult CH rats that contributes to basal vasoconstriction through a mechanism inherent to the VSM, dependent on ROCK-induced Ca^{2+} sensitization but independent of L-type voltage-gated Ca^{2+} channels. Additional studies have revealed an important contribution of O_2^--induced RhoA activation to both receptor-mediated and membrane depolarization-induced myofilament Ca^{2+} sensitization in hypertensive pulmonary arteries from CH rats. Both xanthine oxidase and NOX isoforms have been implicated in the development of pulmonary hypertension and altered pulmonary vasoreactivity in various animal models and represent potential sources of ROS that mediate RhoA-dependent vasoconstriction and associated pulmonary hypertension.

Goals of future studies are to identify the contribution of depolarization-induced ROS generation to myogenic vasoconstriction in the adult hypertensive pulmonary vasculature, establish the sources of O_2^- that mediate enhanced RhoA-dependent Ca^{2+} sensitization following CH, and determine the signaling mechanism linking membrane stretch, receptor activation, and membrane depolarization to RhoA stimulation. Such a mechanism may involve effects of CH to influence the activity or cellular trafficking of components of the ROS-RhoA signaling axis through alterations in the composition of signaling platforms within the lipid bilayer or possible effects of membrane potential to directly regulate enzymatic activity of relevant second messengers. Additional studies are needed to determine distal signaling components of RhoA in pulmonary VSM, including potential roles for actin polymerization and MLC phosphorylation by Ca^{2+}-independent kinases, and to assess the relevance of these signaling pathways to enhanced pulmonary vasoreactivity and hypertension following CH. The concept of depolarization as a Ca^{2+}-independent effector of ROS signaling has potentially broad physiological implications for not only RhoA-induced VSM contraction, cytoskeletal organization, motility, proliferation, and apoptosis, but also for ROS-dependent modulation of a diverse spectrum of intracellular signaling pathways.

References

1. McMurtry IF, Petrun MD, Reeves JT (1978) Lungs from chronically hypoxic rats have decreased pressor response to acute hypoxia. Am J Physiol 235:H104–H109
2. Resta TC, Chicoine LG, Omdahl JL, Walker BR (1999) Maintained upregulation of pulmonary eNOS gene and protein expression during recovery from chronic hypoxia. Am J Physiol 276:H699–H708
3. Walker BR, Resta TC, Nelin LD (2000) Nitric oxide-dependent pulmonary vasodilation in polycythemic rats. Am J Physiol Heart Circ Physiol 279:H2382–H2389
4. Hyvelin JM, Howell K, Nichol A, Costello CM, Preston RJ, McLoughlin P (2005) Inhibition of Rho-kinase attenuates hypoxia-induced angiogenesis in the pulmonary circulation. Circ Res 97:185–191
5. Nagaoka T, Morio Y, Casanova N et al (2004) Rho/Rho kinase signaling mediates increased basal pulmonary vascular tone in chronically hypoxic rats. Am J Physiol Lung Cell Mol Physiol 287:L665–L672
6. Weigand L, Sylvester JT, Shimoda LA (2006) Mechanisms of endothelin-1-induced contraction in pulmonary arteries from chronically hypoxic rats. Am J Physiol Lung Cell Mol Physiol 290:L284–L290
7. Shimoda LA, Sham JSK, Shimoda TH, Sylvester JT (2000) L-type Ca^{2+} channels, resting $[Ca^{2+}]_i$, and ET-1-induced responses in chronically hypoxic pulmonary myocytes. Am J Physiol Lung Cell Mol Physiol 279:L884–L894
8. Nagaoka T, Fagan KA, Gebb SA et al (2005) Inhaled Rho kinase inhibitors are potent and selective vasodilators in rat pulmonary hypertension. Am J Respir Crit Care Med 171:494–499
9. Jernigan NL, Walker BR, Resta TC (2004) Chronic hypoxia augments protein kinase G-mediated Ca^{2+} desensitization in pulmonary vascular smooth muscle through inhibition of RhoA/Rho kinase signaling. Am J Physiol Lung Cell Mol Physiol 287:L1220–L1229
10. Jernigan NL, Walker BR, Resta TC (2008) Reactive oxygen species mediate RhoA/Rho kinase-induced Ca^{2+} sensitization in pulmonary vascular smooth muscle following chronic hypoxia. Am J Physiol Lung Cell Mol Physiol 295:L515–L529
11. Broughton BR, Walker BR, Resta TC (2008) Chronic hypoxia induces Rho kinase-dependent myogenic tone in small pulmonary arteries. Am J Physiol Lung Cell Mol Physiol 294: L797–L806
12. Barman SA (2007) Vasoconstrictor effect of endothelin-1 on hypertensive pulmonary arterial smooth muscle involves Rho-kinase and protein kinase C. Am J Physiol Lung Cell Mol Physiol 293:L472–L479
13. Oka M, Fagan KA, Jones PL, McMurtry IF (2008) Therapeutic potential of RhoA/Rho kinase inhibitors in pulmonary hypertension. Br J Pharmacol 155:444–454
14. Fukumoto Y, Tawara S, Shimokawa H (2007) Recent progress in the treatment of pulmonary arterial hypertension: expectation for rho-kinase inhibitors. Tohoku J Exp Med 211:309–320
15. Abman SH (2007) Recent advances in the pathogenesis and treatment of persistent pulmonary hypertension of the newborn. Neonatology 91:283–290
16. Hirano K (2007) Current topics in the regulatory mechanism underlying the Ca^{2+} sensitization of the contractile apparatus in vascular smooth muscle. J Pharmacol Sci 104:109–115
17. Sauzeau V, Rolli-Derkinderen M, Lehoux S, Loirand G, Pacaud P (2003) Sildenafil prevents change in RhoA expression induced by chronic hypoxia in rat pulmonary artery. Circ Res 93:630–637
18. Nagaoka T, Gebb SA, Karoor V et al (2006) Involvement of RhoA/Rho kinase signaling in pulmonary hypertension of the fawn-hooded rat. J Appl Physiol 100:996–1002
19. Liao JK, Seto M, Noma K (2007) Rho kinase (ROCK) inhibitors. J Cardiovasc Pharmacol 50:17–24
20. Urban NH, Berg KM, Ratz PH (2003) K^+ depolarization induces RhoA kinase translocation to caveolae and Ca^{2+} sensitization of arterial muscle. Am J Physiol Cell Physiol 285:C1377–C1385

21. Sakurada S, Takuwa N, Sugimoto N et al (2003) Ca^{2+}-dependent activation of Rho and Rho kinase in membrane depolarization-induced and receptor stimulation-induced vascular smooth muscle contraction. Circ Res 93:548–556
22. Mita M, Yanagihara H, Hishinuma S, Saito M, Walsh MP (2002) Membrane depolarization-induced contraction of rat caudal arterial smooth muscle involves Rho-associated kinase. Biochem J 364:431–440
23. Woodsome TP, Polzin A, Kitazawa K, Eto M, Kitazawa T (2006) Agonist- and depolarization-induced signals for myosin light chain phosphorylation and force generation of cultured vascular smooth muscle cells. J Cell Sci 119:1769–1780
24. Szaszi K, Sirokmany G, Ciano-Oliveira C, Rotstein OD, Kapus A (2005) Depolarization induces Rho-Rho kinase-mediated myosin light chain phosphorylation in kidney tubular cells. Am J Physiol Cell Physiol 289:C673–C685
25. Broughton BR, Jernigan NL, Walker BR, Resta TC (2007) Superoxide anion mediates enhanced membrane depolarization-induced myofilament calcium-sensitization in small pulmonary arteries following chronic hypoxia. Microcirculation 14:490
26. Broughton BR, Walker BR, Resta TC (2007) Chronic hypoxia augments membrane depolarization-induced myofilament Ca^{2+}-sensitization through stimulation of Rho kinase in small pulmonary arteries. FASEB J 21:A1437
27. Fagan KA, Oka M, Bauer NR et al (2004) Attenuation of acute hypoxic pulmonary vasoconstriction and hypoxic pulmonary hypertension in mice by inhibition of Rho-kinase. Am J Physiol Lung Cell Mol Physiol 287:L656–L664
28. Kataoka C, Egashira K, Inoue S et al (2002) Important role of Rho-kinase in the pathogenesis of cardiovascular inflammation and remodeling induced by long-term blockade of nitric oxide synthesis in rats. Hypertension 39:245–250
29. McNamara PJ, Murthy P, Kantores C et al (2008) Acute vasodilator effects of Rho-kinase inhibitors in neonatal rats with pulmonary hypertension unresponsive to nitric oxide. Am J Physiol Lung Cell Mol Physiol 294:L205–L213
30. Laufs U, Liao JK (1998) Post-transcriptional regulation of endothelial nitric oxide synthase mRNA stability by Rho GTPase. J Biol Chem 273:24266–24271
31. Abe K, Tawara S, Oi K et al (2006) Long-term inhibition of Rho-kinase ameliorates hypoxia-induced pulmonary hypertension in mice. J Cardiovasc Pharmacol 48:280–285
32. Gien J, Seedorf GJ, Balasubramaniam V, Tseng N, Markham N, Abman SH (2008) Chronic intrauterine pulmonary hypertension increases endothelial cell Rho kinase activity and impairs angiogenesis in vitro. Am J Physiol Lung Cell Mol Physiol 295:L680–L687
33. Ghisdal P, Vandenberg G, Morel N (2003) Rho-dependent kinase is involved in agonist-activated calcium entry in rat arteries. J Physiol 551:855–867
34. Ito S, Kume H, Yamaki K et al (2002) Regulation of capacitative and noncapacitative receptor-operated Ca^{2+} entry by rho-kinase in tracheal smooth muscle. Am J Respir Cell Mol Biol 26:491–498
35. Luykenaar KD, Brett SE, Wu BN, Wiehler WB, Welsh DG (2004) Pyrimidine nucleotides suppress KDR currents and depolarize rat cerebral arteries by activating Rho kinase. Am J Physiol Heart Circ Physiol 286:H1088–H1100
36. Mehta D, Ahmmed GU, Paria BC et al (2003) RhoA interaction with inositol 1,4,5-trisphosphate receptor and transient receptor potential channel-1 regulates Ca^{2+} entry. Role in signaling increased endothelial permeability. J Biol Chem 278:33492–33500
37. Takizawa S, Hori M, Ozaki H, Karaki H (1993) Effects of isoquinoline derivatives, HA1077 and H-7, on cytosolic Ca^{2+} level and contraction in vascular smooth muscle. Eur J Pharmacol 250:431–437
38. Wang J, Weigand L, Lu W, Sylvester JT, Semenza GL, Shimoda LA (2006) Hypoxia inducible factor 1 mediates hypoxia-induced TRPC expression and elevated intracellular Ca^{2+} in pulmonary arterial smooth muscle cells. Circ Res 98:1528–1537
39. Lin MJ, Leung GP, Zhang WM et al (2004) Chronic hypoxia-induced upregulation of store-operated and receptor-operated Ca^{2+} channels in pulmonary arterial smooth muscle cells: a novel mechanism of hypoxic pulmonary hypertension. Circ Res 95:496–505

40. Harder DR (1984) Pressure-dependent membrane depolarization in cat middle cerebral artery. Circ Res 55:197–202
41. Schubert R, Lidington D, Bolz SS (2008) The emerging role of Ca^{2+} sensitivity regulation in promoting myogenic vasoconstriction. Cardiovasc Res 77:8–18
42. Belik J (1995) The myogenic response of arterial vessels is increased in fetal pulmonary hypertension. Pediatr Res 37:196–201
43. Belik J (1994) Myogenic response in large pulmonary arteries and its ontogenesis. Pediatr Res 36:34–40
44. Parker TA, Grover TR, Kinsella JP, Falck JR, Abman SH (2005) Inhibition of 20-HETE abolishes the myogenic response during NOS antagonism in the ovine fetal pulmonary circulation. Am J Physiol Lung Cell Mol Physiol 289:L261–L267
45. Tourneux P, Chester M, Grover T, Abman SH (2008) Fasudil inhibits the myogenic response in the fetal pulmonary circulation. Am J Physiol Heart Circ Physiol 295:H1505–H1513
46. Davis MJ, Gilmore JP, Joyner WL (1981) Responses of pulmonary allograft and cheek pouch arterioles in the hamster to alterations in extravascular pressure in different oxygen environments. Circ Res 49:133–140
47. Naik JS, Earley S, Resta TC, Walker BR (2005) Pressure-induced smooth muscle cell depolarization in pulmonary arteries from control and chronically hypoxic rats does not cause myogenic vasoconstriction. J Appl Physiol 98:1119–1124
48. Maclean MR (1999) Endothelin-1 and serotonin: mediators of primary and secondary pulmonary hypertension? J Lab Clin Med 134:105–114
49. Muramatsu M, Rodman DM, Oka M, McMurtry IF (1997) Endothelin-1 mediates nitro-L-arginine vasoconstriction of hypertensive rat lungs. Am J Physiol 272:L807–L812
50. Bonvallet ST, Zamora MR, Hasunuma K et al (1994) BQ123, an ET_A-receptor antagonist, attenuates hypoxic pulmonary hypertension in rats. Am J Physiol 266:H1327–H1331
51. Dempsie Y, Maclean MR (2008) Pulmonary hypertension: therapeutic targets within the serotonin system. Br J Pharmacol 155:455–462
52. Fike CD, Zhang Y, Kaplowitz MR (2005) Thromboxane inhibition reduces an early stage of chronic hypoxia-induced pulmonary hypertension in piglets. J Appl Physiol 99: 670–676
53. Shimoda LA, Sham JSK, Sylvester JT (2000) Altered pulmonary vasoreactivity in the chronically hypoxic lung. Physiol Res 49:549–560
54. Maclean MR, Morecroft I (2001) Increased contractile response to 5-hydroxytryptamine1-receptor stimulation in pulmonary arteries from chronic hypoxic rats: role of pharmacological synergy. Br J Pharmacol 134:614–620
55. Janssen LJ, Lu-Chao H, Netherton S (2001) Excitation-contraction coupling in pulmonary vascular smooth muscle involves tyrosine kinase and Rho kinase. Am J Physiol Lung Cell Mol Physiol 280:L666–L674
56. Nishikawa Y, Doi M, Koji T et al (2003) The role of Rho and Rho-dependent kinase in serotonin-induced contraction observed in bovine middle cerebral artery. Tohoku J Exp Med 201:239–249
57. Sakurada S, Okamoto H, Takuwa N, Sugimoto N, Takuwa Y (2001) Rho activation in excitatory agonist-stimulated vascular smooth muscle. Am J Physiol Cell Physiol 281:C571–C578
58. Homma N, Nagaoka T, Morio Y et al (2007) Endothelin-1 and serotonin are involved in activation of RhoA/Rho kinase signaling in the chronically hypoxic hypertensive rat pulmonary circulation. J Cardiovasc Pharmacol 50:697–702
59. Bedard K, Krause KH (2007) The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol Rev 87:245–313
60. Wedgwood S, Dettman RW, Black SM (2001) ET-1 stimulates pulmonary arterial smooth muscle cell proliferation via induction of reactive oxygen species. Am J Physiol Lung Cell Mol Physiol 281:L1058–L1067
61. Liu Q, Sham JSK, Shimoda LA, Sylvester JT (2001) Hypoxic constriction of porcine distal pulmonary arteries: endothelium and endothelin dependence. Am J Physiol Lung Cell Mol Physiol 280:L856–L865

62. Liu JQ, Folz RJ (2004) Extracellular superoxide enhances 5-HT-induced murine pulmonary artery vasoconstriction. Am J Physiol Lung Cell Mol Physiol 287:L111–L118
63. Reeve HL, Michelakis E, Nelson DP, Weir EK, Archer SL (2001) Alterations in a redox oxygen sensing mechanism in chronic hypoxia. J Appl Physiol 90:2249–2256
64. Elmedal B, de Dam MY, Mulvany MJ, Simonsen U (2004) The superoxide dismutase mimetic, tempol, blunts right ventricular hypertrophy in chronic hypoxic rats. Br J Pharmacol 141:105–113
65. Matsui H, Shimosawa T, Itakura K, Guanqun X, Ando K, Fujita T (2004) Adrenomedullin can protect against pulmonary vascular remodeling induced by hypoxia. Circulation 109:2246–2251
66. Liu JQ, Zelko IN, Erbynn EM, Sham JS, Folz RJ (2006) Hypoxic pulmonary hypertension: role of superoxide and NADPH oxidase (gp91phox). Am J Physiol Lung Cell Mol Physiol 290:L2–L10
67. Grobe AC, Wells SM, Benavidez E et al (2006) Increased oxidative stress in lambs with increased pulmonary blood flow and pulmonary hypertension: role of NADPH oxidase and endothelial NO synthase. Am J Physiol Lung Cell Mol Physiol 290:L1069–L1077
68. Fresquet F, Pourageaud F, Leblais V et al (2006) Role of reactive oxygen species and gp91phox in endothelial dysfunction of pulmonary arteries induced by chronic hypoxia. Br J Pharmacol 148:714–723
69. Brennan LA, Steinhorn RH, Wedgwood S et al (2003) Increased superoxide generation is associated with pulmonary hypertension in fetal lambs: a role for NADPH oxidase. Circ Res 92:683–691
70. Fike CD, Slaughter JC, Kaplowitz MR, Zhang Y, Aschner JL (2008) Reactive oxygen species from NADPH oxidase contribute to altered pulmonary vascular responses in piglets with chronic hypoxia-induced pulmonary hypertension. Am J Physiol Lung Cell Mol Physiol 295:L881–L888
71. Hoshikawa Y, Ono S, Suzuki S et al (2001) Generation of oxidative stress contributes to the development of pulmonary hypertension induced by hypoxia. J Appl Physiol 90:1299–1306
72. Jankov RP, Kantores C, Pan J, Belik J (2008) Contribution of xanthine oxidase-derived superoxide to chronic hypoxic pulmonary hypertension in neonatal rats. Am J Physiol Lung Cell Mol Physiol 294:L233–L245
73. Lyle AN, Griendling KK (2006) Modulation of vascular smooth muscle signaling by reactive oxygen species. Physiology 21:269–280
74. Frey RS, Ushio-Fukai M, Malik A (2009) NADPH oxidase-dependent signaling in endothelial cells: role in physiology and pathophysiology. Antioxid Redox Signal 11(4):791–810
75. Jay DB, Papaharalambus CA, Seidel-Rogol B, Dikalova AE, Lassegue B, Griendling KK (2008) Nox5 mediates PDGF-induced proliferation in human aortic smooth muscle cells. Free Radic Biol Med 45:329–335
76. Guzik TJ, Chen W, Gongora MC et al (2008) Calcium-dependent NOX5 nicotinamide adenine dinucleotide phosphate oxidase contributes to vascular oxidative stress in human coronary artery disease. J Am Coll Cardiol 52:1803–1809
77. Hilenski LL, Clempus RE, Quinn MT, Lambeth JD, Griendling KK (2004) Distinct subcellular localizations of Nox1 and Nox4 in vascular smooth muscle cells. Arterioscler Thromb Vasc Biol 24:677–683
78. Clempus RE, Sorescu D, Dikalova AE et al (2007) Nox4 is required for maintenance of the differentiated vascular smooth muscle cell phenotype. Arterioscler Thromb Vasc Biol 27:42–48
79. Mittal M, Roth M, Konig P et al (2007) Hypoxia-dependent regulation of nonphagocytic NADPH oxidase subunit NOX4 in the pulmonary vasculature. Circ Res 101:258–267
80. Nisbet RE, Graves AS, Kleinhenz DJ et al (2009) The role of NADPH oxidase in chronic intermittent hypoxia-induced pulmonary hypertension in mice. Am J Respir Cell Mol Biol 40(5):601–609
81. Jin L, Ying Z, Webb RC (2004) Activation of Rho/Rho kinase signaling pathway by reactive oxygen species in rat aorta. Am J Physiol Heart Circ Physiol 287:H1495–H1500

82. Al Mehdi AB, Zhao G, Dodia C et al (1998) Endothelial NADPH oxidase as the source of oxidants in lungs exposed to ischemia or high K^+. Circ Res 83:730–737
83. Liu R, Garvin JL, Ren Y, Pagano PJ, Carretero OA (2007) Depolarization of the macula densa induces superoxide production via NAD(P)H oxidase. Am J Physiol Renal Physiol 292: F1867–F1872
84. Sohn HY, Keller M, Gloe T, Morawietz H, Rueckschloss U, Pohl U (2000) The small G-protein Rac mediates depolarization-induced superoxide formation in human endothelial cells. J Biol Chem 275:18745–18750

Polyamine Regulatory Pathways as Pharmacologic Targets in Pulmonary Arterial Hypertension

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Abstract Decades of studies in animal models and in humans with pulmonary artery hypertension have left little doubt that the processes culminating in hypertensive pulmonary vascular remodeling and sustained increases in pulmonary vascular resistance are complex. Modulations in phenotype, proliferative state, and survival of multiple lung vascular cell populations, changes in the local milieu of growth and differentiation factors, and alterations in the extracellular connective tissue environment all seem to contribute to the pathogenesis of the disorder. From a pharmacologic vantage point, identifying which of these is the most suitable target is challenging. Our studies are predicated on the concept that pathways "distal" in the signaling cascades – upon which multiple stimuli dictating vascular cell structure and function converge – might be effective drug targets in PAH. In this regard, we found that the polyamines, putrescine, spermidine, and spermine, a family of low molecular weight organic cations required for cell growth and differentiation, along with their biosynthetic pathways and transmembrane transporters, are altered in rational animal models of pulmonary arterial hypertension. In this article, we summarize these data incriminating polyamines and their regulatory pathways in hypertensive pulmonary vascular disease and advance the contention that polyamine synthesis inhibitors and transport blockers should indeed be considered for clinical trials in human pulmonary arterial hypertension.

Keywords Pulmonary hypertension • polyamines • ornithine decarboxylase • polyamine transport • monocrotaline • hypoxia

1 Pharmacologic Targets in Pulmonary Hypertension

Pulmonary arterial hypertension (PAH) presents a formidable therapeutic challenge. It is insidious and difficult to diagnose. Multiple pharmacologic targets have been

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identified, and studies in experimental animal models would lead to the impression that that there should be a long list of therapeutic options,[1] but there is not. The currently available drug interventions are limited, and none is particularly effective.[2] Pharmacologic therapy of progressive pulmonary hypertension is in need of an advance.

In the history of pharmacology, there are many instances when drugs developed for a given indication have subsequently emerged as a first therapeutic option for conditions unrelated to their initial development. Examples might include the antipyretic and analgesic aspirin for cardiovascular disease; the antineoplastic agent methotrexate for rheumatoid arthritis; the vasodilators minoxidil and sildenafil for hair loss and erectile dysfunction, respectively; and others. Perhaps a class of drugs that would be effective in human PAH has been similarly "hiding in plain sight."

Decades of studies in animal models and in humans with PAH have left little doubt that the processes culminating in hypertensive pulmonary vascular remodeling and sustained increases in pulmonary vascular resistance (PVR) are complex. The involvement of reversible increases in vasomotor tone and structural remodeling of the pulmonary arterial wall relative to elevating PVR is under debate. Reports have advanced the notion that exaggerated vasoconstriction, perhaps linked to increased myogenic tone, plays a previously unsuspected and critical role maintaining elevated resistance.[3,4] In addition, vascular structural changes incriminated in chronic PAH are evident along the entire pulmonary arterial tree.[5,6] The medial layer of muscularized pulmonary arteries is thicker, partly as a result of pulmonary artery smooth muscle cell (PASMC) hypertrophy. A complete ring of smooth muscle also is extended into vessels that are normally partially or nonmuscularized. This increased muscularization seems to be linked to proliferation and differentiation of precursor cells into new smooth muscle. Severe PAH also is associated with intimal occlusive lesions, which may be ascribed to unrestrained endothelial cell (EC) proliferation that mimics certain aspects of tumor growth.[7] Excessive deposition and disorganization of interstitial and basement membrane matrix proteins also contribute to the structural changes.[8] Fibroblasts, PASMCs, ECs, and perhaps other cells may be sources of these matrix proteins.

Not surprisingly, the signaling environment underlying development of chronic PAH is also complex.[9,10] Numerous growth factors, including platelet-derived growth factor (PDGF), transforming growth factor β (TGF-β), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and related receptor tyrosine kinases, vasoactive autacoids, and matrix proteinases all have been incriminated from both experiments on human lung tissue and animal models of PAH. Multiple sequential or parallel-operating second-messenger systems, including various ion channels, calcium, nitric oxide (NO), reactive species, RhoA/Rho kinase, just to name a few, seem to be altered in chronic PAH.[11,12] From a pharmacologic vantage point, identifying which of these is the most suitable target is challenging. The studies described are predicated on the concept that pathways "distal" in the signaling cascades – on which multiple stimuli dictating vascular cell structure and function converge – might be effective drug targets in PAH.

The polyamines putrescine (PUT), spermidine (SPD), and spermine (SPM) are a family of low molecular weight organic cations known to be required for cell growth and differentiation.[13] In light of their role governing fundamental behavior of cells, considerable effort has been directed to developing drugs modulating polyamine regulatory pathways as antineoplastic agents. Polyamine synthesis inhibitors, especially the intensely studied α-difluoromethylornithine (DFMO), polyamine analogues, and transmembrane polyamine transport inhibitors, have all been examined in this respect. While experiments in malignant cells have tended to support the usefulness of polyamine regulatory pathways as molecular targets for anticancer drugs, their performance in clinical trials has been unimpressive.[14] Combination therapy with other antineoplastics seems to hold more promise. For example, there is emerging evidence that DFMO in combination with sulindac suppresses recurrent formation of colorectal polyps.[14] Unrelated to antineoplastic therapy, DFMO also is effective against the African trypanosomal infection causing sleeping sickness.[15] It is fair to say, however, that the potential for polyamine regulatory pathways to serve as isolated targets for pharmacologic intervention has not yet been realized despite decades of investment and study. In this chapter, we review data showing that polyamines and their biosynthetic pathways and transporters are necessary for hypertensive pulmonary vascular remodeling in rational animal models and advance the contention that polyamine synthesis inhibitors and transport blockers should indeed be considered for clinical trials in human PAH.

2 General Aspects of Polyamine Regulation

Cell polyamine contents are governed by multiple interactive pathways.[13] In de novo polyamine synthesis, PUT is synthesized from its precursor, ornithine, via the initial and one of the rate-limiting enzymes in polyamine synthesis, ornithine decarboxylase (ODC). PUT is then converted sequentially to SPD and SPM via two other potentially rate-limiting enzymes, *S*-adenosylmethionine decarboxylase (SAM-DC) and SPD and SPM synthases, respectively. De novo polyamine synthesis can be suppressed pharmacologically by DFMO, a specific inhibitor of ODC. ODC is unusual in terms of its regulation.[16] The protein has one of the shortest half-lives of known mammalian enzymes and is exquisitely sensitive to changes in the abundance of antizyme, a family of at least three 18- to 30-kDa proteins that inhibit ODC and promote its 26S proteasome-dependent degradation.

A second regulatory pathway is transmembrane transport.[17] In some cells, polyamine uptake accompanies proliferation, while it is downregulated during quiescence. In polyamine-depleted cells, transport processes may restore polyamine levels and revitalize cell functions. Polyamine transport activity seems to require ongoing RNA and protein synthesis and, in some cells, may require an intact sodium gradient. There also appear to be considerable differences in terms of the number of polyamine transporters, with some cells expressing a single transporter

with overlapping specificity for the three polyamines and others expressing uptake pathways that are relatively specific for each polyamine. It is unknown whether the multiple transporters are differentially regulated and if so, what biological significance such regulation might be. Like ODC, polyamine uptake is negatively regulated by antizyme.

A third pathway involves polyamine interconversion, catalyzed by the SPD and SPM aminopropyltransferases; another rate-limiting enzyme, SPD/SPM N^1-acetyltransferase (SAT); and polyamine oxidase.[18,19] The last two enzymes are responsible for conversion of SPM back to SPD and SPD to PUT. Finally, it has been speculated for many years that polyamine compartmentalization was an important regulatory pathway. In support of this idea, the abundance of SPM in mitochondria and nuclei is known to change during the cell cycle.[20] It has also been shown that mitochondria express a polyamine "uniporter"[21] that could govern mitochondrial polyamine contents independently of cytosolic changes. Subcellular polyamine distribution has not been explored in pathologic contexts.

3 Polyamine Regulation in the Lungs of Rats With Hypoxia- and Monocrotaline-Induced PAH

Using high-performance liquid chromatography to quantify tissue polyamine contents, we found that PUT, SPD, and SPM were all increased as a function of time in rats with PAH induced by monocrotaline (MCT) and hypoxia (Fig. 24.1). These increases tracked closely the development of PAH and right ventricular hypertrophy in both models.[22,23] Interestingly, and as shown in Fig. 24.2, increases in lung polyamines were associated with disparate changes in the key enzymes governing polyamine biosynthesis; in MCT-treated rats, there were early and substantial increases in ODC and SAM-DC activities,[24] while in chronically hypoxic lungs SAM-DC activity was increased, but ODC activity was persistently depressed.[25]

Unlike the apparent role for de novo polyamine synthesis enzymes in MCT-induced PAH, hypoxic pulmonary hypertension was linked to augmented polyamine transport. In support of this idea, we found in rat lung explants that PUT uptake was augmented, and efflux was reduced in chronic hypoxia.[25] Activities of polyamine interconverting enzymes, SAT, and polyamine oxidase also were elevated.[25] We used autoradiography to delineate cell types displaying increased polyamine transport in hypoxia.[26] Previous reports on the cellular localization of polyamine uptake focused on the normoxic lung and identified the most prominent sites of uptake as alveolar type I and II cells.[27] Uptake by cells of the normoxic pulmonary circulation had not been appreciated. In hypoxia, however, we found increases in the density of [^{14}C]-SPD labeling in both intimal and medial layers of conduit, muscularized, and partially muscularized pulmonary arteries (Fig. 24.3). The extent of [^{14}C]-SPD uptake in main pulmonary artery explant preparations also was elevated in hypoxia, and autoradiography revealed that the increase could be ascribed to augmented

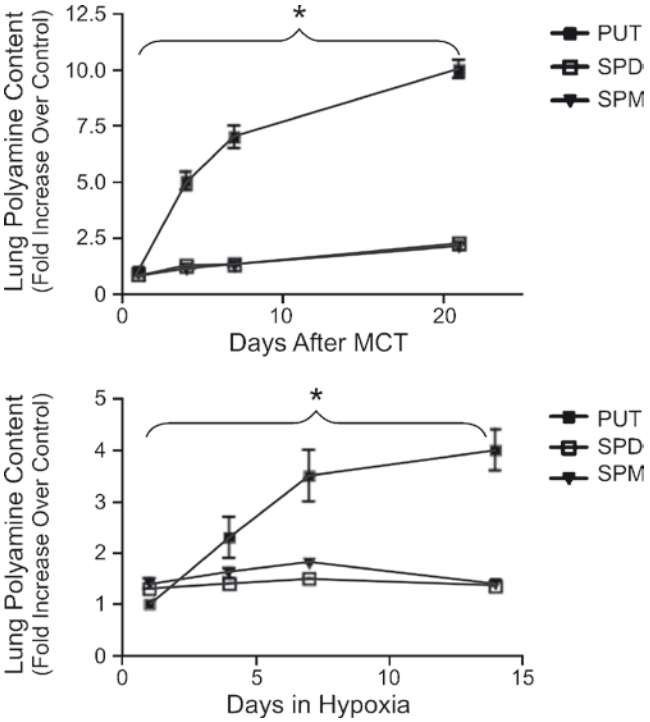

Fig. 24.1 Increases in lung tissue contents of PUT, SPD, and SPM as a function of time in rats with pulmonary arterial hypertension induced by MCT (*top*) and hypoxia (*bottom*). Lung tissue contents of all three polyamines were increased at each time point after treatment with MCT or hypoxia, respectively, in comparison to control rat lung tissue. *Significantly different from control at $p < 0.05$. Adapted with permission[22,23]

labeling of both intimal and medial arterial layers. The hypoxia-induced increase in [^{14}C]-SPD transport was most evident in smooth muscle cells of the media. Viewed collectively, these findings in lung and main pulmonary arterial explant preparations suggested that hypoxia increases SPD uptake in pulmonary artery endothelial cells (PAECs) and PASMCs, most conspicuously in the latter.

We undertook additional studies in rat cultured PAECs and PASMCs with the aims of determining the mechanism of the hypoxic effect on polyamine import and whether there were multiple transporters that were differentially regulated by hypoxia.[28,29] Confluent cultures of both cell types were exposed to normoxic or hypoxic culture conditions and uptake rates for [^{14}C]-PUT, -SPD, and -SPM determined as a function of polyamine concentration. In general terms, polyamine transport pathways in normoxic, control populations of these lung vascular cells resembled other cells described in the literature; they exhibited time, temperature, and concentration dependencies. Polyamine transport in lung vascular cells required ongoing RNA synthesis. Protein synthesis inhibition was associated with a transient increase in polyamine import, presumably as a result of relief from antizyme-

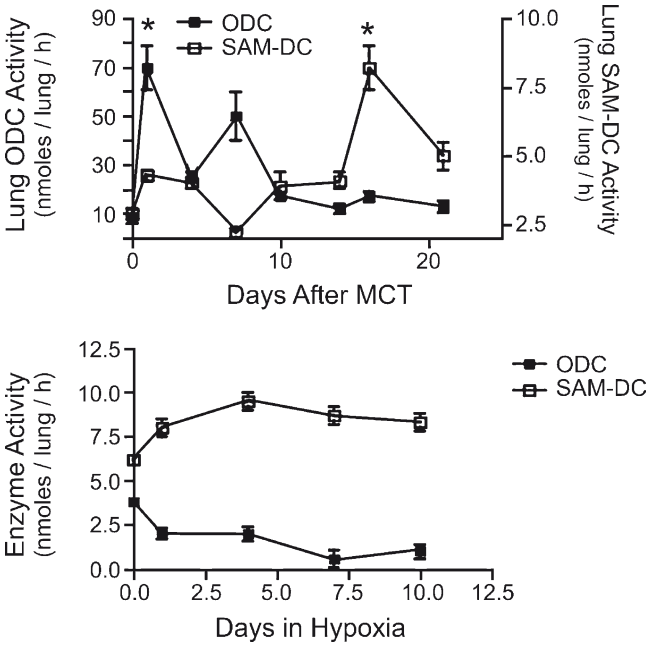

Fig. 24.2 Changes in activities of the key enzymes governing polyamine biosynthesis, ODC and SAM-DC, in lung tissue from rats with MCT-induced (*top*) and hypoxia-induced (*bottom*) PAH. *Significantly different from control at $p < 0.05$. Note that in MCT-treated rats, there were early and substantial increases in ODC and SAM-DC activities, while in chronically hypoxic lungs SAM-DC activity was increased, but ODC activity was persistently depressed. Adapted with permission[24,25]

mediated inhibition, while longer-term protein synthesis inhibition resulted in a reduction in polyamine import. Uptake of PUT, SPD, and SPM could be modeled according to Michaelis–Menten kinetics; values for K_m and V_{max} in the two cell populations are shown in Table 24.1. In normoxic cells, the kinetic parameters are on the same order of magnitude.

In other key respects, however, the polyamine transport pathways operative in cultured rat PAECs and PASMCs differ. For example, in ECs there is relatively little cross competition between the three polyamines for uptake,[28] while in smooth muscle cells, SPD and SPM exhibit cross competition and inhibit PUT uptake, while PUT has minimal effect on the import of SPD and SPM.[29] The sodium dependence of polyamine transport also appears to differ between the cell types.[30] Replacing sodium with choline inhibits uptake of all three polyamines in ECs, while in PASMCs PUT import is more prominently inhibited by sodium depletion than the other polyamines. Finally, the most interesting difference pertains to the transport response to hypoxia. As shown in Table 24.1, PAECs responded to hypoxia with an increase in the V_{max} for transport for all three polyamines, while smooth muscle cells exhibited a selective increase in the V_{max} for PUT uptake, with the values for SPD and SPM unchanged from controls.

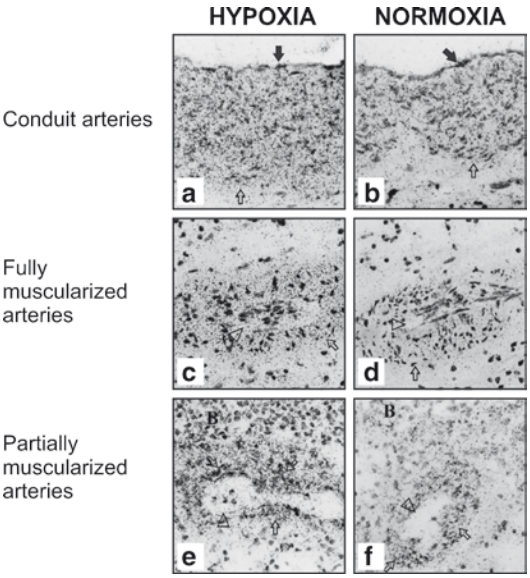

Fig. 24.3 Autoradiographs of [^{14}C]-SPD uptake in rat lung explants. Conduit arteries exhibited prominent [^{14}C]-SPD uptake in the medial layer (*open arrows* demarcate the medial-adventitial border) and a moderate increase in the intima (*black arrow*) under hypoxic conditions (**a**) in comparison to normoxic uptake (**b**). Similar increases in [^{14}C]-SPD uptake were observed in small fully muscularized arteries in hypoxia (**c**) relative to normoxia (**d**); *arrows* delineate medial-adventitial border, *arrowheads* point at the lumen. Medial smooth muscle cells in partially muscularized small arteries (*arrows*) also demonstrated increased SPD uptake in hypoxia (**e**) compared to normoxia (**f**); *arrowheads* point at lumen, bronchus labeled with B. Counterstained with toluidine blue. Magnification: ×420. Adapted with permission[26]

Table 24.1 Values of K_m and V_{max} for polyamine uptake in rat PASMCs and PAECs cultured under control (CON) and hypoxic (HYP) conditions

K_m (μM)						
	PUT		SPD		SPM	
	PASMCs	ECs	PASMCs	ECs	PASMCs	ECs
CON	7.06 ± 3.48	5.10 ± 0.75	2.48 ± 0.66	2.50 ± 0.45	2.85 ± 0.92	5.60 ± 1.15
HYP	14.80 ± 2.97*	6.10 ± 0.75	2.41 ± 0.73	2.80 ± 0.65	2.04 ± 0.77	11.3 ± 3.0*
V_{max} *(pmol/10^6 cells/min)*						
	PUT		SPD		SPM	
	PASMCs	ECs	PASMCs	ECs	PASMCs	ECs
CON	9.14 ± 2.33	5.60 ± 0.30	21.23 ± 1.92	4.60 ± 0.25	17.44 ± 2.04	3.50 ± 0.30
HYP	29.30 ± 3.92*	10.90 ± 0.06	24.72 ± 2.53	8.60 ± 0.65*	19.88 ± 2.39	8.90 ± 1.20*

Values are expressed as mean ± standard error. *Significantly increased ($p < 0.05$) in hypoxic cells relative to cells under control conditions. Adapted with permission[28, 29]

4 Polyamine Synthesis and Transport as Targets for Intervention in PAH

We used DFMO, the site-selective, "suicide" inhibitor of ODC, to determine if increased ODC activity was important for MCT-induced pulmonary hypertension in intact rats.[31,32] As shown in Fig. 24.4, chronic treatment with DFMO prevented the MCT-induced increase in the lung tissue content of all three polyamines. In addition, and also shown in Fig. 24.4, DFMO prevented MCT-induced elevation in pulmonary artery pressure, right ventricular hypertrophy, and medial arterial thickening. Companion studies demonstrated that DFMO also suppressed MCT-induced pulmonary vascular hyperreactivity and edema formation.[31,33] Treatment of rats after the onset of MCT-caused PAH reversed the increased pulmonary arterial pressure and right ventricular hypertrophy.[32] These observations emphasize the importance of ODC and de novo polyamine synthesis in regulating lung polyamine contents and attendant changes in lung structure in the MCT model of chronic pulmonary hypertension.

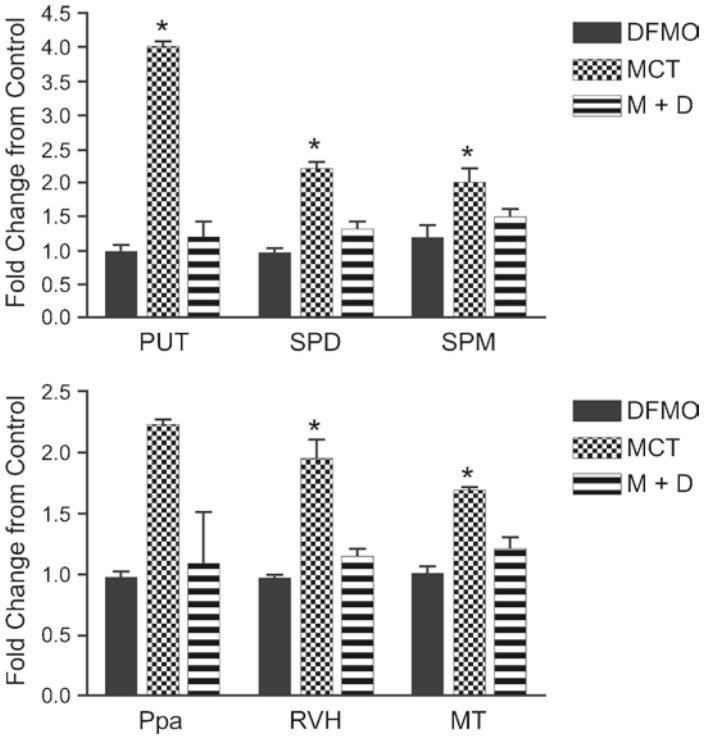

Fig. 24.4 DFMO prevents the MCT-induced increase in the lung tissue content of all three polyamines (*top*) and pathophysiologic alterations (*bottom*), including MCT-induced elevations in pulmonary artery pressure (Ppa), right ventricular hypertrophy (RVH), and medial arterial thickening (MT). Adapted with permission[31,32]

The hypoxia-induced increase in polyamine uptake by pulmonary vascular cells raises the question of whether lung cell polyamine regulatory pathways could serve as targets of pharmacologic intervention in hypoxic pulmonary hypertension. Based on the finding that hypoxia decreases ODC activity, ODC blockade would not seem to be a promising approach. Indeed, as shown in Fig. 24.5, prolonged pretreatment with DFMO modestly attenuated the increase in lung PUT and failed to have an impact on the rise in lung SPM evoked by hypoxia. The effects of DFMO on hypoxia-induced pathophysiologic changes also were unimpressive. There were limited reductions in the extent of PAH and medial arterial thickening, and right ventricular hypertrophy was unaffected by polyamine synthesis blockade.[34]

There are relatively few selective inhibitors of polyamine import. One of the first was a polymeric glutaraldehyde conjugate of SPM (poly-SPM) capable of discriminating between the multiple polyamine import pathways present in PASMCs and other cell types.[35,36] Interestingly, a comparison of the effects of poly-SPM and DFMO on PASMC polyamine content suggested that polyamine transport plays a previously unappreciated role in determining PASMC polyamine contents. We

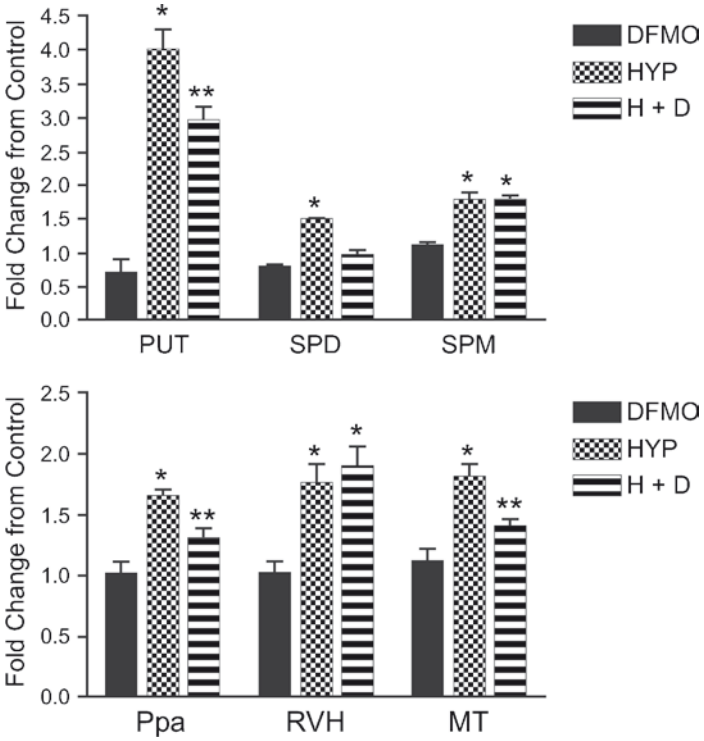

Fig. 24.5 DFMO suppresses, but not does abolish, hypoxia-induced increases in the lung tissue content of the three polyamines (*Top*) and pathophysiologic alterations (*Bottom*), including, hypoxia-induced elevations in pulmonary artery pressure (Ppa), right ventricular hypertrophy (RVH), and medial arterial thickening (MT). Adapted with permission[34]

found that whereas polyamine synthesis inhibition caused partial depletion of cellular SPD with no change in SPM content, the transport inhibitor, either alone or in combination with DFMO, caused prominent reductions in the levels of both SPD and SPM (Table 24.2).

Two lines of evidence support the prospect that polyamine import blockade could serve as a useful strategy in PAH. Using a newer-generation polyamine transport inhibitor, ORI1202, we found that suppression of hypoxia-induced polyamine import blocked p38 MAP kinase activation in hypoxic PASMCs[37] (Fig. 24.6). This observation is of interest because the mitogen-activated protein (MAP) kinase pathway is centrally involved in the response to hypoxia in a variety of cells.[38,39] In companion studies, this time using poly-SPM to prevent the hypoxia-induced increase in polyamine import, we found that noncytotoxic concentrations of the transport inhibitor prevented accumulation of soluble fibronectin in PASMCs (Fig. 24.7). Fibronectin, of course, is a critical extracellular matrix protein whose deposition, along with other components of the extracellular matrix, is grossly altered in PAH.[40]

5 Summary and Future Directions

The work presented showed in two widely used animal models of chronic PAH that polyamines are required for structural remodeling of the pulmonary arterial circulation, for sustained increases in pulmonary artery pressure, and for formation of right ventricular hypertrophy. While cellular mechanisms by which polyamines contribute to PAH are no doubt linked to their critical involvement in fundamental processes such as cell proliferation and differentiation, many questions remain concerning the pathways regulating lung vascular cell polyamine contents and the specific cellular responses so mediated. For example, little is known about ODC regulation in MCT- and hypoxia-induced lung vascular disease. While studies in tumor cell lines would strongly suggest that the prominent increase in ODC noted in lung cells from MCT-treated rats likely involves transcriptional activation of the ODC gene by c-Myc,[41] the mechanism by which ODC is persistently downregulated in hypoxia is less clear. Our results suggest that antizyme is probably not involved,[28] but a more promising concept is that protein–protein interactions between the hypoxia-inducible transcription factor 1 (HIF-1) and c-Myc may suppress the latter's ability to maintain ODC expres-

Table 24.2 Impact of the polyamine import blocker poly-SPM on polyamine contents in cultured rat PASMCs

	Spermidine (mmol/mg protein)	Spermine (mmol/mg protein)
Control	12,600 ± 59	23,059 ± 983
Poly-SPM	4,385 ± 79*	11,437 ± 272*
DFMO	2,973 ± 22*	20,087 ± 153
Poly-SPM + DFMO	1,287 ± 100*	9,790 ± 250*

*Significantly different from control ($p < 0.05$). Adapted with permission[35]

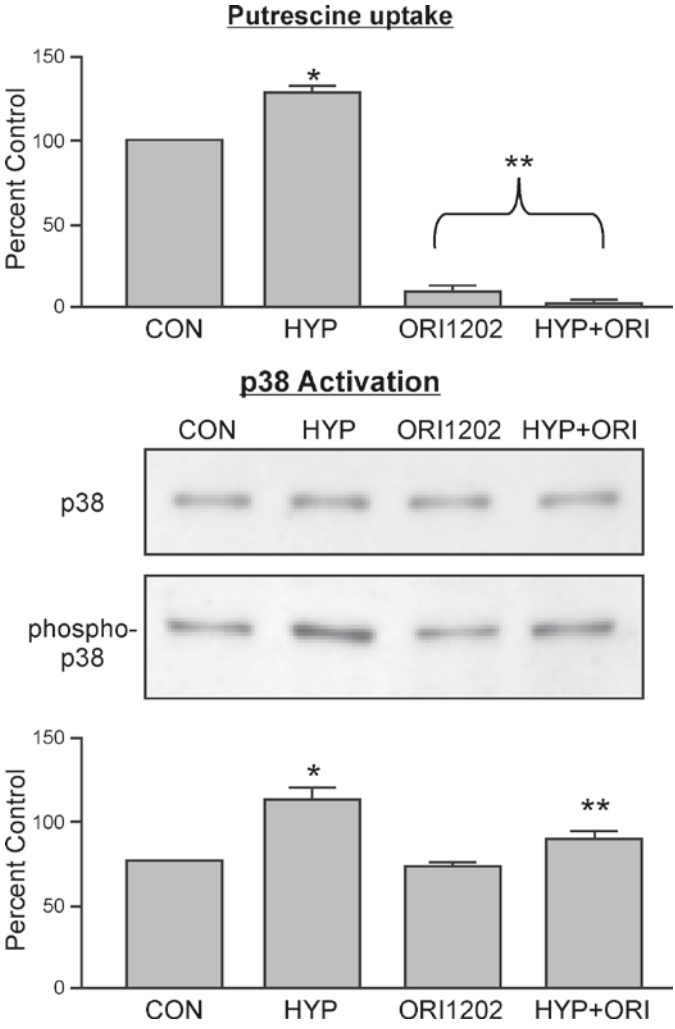

Fig. 24.6 Impact of the polyamine transport inhibitor ORI1202 on hypoxic activation of p38 MAP kinase. *Top*: Uptake of 0.3 μ*M* putrescine in normoxic, control PASMCs (CON), PASMCs cultured in hypoxia for 6 h (HYP), control PASMCs incubated with 30 μ*M* ORI1202, and hypoxic PASMCs incubated with ORI1202. *Middle*: Western analyses of total and phospho-p38 in normoxic, control PASMCs (CON), PASMCs cultured in hypoxia for 3 h (HYP), control PASMCs incubated with 30 μ*M* ORI1202, and hypoxic PASMCs incubated with ORI1202. *Bottom*: Band intensities were quantified by densitometry and expressed as a percentage of control for four experiments. *Significantly different from normoxic control at $p < 0.05$. Identical results were obtained in PASMCs cultured in hypoxia for 24 h (data not shown). Adapted with permission[37]

sion.[42] Obviously, additional studies will be required to address this possibility. In a related context, the mechanisms by which hypoxia augments polyamine transport and, of even more basic import, why there are multiple polyamine transporters that seem to be differentially regulated in discrete cells of the pulmonary

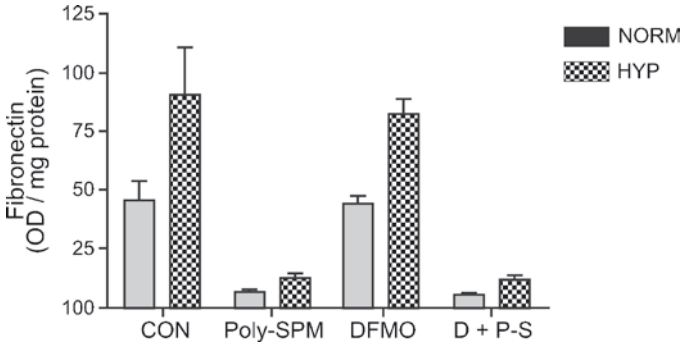

Fig. 24.7 The polyamine uptake inhibitor poly-SPM prevents hypoxia-induced accumulation of soluble fibronectin in PASMCs. n = 4–6 for each experimental group. *Significantly different from normoxic controls at $p < 0.05$

arterial circulation are unresolved issues. The fact that the mammalian polyamine transport proteins have yet to be isolated is a critical obstacle to resolving these deficiencies.[43] Finally, and as noted, while polyamines are required for cell growth and differentiation, emerging evidence suggests that they may govern other, more dynamic cellular processes incriminated in lung vascular remodeling. For example, polyamines regulate mitochondrial function,[44] and abnormalities in mitochondria have been linked to sustained PAH.[45] Polyamines also exert rather potent antioxidant activity,[46] and oxidant stress has been detected in human PAH.[47] Apoptosis, suggested to drive EC death leading to formation of occlusive intimal lesions in PAH,[48] also is governed by polyamines,[49] and whether polyamine-dependent PAEC apoptosis plays a role in intimal lesion formation is an interesting possibility. These are just a few of the unresolved issues relating to involvement of this interesting and ubiquitously distributed family of organic cations in PAH.

The integrated response of the pulmonary circulation to stimuli causing PAH is complex, involving many different cell types, mediators, and transduction pathways. The polyamines, by virtue of their role in multiple signaling events, would seem to be an important point of integration of the many stimuli acting on hypoxic lung cells. Our findings that polyamine transport, rather than de novo polyamine synthesis, seems to be the dominant pathway regulating lung vascular cell polyamines in hypoxia suggest that polyamine transporters may be an isolated target for intervention. The inhibitory effect of polyamine transport inhibitors ORI1202 and poly-SPM on hypoxia-induced p38 MAP kinase phosphorylation and soluble fibronectin release in PASMCs is provocative. If the effector cells in hypoxic pulmonary hypertension rely on augmented polyamine import as a source of signaling molecules while cells not intimately linked to the hypoxic response continue to synthesize polyamines via the actions of ODC, then transport blockade could be a rather selective pharmacologic strategy. Studies in intact animal models would be helpful in resolving this issue and establishing the safety

and efficacy of polyamine transport inhibitors as a therapeutic strategy in hypoxic pulmonary hypertension.

By contrast, MCT-induced pulmonary hypertension seeks to rely on increased ODC activity to produce the polyamine pools needed for pathogenic cell responses. In this regard, DFMO is a safe drug with minimal toxicity. It has been studied in numerous clinical trials and is known to be effective in treating African trypanosome infections and holds promise, in combination with sulindac, for use in preventing colorectal polyps and cancer. One may wonder why DFMO is relatively devoid of toxicity. Although the answer is unknown, it is tempting to speculate that it is the activation of the polyamine transport that compensates for ODC inhibition and thereby minimizes toxicity associated with polyamine depletion.[17] Taking this concept one step further, in disorders primarily driven by increased ODC activity restricted to specific target cell populations, DFMO may be reasonably effective while toxicity is minimized in "normal" cells by transport induction. Such a scenario would explain our results with MCT-induced PAH, which was inhibited and reversed by DFMO without undue toxicity. Against this background, given the facts that DFMO is effective in animal models of PAH, that it is safe in human subjects, and that there are no drugs available that arrest or reverse the course of PAH in humans, it seems reasonable to contemplate clinical trials of DFMO in human subjects with pulmonary hypertension.

References

1. Bauer NR, Moore TM, McMurtry IF (2007) Rodent models of PAH: are we there yet? Am J Physiol Lung Cell Mol Physiol 293:L580–L582
2. Galiè N, Manes A, Negro L, Palazzini M, Bacchi-Reggiani ML, Branzi A (2009) A meta-analysis of randomized controlled trials in pulmonary arterial hypertension. Eur Heart J 30:394–403
3. Oka M, Homma N, Taraseviciene-Stewart L et al (2007) Rho kinase-mediated vasoconstriction is important in severe occlusive pulmonary arterial hypertension in rats. Circ Res 100:923–929
4. Broughton BR, Walker BR, Resta TC (2008) Chronic hypoxia induces Rho kinase-dependent myogenic tone in small pulmonary arteries. Am J Physiol Lung Cell Mol Physiol 294:L797–L806
5. Meyrick B, Reid L (1979) Hypoxia and incorporation of ^{3}H-thymidine by cells of the rat pulmonary arteries and alveolar wall. Am J Pathol 96:51–70
6. Meyrick B, Gamble W, Reid L (1980) Development of Crotalaria pulmonary hypertension: hemodynamic and structural study. Am J Physiol 239:H692–H702
7. Tuder RM, Cool CD, Yeager M, Taraseviciene-Stewart L, Bull TM, Voelkel NF (2001) The pathobiology of pulmonary hypertension. Endothelium. Clin Chest Med 22:405–418
8. Rabinovitch M (2001) Pathobiology of pulmonary hypertension. Extracellular matrix. Clin Chest Med 22:433–449
9. Pak O, Aldashev A, Welsh D, Peacock A (2007) The effects of hypoxia on the cells of the pulmonary vasculature. Eur Respir J 30:364–372
10. Du L, Sullivan CC, Chu D et al (2003) Signaling molecules in nonfamilial pulmonary hypertension. N Engl J Med 348:500–509

11. Mandegar M, Fung YC, Huang W, Remillard CV, Rubin LJ, Yuan JX-J (2004) Cellular and molecular mechanisms of pulmonary vascular remodeling: role in the development of pulmonary hypertension. Microvasc Res 68:75–103
12. Humbert M, Morrell NW, Archer SL et al (2004) Cellular and molecular pathobiology of pulmonary arterial hypertension. J Am Coll Cardiol 43:13S–24S
13. Pegg AE, McCann PP (1982) Polyamine metabolism and function. Am J Physiol 243:C212–C221
14. Gerner EW, Meyskens FL Jr (2004) Polyamines and cancer: old molecules, new understanding. Nat Rev Cancer 4:781–792
15. Wallace HM, Fraser AV, Hughes A (2003) A perspective of polyamine metabolism. Biochem J 376:1–14
16. Pegg AE (2006) Regulation of ornithine decarboxylase. J Biol Chem 281:14529–14532
17. Seiler N, Delcros JG, Moulinoux JP (1996) Polyamine transport in mammalian cells. An update. Int J Biochem Cell Biol 28:843–861
18. Ikeguchi Y, Bewley MC, Pegg AE (2006) Aminopropyltransferases: function, structure and genetics. J Biochem 139:1–9
19. Bewley MC, Graziano V, Jiang J et al (2006) Structures of wild-type and mutant human spermidine/spermine N1-acetyltransferase, a potential therapeutic drug target. Proc Natl Acad Sci U S A 103:2063–2068
20. Rubinstein S, Breitbart H (1994) Cellular localization of polyamines: cytochemical and ultrastructural methods providing new clues to polyamine function in ram spermatozoa. Biol Cell 81:177–183
21. Toninello A, Dalla Via L, Siliprandi D, Garlid KD (1992) Evidence that spermine, spermidine, and putrescine are transported electrophoretically in mitochondria by a specific polyamine uniporter. J Biol Chem 267:18393–18397
22. Olson JW, Hacker AD, Altiere RJ, Gillespie MN (1984) Polyamines and the development of monocrotaline-induced pulmonary hypertension. Am J Physiol 247:H682–H685
23. Olson JW, Hacker AD, Atkinson JE, Altiere RJ, Gillespie MN (1986) Polyamine content in rat lung during development of hypoxia-induced pulmonary hypertension. Biochem Pharmacol 35:714–716
24. Olson JW, Altiere RJ, Gillespie MN (1984) Prolonged activation of rat lung ornithine decarboxylase in monocrotaline-induced pulmonary hypertension. Biochem Pharmacol 33:3633–3637
25. Shiao RT, Kostenbauder HB, Olson JW, Gillespie MN (1990) Mechanisms of lung polyamine accumulation in chronic hypoxic pulmonary hypertension. Am J Physiol 259:L351–L358
26. Babal P, Manuel SM, Olson JW, Gillespie MN (2000) Cellular disposition of transported polyamines in hypoxic rat lung and pulmonary arteries. Am J Physiol Lung Cell Mol Physiol 278:L610–L617
27. Hoet PH, Nemery B (2000) Polyamines in the lung: polyamine uptake and polyamine-linked pathological or toxicological conditions. Am J Physiol Lung Cell Mol Physiol 278:L417–L433
28. Babal P, Ruchko M, Ault-Ziel K, Cronenberg L, Olson JW, Gillespie MN (2002) Regulation of ornithine decarboxylase and polyamine import by hypoxia in pulmonary artery endothelial cells. Am J Physiol Lung Cell Mol Physiol 282:L840–L846
29. Aziz SM, Olson JW, Gillespie MN (1994) Multiple polyamine transport pathways in cultured pulmonary artery smooth muscle cells: regulation by hypoxia. Am J Respir Cell Mol Biol 10:160–166
30. Aziz SM, Lipke DW, Olson JW, Gillespie MN (1994) Role of ATP and sodium in polyamine transport in bovine pulmonary artery smooth cells. Biochem Pharmacol 48:1611–1618
31. Olson JW, Atkinson JE, Hacker AD, Altiere RJ, Gillespie MN (1985) Suppression of polyamine biosynthesis prevents monocrotaline-induced pulmonary edema and arterial medial thickening. Toxicol Appl Pharmacol 81:91–99
32. Olson JW, Orlinska U, Gillespie MN (1989) Polyamine synthesis blockade in monocrotaline-induced pneumotoxicity. Biochem Pharmacol 38:2903–2910
33. Gillespie MN, Dyer KK, Olson JW, O'Connor WN, Altiere RJ (1985) α-Difluoromethylornithine, an inhibitor of polyamine synthesis, attenuates monocrotaline-induced pulmonary vas-

cular hyperresponsiveness in isolated perfused rat lungs. Res Commun Chem Pathol Pharmacol 50:365–378
34. Atkinson JE, Olson JW, Altiere RJ, Gillespie MN (1987) Evidence that hypoxic pulmonary vascular remodeling in rats is polyamine dependent. J Appl Physiol 62:1562–1568
35. Aziz SM, Gosland MP, Crooks PA, Olson JW, Gillespie MN (1995) A novel polymeric spermine conjugate inhibits polyamine transport in pulmonary artery smooth muscle cells. J Pharmacol Exp Ther 274:181–186
36. Aziz SM, Gillespie MN, Crooks PA et al (1996) The potential of a novel polyamine transport inhibitor in cancer chemotherapy. J Pharmacol Exp Ther 278:185–192
37. Ruchko M, Gillespie MN, Weeks RS, Olson JW, Babal P (2003) Putrescine transport in hypoxic rat main PASMCs is required for p38 MAP kinase activation. Am J Physiol Lung Cell Mol Physiol 284:L179–L186
38. Kulisz A, Chen N, Chandel NS, Shao Z, Schumacker PT (2002) Mitochondrial ROS initiate phosphorylation of p38 MAP kinase during hypoxia in cardiomyocytes. Am J Physiol Lung Cell Mol Physiol 282:L1324–L1329
39. Welsh DJ, Peacock AJ, MacLean M, Harnett M (2001) Chronic hypoxia induces constitutive p38 mitogen-activated protein kinase activity that correlates with enhanced cellular proliferation in fibroblasts from rat pulmonary but not systemic arteries. Am J Respir Crit Care Med 164:282–289
40. Vyas-Somani AC, Aziz SM, Arcot SA, Gillespie MN, Olson JW, Lipke DW (1996) Temporal alterations in basement membrane components in the pulmonary vasculature of the chronically hypoxic rat: impact of hypoxia and recovery. Am J Med Sci 312:54–67
41. Auvinen M, Jarvinen K, Hotti A et al (2003) Transcriptional regulation of the ornithine decarboxylase gene by c-Myc/Max/Mad network and retinoblastoma protein interacting with c-Myc. Int J Biochem Cell Biol 35:496–521
42. Huang LE (2008) Carrot and stick: HIF-α engages c-Myc in hypoxic adaptation. Cell Death Differ 15:672–677
43. Sakata K, Kashiwagi K, Igarashi K (2000) Properties of a polyamine transporter regulated by antizyme. Biochem J 347:297–303
44. McCormack JG (1989) Effects of spermine on mitochondrial Ca^{2+} transport and the ranges of extramitochondrial Ca^{2+} to which the matrix Ca^{2+}-sensitive dehydrogenases respond. Biochem J 264:167–174
45. Archer SL, Gomberg-Maitland M, Maitland ML, Rich S, Garcia JG, Weir EK (2008) Mitochondrial metabolism, redox signaling, and fusion: a mitochondria-ROS-HIF-1α-Kv1.5 O_2-sensing pathway at the intersection of pulmonary hypertension and cancer. Am J Physiol Heart Circ Physiol 294:H570–H578
46. Lovaas E, Carlin G (1991) Spermine: an anti-oxidant and anti-inflammatory agent. Free Radic Biol Med 11:455–461
47. Bowers R, Cool C, Murphy RC et al (2004) Oxidative stress in severe pulmonary hypertension. Am J Respir Crit Care Med 169:764–769
48. Taraseviciene-Stewart L, Kasahara Y, Alger L et al (2001) Inhibition of the VEGF receptor 2 combined with chronic hypoxia causes cell death-dependent pulmonary endothelial cell proliferation and severe pulmonary hypertension. FASEB J 15:427–438
49. Poulin R, Pelletier G, Pegg AE (1995) Induction of apoptosis by excessive polyamine accumulation in ornithine decarboxylase-overproducing L1210 cells. Biochem J 311:723–727

5-HT Receptors and K_V Channel Internalization

Angel Cogolludo and Francisco Perez-Vizcaino

Abstract Serotonin (5-HT) and voltage-gated potassium (KV) channels have emerged as two major factors in the pathogenesis of pulmonary arterial hypertension (PAH). In pulmonary artery smooth muscle cells (PASMC), KV channels play a major role in the regulation of pulmonary arterial tone and proliferation. Thus, activation of KV channels leads to vasodilatation and enhances apoptosis, while their inhibition is associated with vasoconstriction and proliferation in PASMC. Moreover, these channels have a prominent role as a common target for pulmonary vasoconstrictors. Modulation of these channels by vasoconstrictors involves the activation of a variety of protein kinases. Here we review the role of localization and internalization as a novel mechanism for acute regulation of KV channels and pulmonary vascular tone by agonists such as serotonin.

Keywords Serotonin • potassium channels • pulmonary arteries • caveola • endocytosis

1 Introduction

Pulmonary circulation is a high-flow and low-pressure system so that under physiological conditions pulmonary artery pressure (PAP) is about one sixth of that in systemic vessels. However, PAP may be increased either as a primary event or more frequently as a result of diverse diseases. *Pulmonary arterial hypertension* (PAH), defined as a sustained increased of mean PAP over 25 mmHg at rest, is characterized by vasoconstriction and remodeling of the pulmonary arteries, causing a progressive increase in pulmonary vascular resistance, leading to right

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ventricular hypertrophy, heart failure, and premature death. The pathophysiology of PAH is still not fully understood, but appears to be associated with an imbalance between vasodilation–apoptosis/vasoconstriction–proliferation in which genetic predisposing and environmental factors together with a number of vasoactive mediators contribute.[1,2] Thus, it is generally accepted that PAH has a multifactorial pathobiology, and interactions between different pathophysiological factors are likely to occur.[3,4] Serotonin (5-HT) and voltage-gated potassium (K_v) channels have emerged as two major factors in PAH pathogenesis. Here, we describe an interaction between these two factors.

2 Serotonin in the Pulmonary Circulation

5-HT has long been suspected to play a role in the pathogenesis of PAH. In the 1960s, an association between the appetite suppressant aminorex (which affects the release or uptake of 5-HT) intake and PAH was identified, and this drug was withdrawn from the market in 1972. In the 1980s, the use of serotoninergic appetite suppressant drugs, mainly fenfluramines, was also associated with an increased risk of developing PAH.[5] Since then, increasing interest in the role of 5-HT in the pathogenesis of PAH has been paid by researchers and clinicians. Currently, several lines of evidence indicate the central role of 5-HT in the pathogenesis of PAH.[1,2,6] First, 5-HT accelerates platelet aggregation, is an effective pulmonary vasoconstrictor, and induces vascular smooth muscle hyperplasia.[2,6,7] Moreover, elevated circulating peripheral serotonin has been associated with the development of PAH.[8,9] In addition, viral-induced overexpression of angiopoietin 1 has been shown to induce PAH, and this is associated with increased production and secretion of 5-HT from endothelial cells.[10]

5-HT targets pulmonary artery smooth muscle cell (PASMC) receptors, namely, $5\text{-HT}_{1B/D}$, 5-HT_{2A}, and 5-HT_{2B}, to cause pulmonary vasoconstriction. On the other hand, 5-HT may enter PASMCs through interaction with the specific 5-HT transporter (5-HTT), resulting in proliferation. Many studies have evidenced the role of 5-HT transporter and receptors in PAH. Thus, 5-HTT overexpression or polymorphisms in the gene encoding 5-HTT are associated with PAH.[6,11] Furthermore, mice lacking 5-HTT or 5-HT receptors (e.g., 5-HT_{1B} or 5-HT_{2B}) show attenuated PAH induced by hypoxia.[7,11,12] Specific pharmacological inhibition of 5-HT_{1B}, 5-HT_{2A}, or 5-HT_{2B} receptors or 5-HTT attenuates or reverses the development of PAH and prolongs survival in animal models of PAH.[7,12–14] The inhibition of tryptophan hydroxylase 1 (Tph-1), the rate-limiting enzyme in the synthesis of 5-HT, has emerged as a possible therapeutic target in PAH.[6]

3 K_v Channels in the Pulmonary Circulation

Potassium channels comprise a diverse and ubiquitous class of membrane proteins that facilitate the diffusion of potassium ions across the plasma membrane. In PASMCs, potassium channels are key determinants in the control of resting

membrane potential E_m and play a major role in the regulation of pulmonary arterial tone.[15,16] Opening of potassium channels leads to membrane hyperpolarization, closure of voltage-gated L-type Ca^{2+} channels, decrease in intracellular Ca^{2+} ($[Ca^{2+}]_i$), and vasodilatation. On the other hand, closure of potassium channels causes membrane depolarization, activation of voltage-gated L-type Ca^{2+} channels, increase in $[Ca^{2+}]_i$, and vasoconstriction. Moreover, activation of potassium channels enhances apoptosis, while their inhibition is associated with proliferation in PASMCs.[17] The five main types of potassium channels identified in PASMCs are K_v channels, large-conductance Ca^{2+}-activated channels (BK_{Ca}), inward rectifiers (K_{IR}), adenosine triphosphate (ATP)-dependent channels (K_{ATP}), and two-pore domain K^+ channels (K_{2P}). Among these, K_v channels have been reported to make a substantial contribution to whole-cell K^+ conductance and resting membrane potential in PASMCs, and their inhibition causes elevation of $[Ca^{2+}]_i$ and contraction of pulmonary arteries.[15,16] It has became clear that two-pore domain K^+ channels, especially TASK-1 channels, also control resting membrane potential in PASMCs,[18] and their inhibition may bring membrane potential to values at which K_v channels are active.

K_v channels exist as tetramers formed by four transmembrane $K_v\alpha$ subunits combined with modulatory cytosolic $K_v\beta$ subunits. In human pulmonary arteries, 22 transcripts of $K_v\alpha$ ($K_v1.1$–1.7, $K_v1.10$, $K_v2.1$, $K_v3.1$, $K_v3.3$, $K_v3.4$, $K_v4.1$–4.2, $K_v5.1$, $K_v6.1$–6.3, $K_v9.1$, $K_v9.3$, $K_v10.1$, and $K_v11.1$) and three of $K_v\beta$ subunits ($K_v\beta1$–3) have been identified by reverse-transcription polymerase chain reaction (RT-PCR).[19] The expression of different K_v channel subunits, including the α ($K_v1.1$, $K_v1.2$, $K_v1.3$, $K_v1.5$, $K_v1.6$, $K_v2.1$, $K_v3.1b$, and $K_v9.3$) and the β ($K_v\beta1.1$, $K_v\beta1.2$, $K_v\beta1.3$, and $K_v\beta2.1$) subunits, has also been verified at the protein level.[20] In addition, heterotetrameric assembly of distinct $K_v\alpha$ subunits can occur. Among the variety of K_v channels expressed in PASMCs, $K_v1.5$ and $K_v2.1$ channels seem to be major contributors to the native K_v current. Likewise, $K_v1.5$ and $K_v2.1$ are oxygen-sensing channels and have been involved in the control of resting membrane potential and in mediating hypoxic pulmonary vasoconstriction.[16] In PASMCs, decreased function of K_v channels is associated with cell proliferation,[17] whereas upregulation of $K_v1.5$ correlates with an increase in apoptosis/proliferation ratio and prevents and reverses PAH.[21] Furthermore, decreased expression or activity and mutations of $K_v1.5$ occurs in human[22] and experimental[16,23] PAH. Therefore, the reduction in K_v channel function and activity results in a more depolarized membrane potential in PASMCs from PAH patients and in an increase in $[Ca^{2+}]_i$, leading to vasoconstriction and proliferation. On the contrary, in vivo gene transfer of $K_v1.5$ restores hypoxic pulmonary vasoconstriction and reduces PAH.[23] In addition, normalization of elevated pulmonary vascular resistance in different models of PAH following treatment with dichloroacetate (an inhibitor of the mitochondrial enzyme pyruvate dehydrogenase kinase) and survivin-targeting gene therapy has been associated with the restoration or activation of K_v channels.[21,24] Altogether, these data support the idea that gene transfer of K_v channels or drugs activating K_v channels may have potential therapeutic value in the management of PAH.

4 Regulation of K_v Channels by Pulmonary Vasoconstrictors

K_v channel inhibition has been involved in the pulmonary constrictor effects induced by a variety of stimuli, such as hypoxia,[16,25] endothelin 1,[26] thromboxane A_2,[27,28] 5-HT,[29] anorectic drugs,[30] and the antiparkinsonian drug pergolide.[31] These studies indicated a prominent role of K_v channels as a common target for pulmonary vasoconstrictors. Interestingly, a number of mechanisms have been involved in the inhibition of PASMC K_v channels. The precise mechanisms involved in hypoxia-induced inhibition of K_v channels are still nuclear, but it has been proposed that the hypoxia-induced closure of K_v channels results from a change in cytoplasmic reactive oxygen species or redox status in PASMCs.[25,32] There is controversy regarding whether hypoxia increases or decreases ROS. Intriguingly, application of the oxidant *t*-butyl hydrogen peroxide increased current flowing through $K_v1.5$ channels cloned from human pulmonary arteries and expressed in CHO (Chinese hamster ovary) cells,[32] while it reduced K_v currents in freshly isolated rat PASMCs.[33]

In human PASMCs, endothelin 1 inhibits K_v currents, and this effect was reduced in the presence of staurosporine or GF-109203X,[26] suggesting the involvement of classic Ca^{2+}-dependent protein kinase C (PKC). On the other hand, we have reported that activation of the atypical PKCζ is involved in K_v channel inhibition and vasoconstriction induced by TXA_2 in rat and piglet pulmonary arteries.[27,28] Moreover, by using KO (knockout) mice we confirmed the role of PKCζ.[34]

5 Modulation of K_v Channels by Serotonin

Figure 25.1 shows that serotonin inhibits K_v current in rat PASMCs in a concentration-dependent manner with a similar potency(EC_{50} values ~2 μmol/L) as that for pulmonary vasoconstriction. Accordingly, 5-HT depolarized membrane potential to a similar extent to that evoked by the K_v channel inhibitor 4-aminopyridine (3 mmol/L). These results strongly suggest that 5-HT-induced membrane depolarization is due to its ability to inhibit K_v channels. However, it cannot be ruled out that 5-HT may inhibit other potassium channels, in addition to K_v channels, involved in PASMC membrane potential, such as TASK channels. In fact, serotonin has been reported to fully abolish TASK-1 currents in motoneurons.[35]

The effects of 5-HT on K_v currents and membrane potential were prevented by the $5\text{-}HT_{2A}$ receptor antagonist ketanserin and by the 5-HTT inhibitor fluoxetine. However, other specific 5-HTT inhibitors such as fluvoxamine or citalopram or the $5\text{-}HT_{1B}$ antagonist SB224289 had no effects on the K_v current-blocking properties of 5-HT.[29] The different behavior of fluoxetine vs. other 5-HTT inhibitors may rely on its relatively high affinity against the $5\text{-}HT_{2A}$ receptor. 5-HT also inhibited the K_v current carried by human cloned $K_v1.5$ channels expressed in Ltk^- cells, which express $5\text{-}HT_{2A}$ receptors. This effect was also prevented by the $5\text{-}HT_{2A}$ receptor antagonist ketanserin. $5\text{-}HT_{2A}$ receptors signal primarily through heterotrimeric

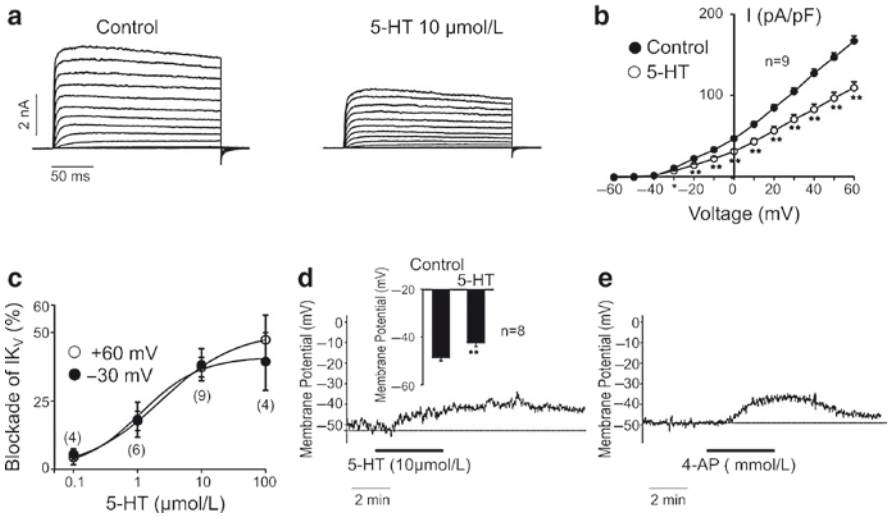

Fig. 25.1 5-HT decreases K_v current recorded in rat PASMCs. (**a**) Current traces are shown for depolarization pulses from –60 to +60 mV (in 10-mV increments) from a holding potential of -60 mV. (**b**) Current–voltage relationships of K_v current measured at the end of the pulse in the absence and the presence of 5-HT (10 µmol/L). (**c**) Concentration-dependent inhibition of K_v current by 5-HT at –30 and +60 mV. (**d** and **e**) Effects of 5-HT and 4-aminopyridine (4-AP) on membrane potential. Data show mean ± SEM (*n* in parenthesis). * and ** indicate $p < 0.05$ and $p < 0.01$ vs. control, respectively. Reprinted with permission[29]

proteins of the $G_{q/11}$ subfamily, activation of phospholipase C, the subsequent formation of diacylglycerol, and activation of classic diacylglycerol-sensitive PKC.[36] Accordingly, the effects of 5-HT on K_v currents were prevented by U73122, a phospholipase C (PLC) inhibitor, and by Gö6976, an inhibitor of classic diacylglycerol-sensitive PKCs.[29] In addition, the effects of 5-HT on K_v channels were prevented by genistein, a widely used tyrosine kinase inhibitor, and by tyrphostin 23, a selective tyrosine kinase inhibitor, whereas the tyrosine phosphatase inhibitor vanadate potentiated the inhibitory effect.[29] Since it is firmly established that K_v channels are regulated by phosphorylation on tyrosine residues, the activation of tyrosine kinases by 5-HT could modulate K_v channels by direct phosphorylation of $K_v1.5$. However, no changes were found at the level of tyrosine or serine phosphorylation in $K_v1.5$ channel protein from cells incubated with 5-HT, ruling out direct phosphorylation of $K_v1.5$ subunits.

6 Signal Compartmentalization

Specific signal transduction requires not only changes in the activity of signaling molecules but also its compartmentalization within specific cellular domains. Cell activation leads to the assembly of multimolecular complexes known as *signalosomes*.

Scaffold or adaptor proteins play a fundamental role in allowing these specific protein-protein interactions and signal compartmentalization.

PKCζ is translocated from a cytosolic to a membrane compartment on stimulation with U46619.[27] A number of scaffold proteins, including p62 (also called ZIP1 or sequestosome 1), Par-4, Par-6, and MEK5[37] are implicated in the specific signal transduction of PKCζ. Interestingly, p62 is required for the specific interaction of PKCζ and the β subunits of K_v channels,[38] and the overexpression of p62 results in a hyperpolarizing shift in the K_v current activation curve.[39] We also found that PKCζ coimmunoprecipitated with $K_v1.5$ channels in rat pulmonary arteries, and this interaction was enhanced by the thromboxane A_2 analog U646619. Moreover, the PKCζ–$K_v1.5$ interaction was absent in lungs from mice deficient in p62.[34] However, the effects of 5-HT appeared to be unrelated to activation of PKCζ.[29]

Caveolae, a subset of lipid rafts, are specialized microdomains in the plasma membrane enriched in sphingolipids and integral membrane proteins (e.g., caveolins) that compartmentalize and integrate numerous signaling events.[40] Caveolin 1 functions as a scaffold protein to allow the specific interactions of signaling molecules within the caveolae. Localization of K_v channels in lipid rafts is under debate. Interestingly, potassium channel localization in lipid rafts seems to be isoform specific. Thus, $K_v1.5$ localizes in caveolae, whereas $K_v2.1$ does so in noncaveolar lipid rafts.[41] McEwen et al.[42] found that disruption of caveolin traps $K_v1.5$ in intracellular compartments and prevents channel expression in the cell surface, indicating that caveolin is necessary for the targeting of $K_v1.5$ channels to lipid raft microdomains. However, this seems to be tissue dependent. Thus, Martinez-Marmol et al.[43] found that while in transfected HEK-293 (human embryonic kidney 293) cells, homo- and heterotetrameric $K_v1.5$ channels targeted to rafts; $K_v1.5$ did not target to rafts in macrophages. 5-HT_{2A} receptors are also associated to caveolae.[44] Interestingly, $K_v1.5$ channels coimmunoprecipitate with both 5-HT_{2A} receptors and caveolin 1 in native rat pulmonary arteries, and these interactions increased after stimulation with 5-HT (Fig. 25.2). Moreover, 5-HT failed to inhibit K_v currents in PASMCs incubated with β-cyclodextrin, a cholesterol-modifying agent that disrupts membrane lipid rafts. These results are in favor of caveolin acting as a protein chaperone for $K_v1.5$ targeting to the lipid raft microdomain.[29] Taken together, these data suggest that pulmonary vasoconstrictors may induce the formation of a signalosome, clustering the signaling proteins with the K_v channel.

A possible role of caveolin 1 in PAH has attracted attention, highlighting the role of protein compartmentalization in cell signaling under pathophysiological conditions. However, controversial results have been obtained. Thus, caveolin 1 knockout animals showed severe lung fibrosis with marked PAH and arterial hypoxemia.[45] Caveolin 1 expression was reduced in pulmonary artery from rats with monocrotaline-induced PAH[46] and in plexiform lesions from humans with PAH.[47] In contrast, caveolin 1 expression was increased in smooth muscle from patients with idiopathic PAH.[48]

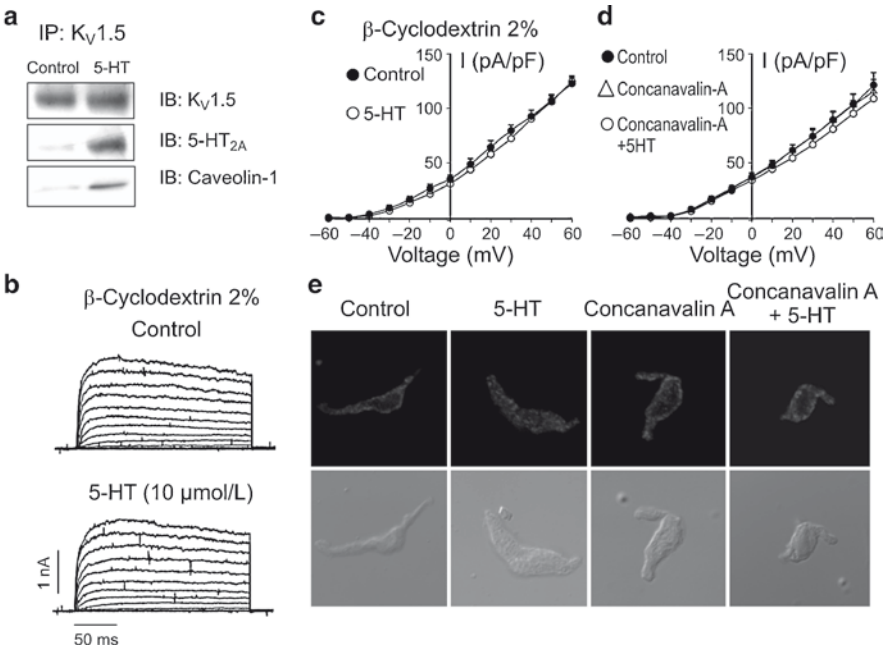

Fig. 25.2 K_V1.5 coimmunoprecipitation with 5-HT_{2A} receptors and caveolin 1 and functional consequences of lipid raft depletion. (**a**) Pulmonary arteries were treated with or without 5-HT (100 μmol/L) for 5 min. Homogenates were immunoprecipitated with anti-K_V1.5 antibody submitted to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and membranes were probed for 5-HT_{2A} receptors (MW ~52 kDa), caveolin 1 (~25 kDa), and K_V1.5. (**b**) Current traces are shown for depolarization pulses from –60 to +60 mV from a holding potential of –60 mV obtained from PASMCs incubated with β-cyclodextrin (2%) for 2 h. (**c**) Current-voltage relationships of $I_{K(V)}$ measured at the end of the pulse in the absence and the presence of 5-HT (10 μmol/L) in β-cyclodextrin-treated cells (n = 4). (**d**) Current-voltage relationships of $I_{K(V)}$ under control conditions and after perfusing with concanavalin A (250 μg/mL) and concanavalin A plus 5-HT (n = 5). (**e**) Confocal images of PASMCs stained with anti-K_V1.5 antibody. PASMCs were incubated in the absence or presence of 5-HT for 5 min; some cells were pretreated with concanavalin A for 15 min. Data show mean ± SEM. Reprinted with permission[29]

7 Role of Internalization

Besides their role in signal transduction, caveolae are involved in endocytotic processes.[40] Nevertheless, there is not much information available regarding potassium channel internalization in vascular smooth muscle cells. It has been reported that oxyhemoglobin reduced K_V current density and the staining of K_V1.5 on the plasma membrane surface, and these effects were suppressed by pharmacological inhibition of tyrosine kinases.[49] There is more information available regarding the role of G protein-coupled receptor (such as 5-HT) internalization following activation. Thus, Bhattacharya et al.[50] reported that internalization of 5-HT_{2A} receptors

began after 2-min activation and was complete within 10 min. These effects were mediated via PLC and classic PKC. Accordingly, we found that in PASMCs $K_v1.5$ channels were partly internalized on 3-min 5-HT_{2A} stimulation with 5-HT (Fig. 25.2). These effects were prevented by concanavalin A, a widely used inhibitor of endocytotic processes. Moreover, in the presence of this drug 5-HT had no effect on K_v currents.

8 Summary

In summary, K_v channels are targeted by a variety of stimuli evoking pulmonary vasoconstriction (Fig. 25.3) and involved in the pathophysiology of pulmonary hypertension. K_v channel activity may be regulated not only by altering the gating of the channel but also by controlling its localization at the membrane level. The recent data are in favor of a role of K_v channel localization and internalization as a novel mechanism for acute regulation of K_v channels and pulmonary vascular tone by agonists such as serotonin.

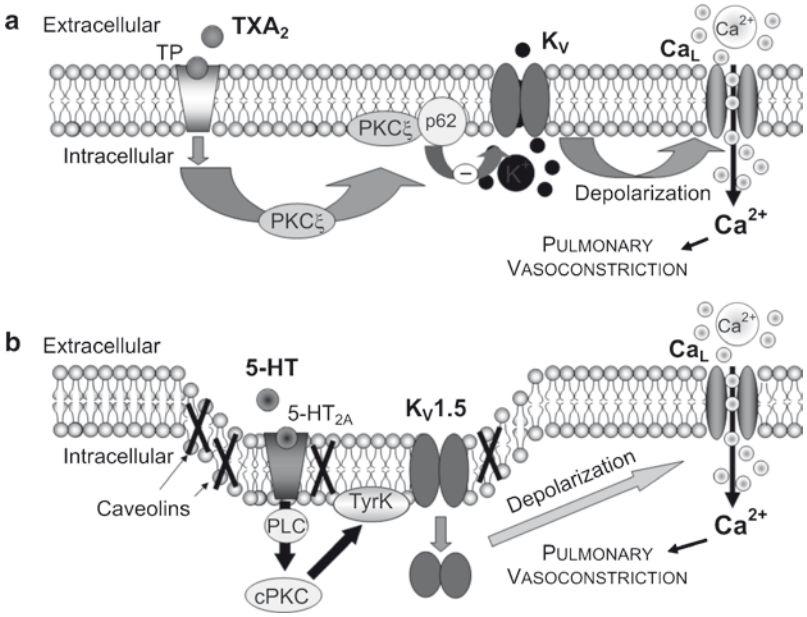

Fig. 25.3 Cartoon illustrating the signaling pathways involved in K_v channel modulation by thromboxane A_2 (**a**) and 5-HT (**b**) in PASMCs. *PLC* phospholipase C, *cPKC* classic protein kinases C, *TyrK* tyrosine kinases

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References

1. Humbert M, Morrell NW, Archer SL et al (2004) Cellular and molecular pathobiology of pulmonary arterial hypertension. J Am Coll Cardiol 43:13S–24S
2. Yuan JX-J, Rubin LJ (2005) Pathogenesis of pulmonary arterial hypertension. Circulation 111:534–538
3. Long L, MacLean MR, Jeffery TK et al (2006) Serotonin increases susceptibility to pulmonary hypertension in BMPR2-deficient mice. Circ Res 98:818–827
4. Fantozzi I, Platoshyn O, Wong AH et al (2006) Bone morphogenetic protein-2 upregulates expression and function of voltage-gated K+ channels in human pulmonary artery smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 291:L993–L1004
5. Atanassoff PG Weiss BM Schmid ER Tornic M (1992) Pulmonary hypertension and dexfenfluramine Lancet 339:436
6. Dempsie Y, MacLean MR (2008) Pulmonary hypertension: therapeutic targets within the serotonin system. Br J Pharmacol 155:455–462
7. Launay JM, Hervé P, Peoch K et al (2002) Function of the serotonin 5-hydroxytryptamine 2B receptor in pulmonary hypertension. Nat Med 8:1129–1135
8. Hervé P, Launay JM, Scrobohaci ML et al (1995) Increased plasma serotonin in primary pulmonary hypertension. Am J Med 99:249–254
9. Eddahibi S, Humbert M, Fadel E et al. (2001) Serotonin transporter overexpression is responsible for pulmonary artery smooth muscle hyperplasia in primary pulmonary hypertension. J Clin Invest 108:1141–1150
10. Sullivan CC, Du L, Chu D et al (2003) Induction of pulmonary hypertension by an angiopoietin 1/TIE2/serotonin pathway. Proc Natl Acad Sci U S A 100:12331–12336
11. Eddahibi S, Hanoun N, Lanfumey L et al (2000) Attenuated hypoxic pulmonary hypertension in mice lacking the 5-hydroxytryptamine transporter gene. J Clin Invest 105:1555–1562
12. Keegan A, Morecroft I, Smillie D, Hicks MN, MacLean MR (2001) Contribution of the 5-HT_{1B} receptor to hypoxia-induced pulmonary hypertension: converging evidence using 5-HT_{1B}-receptor knockout mice and the $5\text{-HT}_{1B/1D}$-receptor antagonist GR127935. Circ Res 89:1231–1239
13. Guignabert C, Raffestin B, Benferhat R et al (2005) Serotonin transporter inhibition prevents and reverses monocrotaline-induced pulmonary hypertension in rats. Circulation 111:2812–2819
14. Hironaka E, Hongo M, Sakai A et al (2003) Serotonin receptor antagonist inhibits monocrotaline-induced pulmonary hypertension and prolongs survival in rats. Cardiovasc Res 60:692–699
15. Yuan X-J (1995) Voltage-gated K^+ currents regulate resting membrane potential and $[Ca^{2+}]_i$ in pulmonary arterial myocytes. Circ Res 77:370–378
16. Archer S, Souil E, Dinh-Xuan AT et al (1998) Molecular identification of the role of voltage-gated K^+ channels, $K_v1.5$ and $K_v1.2$, in hypoxic pulmonary vasoconstriction and control of resting membrane potential in rat pulmonary artery myocytes. J Clin Invest 101:2319–2330
17. Burg ED, Remillard CV, Yuan JX-J (2008) Potassium channels in the regulation of pulmonary artery smooth muscle cell proliferation and apoptosis: pharmacotherapeutic implications. Br J Pharmacol 153(Suppl 1):S99–S111
18. Gurney AM, Osipenko ON, MacMillan D, McFarlane KM, Tate RJ, Kempsill FE (2003) Two-pore domain K channel, TASK-1, in pulmonary artery smooth muscle cells. Circ Res 93:957–964
19. Platoshyn O, Remillard CV, Fantozzi I et al (2004) Diversity of voltage-dependent K^+ channels in human pulmonary artery smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 287:L226–L238

20. Coppock EA, Martens JR, Tamkun MM (2001) Molecular basis of hypoxia-induced pulmonary vasoconstriction: role of voltage-gated K^+ channels. Am J Physiol Lung Cell Mol Physiol 281:L1–L12
21. McMurtry MS, Bonnet S, Wu X et al (2004) Dichloroacetate prevents and reverses pulmonary hypertension by inducing pulmonary artery smooth muscle cell apoptosis. Circ Res 95:830–840
22. Yuan X-J Wang J Juhaszova M Gaine SP Rubin L (1998) Attenuated K+ channel gene transcription in primary pulmonary hypertension. Lancet 351:726–727
23. Pozeg ZI, Michelakis ED, McMurtry MS et al (2003) In vivo gene transfer of the O_2-sensitive potassium channel $K_v1.5$ reduces pulmonary hypertension and restores hypoxic pulmonary vasoconstriction in chronically hypoxic rats. Circulation 107:2037–2044
24. McMurtry MS, Archer SL, Altieri DC et al (2005) Gene therapy targeting survivin selectively induces pulmonary vascular apoptosis and reverses pulmonary arterial hypertension. J Clin Invest 115:1479–1491
25. Archer SL, Huang J, Henry T, Peterson D, Weir EK (1993) A redox-based O_2 sensor in rat pulmonary vasculature. Circ Res 73:1100–1112
26. Shimoda LA, Sylvester JT, Sham JSK (1998) Inhibition of voltage-gated K^+ current in intrapulmonary arterial myocytes by endothelin-1. Am J Physiol 274:L842–L843
27. Cogolludo A, Moreno L, Boscá L, Tamargo J, Pérez-Vizcaino F (2003) Thromboxane A_2-induced inhibition of voltage-gated K^+ channels and pulmonary vasoconstriction. Role of protein kinase Cζ. Circ Res 93:656–663
28. Cogolludo A, Moreno L, Lodi F, Tamargo J, Perez-Vizcaino F (2005) Postnatal maturational shift from PKCζ and voltage-gated K^+ channels to RhoA/Rho kinase in pulmonary vasoconstriction. Cardiovasc Res 66:84–93
29. Cogolludo A, Moreno L, Lodi F et al (2006) Serotonin inhibits voltage-gated K^+ currents in pulmonary artery smooth muscle cells: role of 5-HT_{2A} receptors, caveolin-1, and $K_v1.5$ channel internalization. Circ Res 98:931–938
30. Wang J, Juhaszova M, Conte JV, Gaine SP, Rubin LJ, Yuan JX-J (1998) Action of fenfluramine on voltage-gated K^+ channels in human pulmonary artery smooth-muscle cells. Lancet 352:290
31. Hong Z, Smith AJ, Archer SL et al (2005) Pergolide is an inhibitor of voltage-gated potassium channels, including $K_v1.5$, and causes pulmonary vasoconstriction. Circulation 112:1494–1499
32. Michelakis ED, Thébaud B, Weir EK, Archer SL (2004) Hypoxic pulmonary vasoconstriction: redox regulation of O_2-sensitive K^+ channels by a mitochondrial O_2-sensor in resistance artery smooth muscle cells. J Mol Cell Cardiol 37:1119–1136
33. Cogolludo A, Frazziano G, Cobeño L et al (2006) Role of reactive oxygen species in K_v channel inhibition and vasoconstriction induced by TP receptor activation in rat pulmonary arteries. Ann N Y Acad Sci 1091:41–51
34. Moreno L, Frazziano G, Cogolludo A, Cobeño L, Tamargo J, Perez-Vizcaino F (2007) Role of protein kinase Czeta and its adaptor protein p62 in voltage-gated potassium channel modulation in pulmonary arteries. Mol Pharmacol 72:1301–1309
35. Talley EM, Lei Q, Sirois JE, Bayliss DA (2000) TASK-1, a two-pore domain K^+ channel, is modulated by multiple neurotransmitters in motoneurons. Neuron 25:399–410
36. Tamir H, Hsiung SC, Yu PY et al (1992) Serotonergic signalling between thyroid cells: protein kinase C and 5-HT2 receptors in the secretion and action of serotonin. Synapse 12:155–168
37. Moscat J, Diaz-Meco MT (2000) The atypical protein kinase Cs. Functional specificity mediated by specific protein adapters. EMBO Rep 1:399–403
38. Gong J, Xu J, Bezanilla M, van Huizen R, Derin R, Li M (1999) Differential stimulation of PKC phosphorylation of potassium channels by ZIP1 and ZIP2. Science 285:1565–1569
39. Kim Y, Uhm DY, Shin J, Chung S (2004) Modulation of delayed rectifier potassium channel by protein kinase C zeta-containing signaling complex in pheochromocytoma cells. Neuroscience 125:359–368
40. Shaul PW, Anderson RG (1998) Role of plasmalemmal caveolae in signal transduction. Am J Physiol 275:L843–L851

41. Martens JR, Sakamoto N, Sullivan SA, Grobaski TD, Tamkun MM (2001) Isoform-specific localization of voltage-gated K^+ channels to distinct lipid raft populations. Targeting of $K_v1.5$ to caveolae. J Biol Chem 276:8409–8414
42. McEwen DP, Li Q, Jackson S, Jenkins PM, Martens JR (2008) Caveolin regulates $K_v1.5$ trafficking to cholesterol-rich membrane microdomains. Mol Pharmacol 73:678–685
43. Martínez-Mármol R, Villalonga N, Solé L et al (2008) Multiple $K_v1.5$ targeting to membrane surface microdomains. Cell Physiol 217:667–673
44. Allen JA, Yadav PN, Roth BL (2008) Insights into the regulation of 5-HT_{2A} serotonin receptors by scaffolding proteins and kinases. Neuropharmacology 55:961–968
45. Zhao YY, Liu Y, Stan RV et al (2002) Defects in caveolin-1 cause dilated cardiomyopathy and pulmonary hypertension in knockout mice. Proc Natl Acad Sci U S A 99:11375–11380
46. Huang J, Kaminski PM, Edwards JG et al (2008) Pyrrolidine dithiocarbamate restores endothelial cell membrane integrity and attenuates monocrotaline-induced pulmonary artery hypertension. Am J Physiol Lung Cell Mol Physiol 294:L1250–L1259
47. Achcar RO, Demura Y, Rai PR et al (2006) Loss of caveolin and heme oxygenase expression in severe pulmonary hypertension. Chest 129:696–705
48. Patel HH, Zhang S, Murray F et al (2007) Increased smooth muscle cell expression of caveolin-1 and caveolae contribute to the pathophysiology of idiopathic pulmonary arterial hypertension. FASEB J 21:2970–2979
49. Ishiguro M, Morielli AD, Zvarova K, Tranmer BI, Penar PL, Wellman GC (2006) Oxyhemoglobin-induced suppression of voltage-dependent K^+ channels in cerebral arteries by enhanced tyrosine kinase activity. Circ Res 99:1252–1260
50. Bhattacharya S, Puri S, Miledi R, Panicker MM (2002) Internalization and recycling of 5-HT_{2A} receptors activated by serotonin and protein kinase C-mediated mechanisms. Proc Natl Acad Sci U S A 99:14470–14475

Part VI
Targeting Ion Channels and Membrane Receptors in Developing Novel Therapeutic Approaches for Pulmonary Vascular Disease

KCNQ Potassium Channels: New Targets for Pulmonary Vasodilator Drugs?

Alison M. Gurney, Shreena Joshi, and Boris Manoury

Abstract Smooth muscle cells regulate the diameter of pulmonary arteries and the resistance to blood flow in the pulmonary circulation. These cells are normally relaxed to maintain low intrinsic vessel tone, but are contracted in pulmonary arterial hypertension (PAH). Potassium channels in the smooth muscle cell help to maintain low tone by polarising the membrane and preventing Ca^{2+} influx through voltage-operated Ca^{2+} channels. There is a loss of K^+ channel activity in PAH, so drugs that open K^+ channels are predicted to have a beneficial effect, provided their action can be restricted to the pulmonary circulation. Here we review the myriad of K^+ channels that are expressed in pulmonary arteries and suggest the roles that each might play in regulating pulmonary artery tone. We conclude that members of the KCNQ family of K^+ channels, the most recent K^+ channels to be discovered in pulmonary artery, may be a useful therapeutic target for the treatment of PAH. KCNQ channels appear to be preferentially expressed in pulmonary arteries and drugs that modulate their activity have potent effects on pulmonary artery tone.

Keywords pulmonary artery • pulmonary arterial hypertension • membrane potential • KCNQ • Kv1.7 • K_{2P} • flupirtine • retigabine

1 Introduction

The contractile activity of the smooth muscle cells (SMCs) in the walls of small intrapulmonary arteries is a major determinant of the diameter of the vessels and the resistance to blood flow in the pulmonary circulation. Smooth muscle tone

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therefore plays an important role in the regulation of pulmonary arterial pressure and the distribution of blood around the lung. At any time, the contractile state of pulmonary artery smooth muscle depends on the interplay among neurotransmitters, circulating hormones and endothelium-derived agents, each of which exerts a constrictor or dilator effect. The SMC integrates these signals to provide the appropriate level of tone. In the absence of stimulation, the SMCs are relaxed, giving rise to low intrinsic tone in the pulmonary arteries, which helps to maintain the pressure in the pulmonary circulation at a low level. An important factor that helps to keep the cells relaxed is the steady efflux of K^+ ions from the cell through K^+ channels in the plasma membrane. By polarising the cell membrane, this keeps voltage-gated Ca^{2+} channels closed, thereby preventing Ca^{2+} in the extracellular space from entering the cell and causing contraction (Fig. 26.1).

Pulmonary arterial hypertension (PAH) is associated with a loss of K^+ channel expression and activity, both in animal models[1,2] and human patients.[3,4] Moreover, replacing the lost K^+ channel activity, by in vivo transfer of the $K_V1.5$ K^+ channel gene, was reported to have a beneficial effect in a rat model of hypoxia-induced PAH.[5] In the same way, drugs that open K^+ channels have the potential to act as

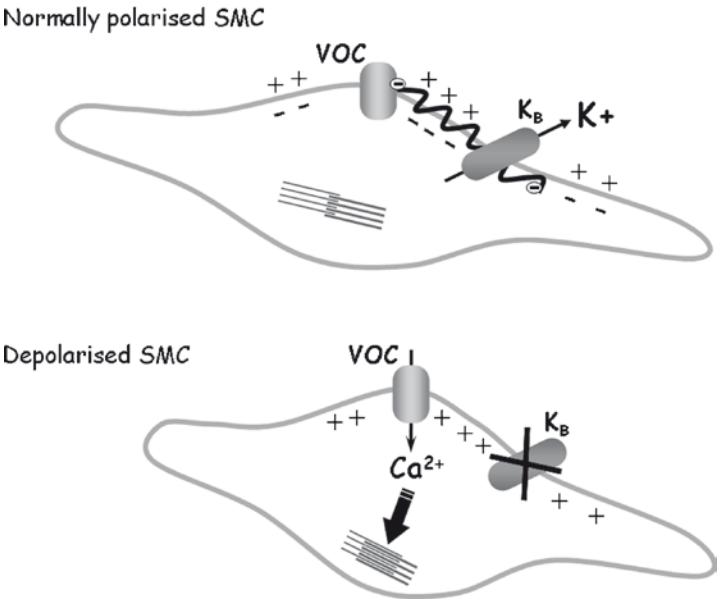

Fig. 26.1 In the normally polarised pulmonary artery smooth muscle cell (SMC), the resting efflux of K^+ through background K^+ channels (K_B) maintains a negative potential across the resting membrane (*upper panel*). This prevents the opening of voltage-operated Ca^{2+} channels (VOCs), thereby inhibiting Ca^{2+} influx, maintaining a low resting cytoplasmic Ca^{2+} concentration and preventing contraction. The inhibition or downregulation of K_B channels blocks K^+ efflux, allowing the membrane to depolarise (*lower panel*). This removes the inhibition of VOCs so that Ca^{2+} can permeate into the cell to raise the cytoplasmic Ca^{2+} concentration and promote contraction

pulmonary vasodilators and could prove useful in the treatment of PAH.[6] Unfortunately, the K^+ channel openers that have been investigated to date also cause systemic vasodilation because the K^+ channels they activate are also expressed and functionally active in the systemic circulation. Pulmonary artery smooth muscle does, however, express a large number of K^+ channel genes, several of which have been implicated in the regulation of pulmonary artery tone. This raises the possibility that particular subtypes of K^+ channel may be selectively or preferentially expressed in the pulmonary circulation, and these could provide molecular targets for drug discovery. Our understanding of pulmonary artery K^+ channels is, however, rudimentary; a better understanding is required not just of the K^+ channels expressed in pulmonary arteries but also the precise functions they serve. This review considers how the properties of pulmonary artery K^+ channels determine their functional roles. There is a particular focus on KCNQ (K_V7) potassium channels, the most recently described channels in the pulmonary circulation, which are accessible to pharmacological manipulation[7] and are promising molecular targets in the search for new drugs to treat PAH.[8]

2 Potassium Channels Expressed in Pulmonary Artery

K^+ channels are membrane-spanning proteins that gate the flow of K^+ ions across the plasma membrane. When open, they are selectively permeable to K^+, although the stimulus to open the channel differs between different types of K^+ channel. Each K^+ channel protein contains a pore-forming α-subunit, often associated with auxiliary subunits that influence its biophysical and pharmacological properties. The human genome contains over 70 different genes that encode K^+ channel α-subunits, and they are classified according to their structural and functional properties (Fig. 26.2). The three main families are distinguished by the number of domains (six, four or two) in the protein that span the plasma membrane.[9]

The six trans-membrane (TM) family includes subfamilies of the classical, voltage-gated (K_V), delayed rectifiers (K_V1–4), members of which are denoted, for example, as $K_V1.x$. Of the delayed rectifier subunits, $K_V1.5$ and $K_V2.1$ have been investigated most in the pulmonary artery.[3,10] There is, however, evidence for the expression of many more of these subunits, including $K_V1.1$–1.4, $K_V1.6$, $K_V3.1$, $K_V3.3$, $K_V3.4$ and $K_V4.1$–4.3.[11–18] The 6-TM family also includes the Ca^{2+}-activated channels ($K_{Ca}1$–5), of which the expression of $K_{Ca}1.1$ and $K_{Ca}2.2$–2.4 has been reported in human pulmonary artery.[17] Another class of 6-TM channels is formed by the ether à go-go (EAG) α-subunits, K_V10–12, named for a *Drosophila* mutant showing leg-shaking behaviour on exposure to ether. Pulmonary artery smooth muscle expresses two members of this family, $K_V10.1$ and $K_V11.1$,[17] but their functional roles have not been investigated. Most recently, rat pulmonary artery smooth muscle cells (PASMCs) were shown to express three members of the KCNQ (K_V7) subfamily of 6-TM channels: $K_V7.1$, -7.4 and -7.5.[19] Evidence is accumulating that these channels, especially $K_V7.4$, play a major role in regulating the tone of pulmonary arteries.[8,19,20]

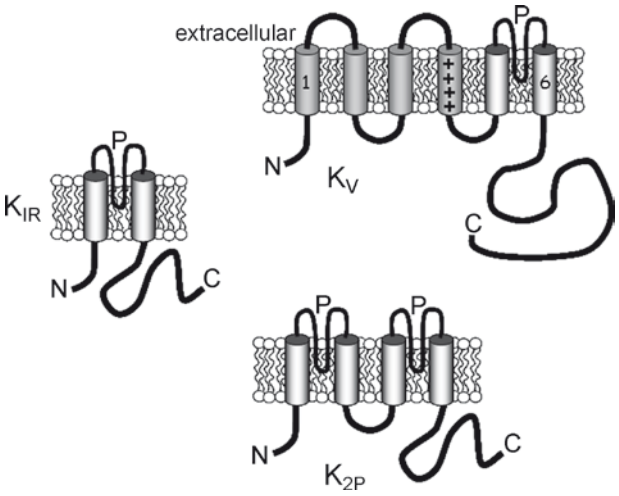

Fig. 26.2 Structural features of voltage-gated (K_V), inward rectifier (K_{IR}) and two-pore-domain (K_{2P}) channels showing the amino (N) and carboxy (C) termini, the pore-forming domains (P) and the highly charged voltage-sensing region in TM4. For orientation TM domains 1 and 6 are labelled in K_V. The two TM domains in K_{IR} are equivalent to TM5 and -6 in K_V, while K_{2P} resembles two K_{IR} channels in tandem

In the 6-TM family of α-subunits, the K^+ channel pore is formed by a "P" loop, located between TM5 and TM6 (Fig. 26.2). Four α-subunits come together as a tetramer to form a functional channel, each subunit contributing to the pore. The functional channel may be homomeric, containing only one type of α-subunit, but heteromeric channels may also be formed from subunits within a subfamily. Voltage sensing by 6-TM channels is conferred by the TM4 region, which is rich in positively charged basic residues. In contrast, the 4-TM family of α-subunits lacks this charged domain, so they are essentially voltage independent. Another feature of 4-TM channels is that each subunit contains two pore domains in tandem (Fig. 26.2), so that only two α-subunits are required to form a functional channel. They are consequently known as two (or tandem) pore domain (K_{2P}) channels. Subfamilies of K_{2P} channels include TWIK (two-pore-domain weakly inward rectifying K^+); TREK (TWIK-related K^+); TASK (TWIK-related, acid-sensitive K^+); TALK (TWIK-related alkaline activated K^+); THIK (tandem pore domain halothane-inhibited K^+); TRAAK (TWIK-related arachidonic acid activated K^+) and TRESK (TWIK-related spinal cord K^+). TWIK-2, TREK-1, TREK-2, TASK-1, TASK-2 and THIK-1 have all been reported to be expressed in pulmonary arteries,[21–23] although to date functional roles in pulmonary artery have only been reported for TASK channels. Functional K_{2P} channels may be formed by homomeric or heteromeric dimers of α-subunits from the same subfamily. Since these channels can be activated at all membrane potentials, they are often referred to as "background" or "leak" channels and are expected to contribute to the resting membrane potential in many cell types.

The 2-TM family of K^+ channels is known as the inward rectifiers because, unlike other K^+ channels, they preferentially support the influx of K^+ at negative membrane potentials. The main subfamilies are denoted $K_{IR}1$–6. The $K_{IR}3$ subfamily gives rise to G protein-activated inward rectifier channels, while the $K_{IR}6$ subfamily forms adenosine triphosphate (ATP)-sensitive K^+ (K_{ATP}) channels when combined with sulphonylurea receptors (SURs). The presence of K_{ATP} channels in pulmonary artery is well established,[24] and they appear to be formed by $K_{IR}6.1$ subunits in a complex with SUR2B.[25] Cultured human PASMCs were also found to express messenger RNA (mRNA) for $K_{IR}2.1$, -2.2 and -2.4, with $K_{IR}2.1$ and -2.4 thought to be functionally active.[26] As this is the only report of inward rectifiers in pulmonary artery, it remains to be seen if it is a consequence of cell culture or a difference between human and other species.

3 Potassium Channels, Membrane Potential, and Excitability

Why so many K^+ channels are expressed in pulmonary arteries is not clear, especially as many of the voltage-gated channels show substantial activation only when the membrane is depolarised to levels that are not reached in physiological conditions. Such wide-ranging expression does, however, suggest an important role in the pulmonary circulation.

Potassium channels serve two major functions in smooth muscle: They help to set the resting membrane potential, and they determine membrane excitability. Each function is likely to be mediated by different K^+ channels, with properties honed to equip them for their specific role. As the resting membrane potential depends on a steady leak of K^+ out of the cell, the K^+ channels that serve this role must be continuously open at voltages around the resting level, between -60 and -50 mV.[27,28] Blockers of these channels would inhibit the resting K^+ conductance and cause membrane depolarisation. Voltage-dependent K^+ channels control membrane excitability. PASMCs are electrically silent, with depolarising stimuli able to evoke step changes in membrane potential but not action potentials.[27–29] Depolarisation to above -35 mV activates both Ca^{2+} and K^+ channels, the former promoting further depolarisation, Ca^{2+} entry and contraction, while the latter act to repolarise the membrane and limit excitability. The K^+ channel currents recorded from PASMCs are large compared with Ca^{2+} channel currents: In rabbit cells, voltage steps to 0 mV activated 400-pA K^+ currents[30] compared with 43-pA Ca^{2+} currents[31] under the same conditions. The large K^+ current would provide a strong repolarising influence, preventing the smaller Ca^{2+} current from initiating an action potential. Voltage-activated K^+ channels therefore limit excitability and maintain low intrinsic tone. As low tone is an essential feature of pulmonary arteries, perhaps the large number of K^+ channel genes expressed in pulmonary arteries reflects a high degree of safety in the system to protect the vessels from depolarising inputs that might otherwise evoke vasospasm.

3.1 The Classical Delayed Rectifier K^+ Channels in Pulmonary Artery

The delayed rectifiers open within milliseconds of the membrane depolarising so are well suited to a role in responding to and preventing excitation. $K_V1.5$ and $K_V2.1$ are the only delayed rectifier channels to have received serious attention in relation to PAH and the regulation of pulmonary artery tone, and their proposed roles have been reviewed many times.[4,10,32] There is considerable evidence that the expression and activity of both these channels is reduced in hypoxic rats with PAH, and $K_V1.5$ expression is reduced in patients with idiopathic PAH.[33] Single-nucleotide polymorphisms in the $K_V1.5$ gene have also been identified in these patients.[3] $K_V1.5$ and $K_V2.1$ are frequently ascribed roles in setting the resting potential, such that loss of activity would depolarise the smooth muscle, promoting pulmonary vasoconstriction and hypertension.[3,10–12,34] This does not fit easily, however, with their known biophysical properties. They are more likely to act as detectors of depolarisation and limit excitation, so that loss of expression leads to enhanced excitability or reactivity.

The relatively depolarised voltage thresholds for activation of $K_V1.5$ and $K_V2.1$ and their high sensitivity to block by 4-aminopyridine[35] argue strongly against a major role for these channels in setting the resting potential. $K_V1.5$ opens positive to –50 mV and is blocked by sub-millimolar concentrations of 4-aminopyridine, whereas high millimolar concentrations of the drug are required to depolarise PASMCs.[36] The similar membrane potentials recorded from wild-type mice (–44 mV) and mice lacking the $K_V1.5$ subunit (–40 mV), despite a 30% reduction in delayed rectifier current,[37] are further evidence against such a role. Moreover, $K_V1.5$ knockout mice showed no sign of PAH,[38] implying that loss of $K_V1.5$ per se does not underlie disease.

The voltage threshold for opening $K_V2.1$ is even more positive.[35] It is 50% blocked by 1 m*M* 4-aminopyidine, which has little effect on membrane potential or artery tone.[36] On the other hand, $K_V2.1$ subunits in pulmonary artery are likely to be present in a complex with electrically silent $K_V9.3$ subunits, which shift the threshold for activation closer to –50 mV and reduce 4-aminopyridine sensitivity.[13] All the same, like $K_V1.5$, the $K_V2.1/K_V9.3$ complex would be closed at membrane potentials around –50 mV or below. So, rather than mediating depolarisation, the loss of $K_V2.1$ expression is likely to render the SMCs more excitable and able to sustain depolarisation and voltage-gated Ca^{2+} entry. Unfortunately, $K_V1.5$ and $K_V2.1$ channels are widely expressed in the cardiovascular and nervous systems, so they are poor candidates for drug discovery aimed at therapies for PAH.

3.2 Two-Pore-Domain K^+ Channels in Pulmonary Artery

The K_{2P} channels were discovered about a decade ago and are a growing area of interest because their biophysical properties suit them well to a role in setting resting potential. Evidence is growing that TASK channels are regulators of resting

potential in pulmonary artery smooth muscle, and they are candidates for a role in oxygen sensing.[31,38] TASK-1 channels are expressed in rabbit, rat, mouse and human PASMCs.[22,23,39,40] Moreover, small-interfering RNA (siRNA) knockdown of TASK-1 in human PASMCs, by RNA interference, caused substantial depolarisation (from –40 to –25 mV) and loss of the acute depolarising response to hypoxia.[40] Although TASK-2 is also expressed in rat pulmonary artery, siRNA targeted against TASK-2 caused only a small depolarisation.[41] Together, TASK-1 and -2 could, however, make a substantial contribution to the resting potential.

TASK channels are of particular interest because of their pH sensitivity, which could lead pulmonary arteries to constrict in response to acidosis and dilate to alkalosis. Such conditions can occur in physiological or patho-physiological situations, which may be caused by hypo- or hyper-ventilation or local ischemia due, for example, to pulmonary embolism. TASK-1 channels are also of interest because they appear to be targets for a number of endogenous agents that regulate pulmonary artery tone. The prostacyclin analogue treprostinil was found to enhance the TASK-1-dependent background K^+ current in human PASMCs at clinically relevant concentrations via a mechanism involving cyclic adenosine monophosphate (cAMP)-dependent phosphorylation.[40] The beneficial effects of prostanoids in the therapy of PAH may therefore be explained, at least in part, by an action on TASK-1 channels. Likewise, TASK-1 channels have been proposed to underlie the cAMP-dependent, β-adrenoceptor-mediated hyperpolarisation and relaxation of pulmonary arteries.[42] In contrast, endothelin, at clinically relevant concentrations, was reported to inhibit TASK-1 channels in primary cultured human PASMCs, through an action dependent on endothelin A (ET_A) receptors, phospholipase C and protein kinase C activity.[43] This could represent a mechanism by which ET activates depolarisation and promotes PAH.

RNA interference techniques have been instrumental in elucidating the role of TASK channels in PASMCs. To fully understand their contribution to artery function in vivo requires inhibition of TASK channel activity in more complex, intact preparations. The lack of selective pharmacological tools for TASK channels has made their roles difficult to dissect. Drugs that modulate TASK channel activity have little effect on pulmonary artery tone,[23] but the only drugs available to study TASK channels are highly non-selective and can affect pathways regulating smooth muscle tone that might counteract depolarisation. Thus, the results of pharmacological studies have shed little light on TASK channel function. Although RNA interference was used successfully in intact pulmonary arteries to investigate TASK-2 function, in our hands this technique has proved to be fraught with difficulty. Mice lacking the TASK-1 gene appear to have unaltered pulmonary artery function (data unpublished), but it is not known if this represents a species difference.

A functional role has been proposed for TREK-1 in the systemic circulation, where it may have a critical role in mediating the vasodilator response of resistance arteries to polyunsaturated fatty acids.[38,44] Although TREK channels are expressed in pulmonary artery, no one has investigated their function there yet. The finding that TREK-2 is expressed in pulmonary but not mesenteric artery, whereas TRAAK and

TREK-1 are present in mesenteric but not pulmonary artery,[23] suggests different roles for these channels in vessel function, which may be relevant to the differential effect of hypoxia on pulmonary and systemic vessels.

3.3 The KCNQ (K_V7) Voltage-Gated K^+ Channels in Pulmonary Artery

KCNQ genes express K^+ channels with properties appropriate for a role in regulating resting membrane potentials and members of the KCNQ family are known to play such a role in neurones. They are voltage-gated channels, but the threshold for activation is substantially more negative than other types of K_V channel (between –60 and –80 mV). They also activate more slowly than other K_V channels, taking hundreds of milliseconds or even seconds to fully activate, and do not inactivate during sustained depolarisation. These properties are remarkably similar to the background K^+ conductance recorded from PASMCs.[45] The first indication that KCNQ channels might be important in pulmonary artery was the finding that two selective blockers (XE991 and linopirdine) are potent pulmonary vasoconstrictors, with EC_{50} values of 0.4 and 1 μM.[20] Importantly, they constrict pulmonary arteries at concentrations having little or no effect on a wide range of other blood vessels (Fig. 26.3a). The vasoconstriction was abolished by the Ca^{2+} antagonist nifedipine and by hyperpolarising the cell with the K_{ATP} channel opener levcromakalim, indicating the involvement of membrane depolarisation and voltage-gated Ca^{2+} influx. We found that two selective activators of KCNQ channels (retigabine and flupirtine) are potent pulmonary vasodilators,[19] and we have since identified further activators with the same effect (data unpublished). Suppression of the dilation by raised extracellular K^+ concentrations suggested the involvement of K^+ channels. In accord with their effects on vessel tone, the KCNQ channel blockers inhibited a background K^+ conductance in PASMCs and caused membrane depolarisation, while the activators produced the opposite effects.[19]

Of the five KCNQ genes, PASMCs were found to express three (KCNQ1, -4 and -5), although the most highly expressed is KCNQ4.[19] Although arteries from other vascular beds expressed the same transcripts, they were present at much lower levels than in pulmonary artery, further implying a pulmonary-specific role. The effects of KCNQ blockers on more intact preparations are consistent with this. Linopirdine raised pulmonary arterial pressure in the isolated, saline-perfused rat lung, and when administered in vivo it raised pulmonary arterial pressure without affecting systemic blood pressure.[19] We have still to identify the precise molecular makeup of the KCNQ channels mediating the effects of KCNQ modulators on pulmonary artery. Nevertheless, the drugs are sufficiently selective for KCNQ channels, and sufficiently potent on pulmonary arteries, to suggest that KCNQ channels play a major and specific role in pulmonary arteries.

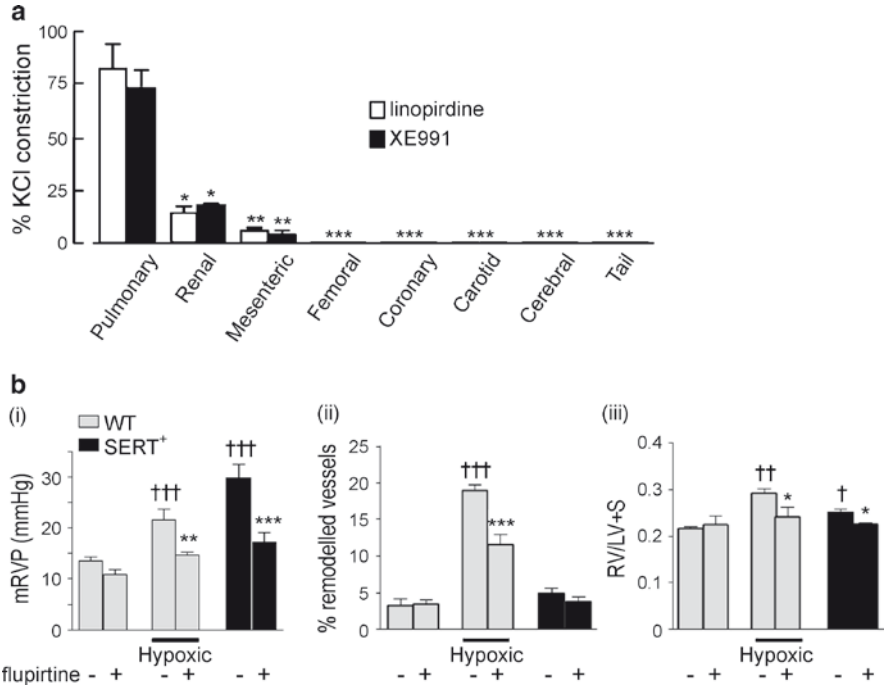

Fig. 26.3 Evidence for an important functional role for KCNQ channels. (**a**) Constrictor responses evoked by 10 μ*M* linopirdine and 1 μ*M* XE991 in a range of blood vessels, amplitudes plotted as a percentage of the contraction evoked by 50 m*M* KCl in the same tissues. *Value significantly less than pulmonary artery (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). Reproduced with permission.[19] (**b**) (i) Mean RVP (mRVP), (ii) pulmonary vascular remodelling and (iii) ratio of right ventricle (RV) weight to the combined weight of left ventricle (LV) and septum (S) in wild-type (WT) and SERT+ mice. Half of the WT mice were maintained for 2 weeks in a hypobaric/hypoxic environment (equivalent to 10% O_2). Half of the animals in each group received 30 mg kg^{-1} day^{-1} oral flupirtine (+), and the other half received the equivalent volume of vehicle (–). Data represent the mean ± SE mean of six to eight experiments. *Value significantly less than corresponding value in vehicle-treated mice (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). †Value significantly greater than corresponding value in normoxic WT mice (†$P < 0.05$, ††$P < 0.01$, †††$P < 0.001$). Adapted with permission[8]

3.4 Other K+ Channels in Pulmonary Artery

In addition to the K^+ channels described, pulmonary artery myocytes express K_{Ca} and K_{ATP} channels,[17,25] but neither of these channel types appears to be open at the resting potential under physiological conditions. The main activator of K_{Ca} channels is elevations in the cytoplasm Ca^{2+} concentration, and this provides negative-feedback regulation of Ca^{2+} influx by hyperpolarising the membrane during active contraction. This limits the vasoconstriction. The roles of K_{Ca} channels in PAH have

not been widely studied. Although a splice variant of the $K_{Ca}1.1$ channel is inhibited by acute hypoxia,[46] pulmonary hypertensive rats exposed to chronic hypoxia showed increased $K_{Ca}1$ expression.[47] This is likely to be a protective mechanism that counteracts the vasoconstriction seen in PAH. K_{ATP} channels are well placed to mediate hyperpolarisation or repolarisation, with consequent dilation of pulmonary arteries, in response to changes in the metabolic state of the cell or endogenous or exogenous agents that act as K^+ channel openers.[24] Possible alterations in K_{ATP} channel activity in PAH have received little attention, but the channels have been considered as possible drug targets for treating the disease.[6] Unfortunately, the lack of specificity for pulmonary artery K_{ATP} channels means that openers of these channels lack selectivity for the pulmonary circulation.

4 KCNQ Channels as a Molecular Target for Pulmonary Vasodilators

KCNQ channels provide an opportunity for therapeutic intervention. Unlike most K^+ channels, KCNQ channels have proved to be druggable, in that we know they can be modulated by a number of small-molecule drugs.[7] There is extensive evidence that at least two KCNQ activators have only minor side effects and are safe when administered to humans.[48,49] Indeed, since the early 1980s oral flupirtine has been approved as a treatment for pain in Europe, and in the United States it is currently in phase II trials for the treatment of fibromyalgia and ocular complications of diabetes. The more potent analogue retigabine successfully completed phase II trials for partial onset seizures,[48] and the results of phase III trials are expected soon. Pulmonary arterial pressure was not monitored in these trials, but none reported cardiovascular side effects, including changes in systemic blood pressure.

In an attempt to elucidate the potential of KCNQ activators in the treatment of PAH, we collaborated with Professor Mandy MacLean to investigate the effects of oral flupirtine in two independent mouse models.[8] In one model, PAH developed secondary to chronic hypoxia. In the other, PAH developed spontaneously due to over-expression of the serotonin transporter ($SERT^+$ mice). Animals were chronically dosed with 30 mg kg^{-1} flupirtine daily and indices of PAH measured at the end of the experimental period. As illustrated in Fig. 26.3b, flupirtine had no effect on wild-type mice exposed to a normoxic environment, but it prevented the indices of PAH caused by chronic hypoxia. Moreover, it reversed the pre-existing PAH in $SERT^+$ mice. The data provide proof of principle that KCNQ channel activators could provide an effective vasodilator for the treatment of PAH. These drugs clearly have neuronal effects, but improved understanding of the molecular makeup of the particular KCNQ channels in PASMCs will aid in the development of KCNQ activators with even greater selectively for the pulmonary circulation.

5 Summary

The resting potential of PASMCs is an important regulator of the PASMC cytoplasmic Ca^{2+} concentration and pulmonary vascular tone. The membrane potential is depolarised in PAH due to loss of K channel expression. The molecular correlates of the main K^+ channel involved in setting the resting potential is not yet certain, but several are likely to contribute. KCNQ channels are the most recent to receive attention regarding this role. Since KCNQ channels appear to be preferentially expressed in pulmonary arteries and are amenable to pharmacological manipulation, they represent a potentially new molecular target for the development of pulmonary-selective vasodilators.

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References

1. Wang J, Juhaszova M, Rubin LJ, Yuan XJ (1997) Hypoxia inhibits gene expression of voltage-gated K^+ channel α-subunits in pulmonary artery smooth muscle cells. J Clin Invest 100:2347–2353
2. Osipenko ON, Alexander D, MacLean MR, Gurney AM (1998) Influence of chronic hypoxia on the contributions of non-inactivating and delayed rectifier K currents to the resting potential and tone of rat pulmonary artery smooth muscle. Br J Pharmacol 124:1335–1337
3. Remillard CV, Tigno DD, Platoshyn O et al (2007) Function of $K_V1.5$ channels and genetic variations of KCNA5 in patients with idiopathic pulmonary arterial hypertension. Am J Physiol Cell Physiol 292:C1837–C1853
4. Burg ED, Remillard CV, Yuan JX-J (2008) Potassium channels in the regulation of pulmonary artery smooth muscle cell proliferation and apoptosis: pharmacotherapeutic implications. Br J Pharmacol 153:S99–S111
5. Pozeg ZI, Michelakis ED McMurtry MS et al (2003) In vivo gene transfer of the O_2-sensitive potassium channel $K_V1.5$ reduces pulmonary hypertension and restores hypoxic pulmonary vasoconstriction in chronically hypoxic rats. Circulation 107:2037–2044
6. Wanstall JC (1996) The pulmonary vasodilator properties of potassium channel opening drugs. Gen Pharmacol 27:599–605
7. Dalby-Brown W, Hansen HH, Korsgaard MP, Mirza N, Olesen SP (2006) K_V7 channels: function, pharmacology and channel modulators. Curr Top Med Chem 6:999–1023
8. Morecroft I, Murray A, Nilsen M, Gurney AM, MacLean MR (2009) The K_V7 potassium channel activator flupirtine prevents pulmonary hypertension in two independent mouse models. Br J Pharmacol 157:1241–1249
9. Alexander SPH, Mathie A, Peters JA (2008) Guide to receptors and channels (GRAC). Br J Pharmacol 153:S130–S132
10. Moudgil R, Michelakis ED, Archer SL (2006) The role of K^+ channels in determining pulmonary vascular tone, oxygen sensing, cell proliferation, and apoptosis: implications in hypoxic pulmonary vasoconstriction and pulmonary arterial hypertension. Microcirculation 13:615–632
11. Archer SL, Souil E, Dinh-Xuan AT et al (1998) Molecular identification of the role of voltage-gated K^+ channels, $K_V1.5$ and $K_V2.1$, in hypoxic pulmonary vasoconstriction and control of resting membrane potential in rat pulmonary artery myocytes. J Clin Invest 101:2319–2330

12. Yuan XJ, Wang J, Juhaszova M, Golovina VA, Rubin LJ (1998) Molecular basis and function of voltage-gated K^+ channels in pulmonary arterial smooth muscle cells. Am J Physiol 274:L621–L635
13. Patel AJ, Lazdunski M, Honoré E (1997) $K_V2.1/K_V9.3$, a novel ATP-dependent delayed-rectifier K^+ channel in oxygen-sensitive pulmonary artery myocytes. EMBO J 16:6615–6625
14. Osipenko ON, Tate RJ, Gurney AM (2000) Potential role for $K_V3.1b$ channels as oxygen sensors. Circ Res 86:534–540
15. Davies AR, Kozlowski RZ (2001) K_V channel subunit expression in rat pulmonary arteries. Lung 179:147–161
16. Coppock EA, Tamkun MM (2001) Differential expression of K_V channel α- and β-subunits in the bovine pulmonary arterial circulation. Am J Physiol Lung Cell Mol Physiol 281:L1350–L1360
17. Platoshyn O, Remillard CV, Fantozzi I et al (2004) Diversity of voltage-dependent K^+ channels in human pulmonary artery smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 287:L226–L238
18. Iida H, Jo T, Iwasawa K et al (2005) Molecular and pharmacological characteristics of transient voltage-dependent K^+ currents in cultured human pulmonary arterial smooth muscle cells. Br J Pharmacol 146:49–59
19. Joshi S, Sedivy V, Hodyc D, Herget J, Gurney AM (2009) KCNQ modulators reveal a key role for KCNQ potassium channels in regulating the tone of rat pulmonary artery smooth muscle. J Pharmacol Exp Ther 329:368–376
20. Joshi S, Balan P, Gurney AM (2006) Pulmonary vasoconstrictor action of KCNQ potassium channel blockers. Respir Res 7:31–41
21. Koh SD, Monaghan K, Sergeant GP et al (2001) TREK-1 regulation by nitric oxide and cGMP-dependent protein kinase. An essential role in smooth muscle inhibitory neurotransmission. J Biol Chem 276:44338–44346
22. Gurney AM, Osipenko ON, MacMillan D, McFarlane KM, Tate RJ, Kempsill FE (2003) Two-pore domain K channel, TASK-1, in pulmonary artery smooth muscle cells. Circ Res 93:957–964
23. Gardener MJ, Johnson IT, Burnham MP, Edwards G, Heagerty AM, Weston AH (2004) Functional evidence of a role for two-pore domain potassium channels in rat mesenteric and pulmonary arteries. Br J Pharmacol 142:192–202
24. Clapp LH, Davey R, Gurney AM (1993) ATP-sensitive K^+ channels mediate vasodilation produced by lemakalim in rabbit pulmonary artery. Am J Physiol 264:H1907–H1915
25. Cui Y, Tran S, Tinker A, Clapp LH (2002) The molecular composition of K_{ATP} channels in human pulmonary artery smooth muscle cells and their modulation by growth. Am J Respir Cell Mol Biol 26:135–143
26. Tennant BP, Cui Y, Tinker A, Clapp LH (2006) Functional expression of inward rectifier potassium channels in cultured human pulmonary smooth muscle cells: evidence for a major role of Kir2.4 subunits. J Membr Biol 213:19–29
27. Casteels R, Kitamura K, Kuriyama H, Suzuki H (1977) Excitation–contraction coupling in the smooth muscle cells of the rabbit main pulmonary artery. J Physiol 271:63–79
28. Suzuki H, Twarog BM (1982) Membrane properties of smooth muscle cells in pulmonary arteries of the rat. Am J Physiol 242:H900–H906
29. Gurney AM (2004) Functional roles of ion channels in the regulation of membrane potential and pulmonary vascular tone. In: Yuan JX-J (ed) Ion channels in the pulmonary vasculature (lung biology in health and disease). Dekker, New York, pp 447–461
30. Clapp LH, Gurney AM (1991) Outward currents in rabbit pulmonary artery cells dissociated with a new technique. Exp Physiol 76:677–693
31. Gurney AM, Joshi S (2006) The role of twin pore domain and other K^+ channels in hypoxic pulmonary vasoconstriction. Novartis Found Symp 272:218–228
32. Clapp LH, Gurney AM (1991) Modulation of calcium movements by nitroprusside in isolated vascular smooth muscle cells. Pflügers Arch 418:462–470
33. Yuan XJ, Wang J, Juhaszova M, Gaine SP, Rubin LJ (1998) Attenuated K^+ channel gene transcription in primary pulmonary hypertension. Lancet 351:726–727

34. Archer SL, Wu XC, Thébaud B et al (2004) Preferential expression and function of voltage-gated, O_2-sensitive K^+ channels in resistance pulmonary arteries explains regional heterogeneity in hypoxic pulmonary vasoconstriction: ionic diversity in smooth muscle cells. Circ Res 95:308–318
35. Coetzee WA, Amarillo Y, Chiu J et al (1999) Molecular diversity of K^+ channels. Ann N Y Acad Sci 868:233–285
36. Osipenko ON, Alexander D, MacLean MR, Gurney AM (1998) Influence of chronic hypoxia on the contributions of non-inactivating and delayed rectifier K currents to the resting potential and tone of rat pulmonary artery smooth muscle. Br J Pharmacol 124:1335–1337
37. Archer SL, London B, Hampl V et al (2001) Impairment of hypoxic pulmonary vasoconstriction in mice lacking the voltage-gated potassium channel $K_V1.5$. FASEB J 15:1801–1803
38. Gurney A, Manoury B (2009) Two-pore potassium channels in the cardiovascular system. Eur Biophys J 38:305–318
39. Gurney AM, Hunter E (2005) The use of small interfering RNA to elucidate the activity and function of ion channel genes in an intact tissue. J Pharmacol Toxicol Methods 51:253–262
40. Olschewski A, Li Y, Tang B et al (2006) Impact of TASK-1 in human pulmonary artery smooth muscle cells. Circ Res 98:1072–1080
41. Gönczi M, Szentandrássy N, Johnson IT, Heagerty AM, Weston AH (2006) Investigation of the role of TASK-2 channels in rat pulmonary arteries; pharmacological and functional studies following RNA interference procedures. Br J Pharmacol 147:496–505
42. Bieger D, Parai K, Ford CA, Tabrizchi R (2006) β-Adrenoceptor mediated responses in rat pulmonary artery: putative role of TASK-1 related K channels. Naunyn Schmiedebergs Arch Pharmacol 373:186–196
43. Tang B, Li Y, Nagaraj C et al (2009) Endothelin-1 inhibits background two-pore domain channel TASK-1 in primary human pulmonary artery smooth muscle cells. Am J Respir Cell Mol Biol 41:467–483
44. Bryan RM Jr, Joseph BK, Lloyd E, Rusch NJ (2007) Starring TREK-1: the next generation of vascular K^+ channels. Circ Res 101:119–121
45. Evans AM, Osipenko ON, Gurney AM (1996) Properties of a novel K^+ current that is active at resting potential in rabbit pulmonary artery smooth muscle cells. J Physiol 496:407–420
46. McCartney CE, McClafferty H, Huibant JM, Rowan EG, Shipston MJ, Rowe IC (2005) A cysteine-rich motif confers hypoxia sensitivity to mammalian large conductance voltage- and Ca-activated K (BK) channel α-subunits. Proc Natl Acad Sci U S A 102:17870–17876
47. Resnik E, Herron J, Fu R, Ivy DD, Cornfield DN (2006) Oxygen tension modulates the expression of pulmonary vascular BKCa channel α- and β-subunits. Am J Physiol Lung Cell Mol Physiol 290:L761–L768
48. Porter RJ, Partiot A, Sachdeo R, Nohria V, Alves WM (2007) Randomized, multicenter, dose-ranging trial of retigabine for partial-onset seizures. Neurology 68:1197–1204
49. Li C, Ni J, Wang Z et al (2008) Analgesic efficacy and tolerability of flupirtine vs. tramadol in patients with subacute low back pain: a double-blind multicentre trial. Curr Med Res Opin 24:3523–3530

Receptor Tyrosine Kinase Inhibitors in Rodent Pulmonary Hypertension

Liliana Moreno-Vinasco and Joe G. N. Garcia

Abstract Pulmonary hypertension (PH) is a disorder characterized by vascular remodeling and proliferation, a phenotype dependent upon unimpeded growth factor and kinase pathway activation with strong similarities to malignant tumors. This chapter details our novel application of the multikinase inhibitor, sorafenib, in rodent models of PH to improved hemodynamic parameters and attenuates PH structural changes[1]. Sorafenib is a Raf kinase inhibitor and our biochemical and genomic evidence supported the potential involvement of the MAPK cascade system and TGFB3 in PH development and the response to therapy. Integration of expression genomic analyses coupled with intense bioinformatics identified gene expression and ontology signatures in the development of PH and implicated the role of cytoskeletal protein such as caldesmon or nmMLCK as potentially key participants in PH-induced vascular remodeling and proliferation. Our studies suggest the PKI sorafenib as a potentially novel treatment for severe PH with the MAPK cascade a potential canonical target profoundly effecting vascular cytoskeletal rearrangements and remodeling[1].

Keywords Vascular remodeling • sorafenib • endothelium • cytoskeleton • caldesmon.

1 Introduction

Pulmonary hypertension (PH) and cancer pathology share growth factor- and mitogen-activated protein kinase (MAPK) stress-mediated signaling pathways that result in endothelial and smooth muscle cell (SMC) dysfunction and angioproliferative vasculopathy. Protein kinase inhibitors (PKIs) potentially target angiogenic growth factors such as vascular endothelial growth factor receptors (VEGFR-1–3), platelet-derived growth factor receptors (PDGFR-α and -β) as well as key signaling

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intermediates involved in mediating MAP (mitogen-activated protein) kinase pathway activation such as Raf-1-kinase. We conducted studies with the protein kinase inhibitor (PKI) sorafenib as a potentially novel treatment for severe PH with the MAPK cascade a potential canonical target profoundly effecting vascular cytoskeletal rearrangements and remodeling.[1]

2 Overview of Pulmonary Arterial Hypertension

Pulmonary arterial hypertension (PH) is a lethal syndrome characterized by obstruction of the pulmonary vasculature due to excessive cell proliferation, impaired apoptosis, and vasoconstriction. PH is a disease of small pulmonary arteries (PAs) characterized by intimal hyperplasia, medial hypertrophy, a thickened adventitia, and endothelial proliferative plexiform lesions.[2–4] PH occurs in idiopathic and familial forms and is most commonly associated with connective tissue diseases, anorexigen use, HIV, or congenital heart disease. PH typically appears in the third to fifth decade, with increased mortality rates (~50% at 5 years) due to right heart failure as a consequence of increased pulmonary vascular resistance (PVR).[2] Elevated PVR reflects reduced cross-sectional area of the vascular bed caused by obstructive vascular remodeling and vasoconstriction. PH is clinically characterized by a progressive increase in pulmonary arterial pressure (PAP) with a mean pressure of greater than 25 mmHg at rest or 30 mmHg during exercise.[5] PH can be categorized by the spectrum of its clinical presentation: mild-to-moderate PH, for which PAP is greater than 25 mmHg, or severe PH, for which PAP is greater than 50 mmHg. Severe PH manifests as both an acute and chronic presentation, with acute PH inducing a sudden increase in right ventricle (RV) afterload, with increased end-diastolic volume, and reduced RV ejection fraction.[6] Chronic PH leads to progressive RV systolic pressure overload, which leads to dilated RV dysfunction and failure and ultimately death. In 1998, the World Health Organization devised a more clinically useful classification that divides PH into distinct categories, including PH-associated primary pulmonary hypertension (PPH) (familial and sporadic) and PH linked with respiratory system disorders and hypoxemia, among others.[5]

3 Genetic Studies in Pulmonary Hypertension

The frequency of chronic PH and secondary PH are not known but has been estimated at an annual incidence of 1–2 per million.[7] Mutations of the bone morphogenetic protein receptor type 2 gene (*BMPR2*) have been identified in about 50% of cases of familial PH, with only 20% of individuals with a *BMPR2* mutation developing PH. Clearly, other genetic polymorphisms and environmental factors are necessary to initiate the pathological sequence that leads to disease. As external stimuli coupled with undefined genetic susceptibility are likely responsible for the majority of PH cases,[5,8–11] this complexity lends itself to the use of high-throughput technologies such as gene microarrays, allowing efficient and accu-

rate simultaneous assessment of the expression of thousands of genes. This technology has been most successfully employed in the investigation of cancer, including the classification of histologically indistinct tumor types with different natural histories.[12] Gene microarray strategies permit analysis of the expression profile of lung tissue obtained from patients with PH and the comparison of the gene profile in diseased lungs with that found in the normal lung.[9]

4 New Concepts in PH Pathophysiology – Neoplastic Vasculopathy

The endothelium is dysfunctional in PH and represents one of the key cell types to be studied. An early proapoptotic endothelial insult may promote PH by damaging normal endothelium, thereby selecting apoptosis-resistant clones that ultimately form characteristic plexiform lesions.[4] Drawn from drug discovery studies is the observation that severe PH and cancer pathophysiology share common signal transduction pathways leading to abnormal endothelial cell (EC) and SMC interactions and angioproliferative vasculopathy.[4] In primary PH, the lung ECs expand in a monoclonal pattern and contain an inactivating mutation of the transforming growth factor receptor II.[13] Severe PH can also present with unique tumorlets of ECs that obliterate medium-sized precapillary arteries. The hyperproliferating ECs often form structures known as plexiform lesions and express angiogenic factors, including vascular endothelial growth factor (VEGF) and its receptor, VEGF receptor 2 (VEGFR-2, KDR).[4] Interestingly, the VEGFR-2 inhibitor known as sugen or SU5416 (SU) has been described to augment PH in combination with chronic hypoxia in the rat model and mimic the precapillary arterial EC proliferation, plexiform lesions, and vascular remodeling and hemodynamic effects of severe PH in humans.[4] The vascular changes are not reversible on reoxygenation and ultimately evolve into right heart failure and death.[4]

5 Receptor Tyrosine Kinase Inhibitors as Novel Therapies for Pulmonary Hypertension

Therapeutic options targeted to specific molecular PH mechanisms are sparse but include epoprostenol (Flolan) and iloprost, both prostocyclin (PGI_2) analogues, whereas the mainstay of current therapy consists of the use of a combination of agents, including supplemental oxygen, diuretics, anticoagulants, calcium channel blockers, prostanoids, statins, endothelin receptor antagonists, phosphodiesterase 5 inhibitors, or surgical procedures.[6,14,15] Despite these advances, PH remains a devastating disease as most approved therapies are expensive, do not reverse the disease remodeling, and consequently offer only limited benefit to exercise capacity. Thus, there is a strong rationale to consider novel therapies related to pathogenic mechanisms such as tyrosine kinase inhibitors.[16]

Protein phosphorylation is a major posttranslational modification and regulatory (activation, inhibition) mechanism that controls multiple cell functions (transcription,

cell growth, proliferation, differentiation, apoptosis, cell cycle) and is catalyzed by a large family of adenosine triphosphate (ATP) phosphotransferases or protein kinases (PKs), which phosphorylate tyrosine (Tyr), serine (Ser), or threonine (Thr) residues. However, PKs potentially undergo abnormal activity by activating cell growth pathways, leading to tumor development. Given their critical role, PKs are now a wide therapeutic target, and several different receptor tyrosine kinase (RTK) inhibitors have been tested in clinical trials, mostly for cancer, and their use has been expanded to rheumatoid arthritis, cardiovascular diseases, diabetes, and more recently PH.[1,6,17]

5.1 Protein Kinase Inhibitor Effects on Growth Factors and Angiogenesis

RTKs are cell surface receptors that, on binding to several growth factors, activate a cascade of events that ultimately induce cell growth and proliferation. These growth factors include, among many, epidermal growth factor (EGF), insulin growth factor (IGF), and VEGF. On ligation, RTKs form dimers that activate intracellular PK domains, resulting in PK signaling cascades. For example, the RTK/phosphoinositide 3-kinase (PI3K) pathway activates downstream targets such as pyruvate dehydrogenase kinase-isomerase 1 (PDK-1), protein kinase B (AKT), and activation of the transcription factors IκB and nuclear factor κB (NFκB).[15] The use of small-molecule PKIs has now expanded as these molecules are competitive receptor antagonists, thereby inhibiting downstream effects. Currently, only eight small-molecule PKIs are approved in the United States, all for cancer treatment: Gleevec (imitinib mesylate), Iressa (gefitinib), Tarceva (erlotinib HCl), Sutent (sunitibin malate), Sprycel (dastinib), Tykerb (lapatinib ditosylate), Nexavar (sorafenib tosylate), and Tasigna (nilotinib HCl monohydrate). Gleevec, dastinib, and nilotinib are inhibitors of the oncogene BCR (Breakpoint cluster region)–ABL (Abelson murine leukemia viral, oncogene homolog 1) fusion PK in chronic myeloid leukemia, whereas Iressa, Tarceva, and Tykerb are EGF receptor (EGFR) family member inhibitors, and Nexavar or sorafenib is a multikinase inhibitor.[15] Sorafenib is an oral multikinase inhibitor of the PKs (protein kinases) PKC (protein kinase C)/Ras (Ras sarcoma), Raf, MEK-1,2(mitogen-activated protein kinase/extracellular signal regulated kinase 1,2), extracellular-regulated kinase (ERK) 1 and 2, and Ets-like transcription factor-1 (Elk-1) signaling pathway involved in tumor angiogenesis that has been approved by the Food and Drug Administration (FDA) for intervention of solid tumors in patients with advanced renal cell carcinoma (RCC) and those with unresectable hepatocellular carcinoma (HCC) and currently is undergoing phase II and III clinical evaluation for such other tumors as melanoma and non-small-cell lung cancer.

Sorafenib, previously known as BAY 43-9006 compound, was originally identified as a Raf kinase inhibitor in vitro[18] and subsequently as an inhibitor of ERK phosphorylation but not ERK signaling[18–20] in solid tumors. In addition to targeting Raf Ser/Thr kinases, sorafenib also inhibits the Tyr kinase activity of the proangiogenic VEGFR-1, -2, -3 and the platelet-derived growth factor receptor B (PDGFR-B) on human vascular ECs, fibroblasts, and vascular SMCs.[21] Thus, sorafenib is now considered a multikinase inhibitor and modifier of tumor growth

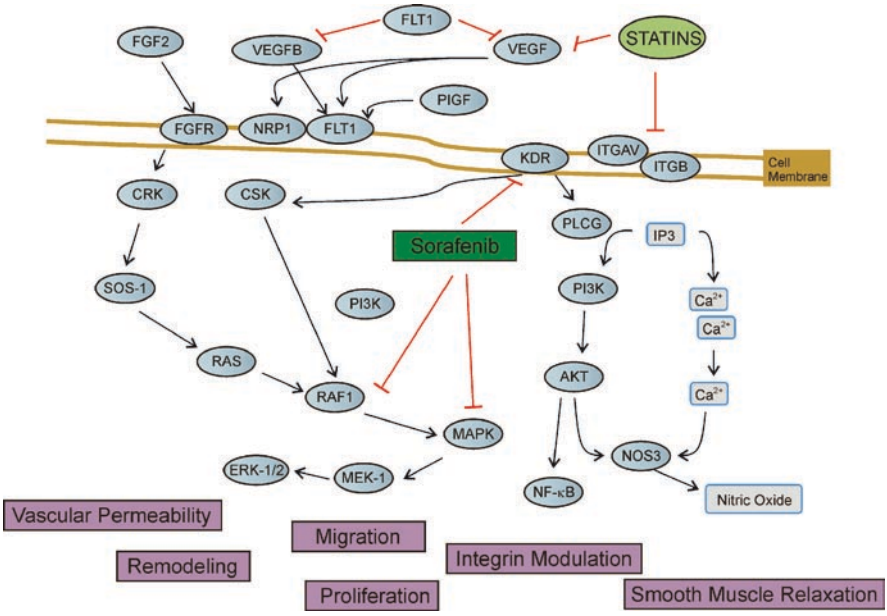


Fig. 27.1 Tyrosine kinase (TYK) targeting growth factor and proliferation pathways. Regulation of endothelial cell functions (migration, proliferation, vascular permeability, remodeling) is determined by the activity of protein kinases on the endothelium. Growth factor, angiogenesis/proliferation, and integrin pathways lead to a chain of events that ultimately produce changes in vascular permeability or remodeling. Thus, modulation with therapeutic agents such as statins and sorafenib corresponds to protein tyrosine kinase (PTK), which inhibits the RAS/RAF-1/MAPK pathway, and vascular endothelium growth factor receptor-2 (VEGFR-2) (KDR) blocker prevents vascular remodeling and proliferation. Statins have also been shown to modulate integrin signaling and VEFGR pathways to ultimately affect vascular function

and progression (Fig. 27.1) with a less-well-elucidated role in apoptosis on tumor endothelium. Downregulation of myeroid cell leukemia-1 protein (Mcl-1) by sorafenib is associated with the release of cytochrome *c* from mitochondria, caspase activation, and apoptosis, possibly via a MEK/ERK-independent mechanism.[19,21] In the clinic, sorafenib demonstrated significant antitumor activity, increasing the medium overall survival,[22] and decreased the time to progression in patients with advanced RCC. Overall, sorafenib is well tolerated in patients with solid tumors and even when combined with other chemotherapy.

5.2 Sorafenib Effects on Hemodynamic Indices and Vascular Morphology in Rodent PH

Genomic studies for generation of biomarkers still remain to be elucidated for sorafenib in cancer as well as for other sorafenib-responsive entities such as PH. We utilized two preclinical PH rodent models (hypoxia- and hypoxia/SU5416-

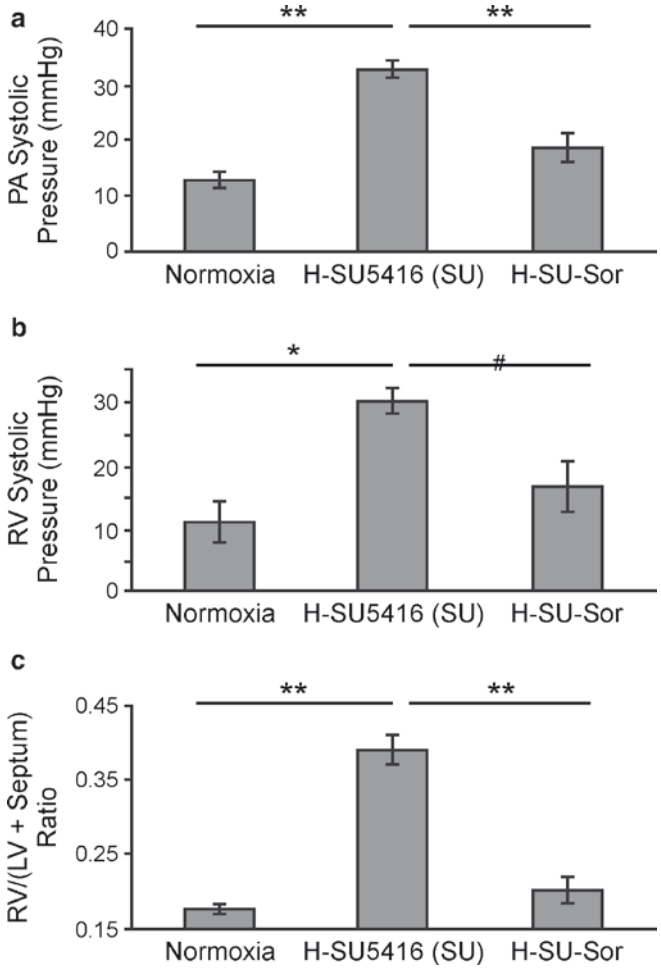


Fig. 27.2 Effect of sorafenib on hemodynamic measurements. (**a**, **b**) PA and RV systolic pressures, respectively, obtained in Dahl SS rodents at 3.5-week exposure to normoxia ($n = 7$), hypoxia/SU5416 (H-SU5416, $n = 5$), or hypoxia/SU5416/sorafenib treatments (H-SU-Sor, $n = 5$). (**c**) RV hypertrophy (RVH) measurements across all of the tested conditions represented by the ratio of RV mass divided by the sum of the mass of left ventricle (LV) plus septum (LV + septum). $^{*}p < 0.01$, $^{**}p < 0.001$

exposed rats)[4] for the study of sorafenib effects on the development of PH. The effect of sorafenib on PA remodeling and RV hemodynamics induced by hypoxia/SU5416 demonstrates significant reductions in PA pressures, RV pressures (Fig. 27.2a, b), and RV hypertrophy (Fig. 27.2c).[1] Representative histological observations demonstrated remarkable reductions in medial wall thickening, fibrosis, and

luminal obliteration with plexiform-like lesions in hypoxia/SU5416-exposed rat lungs when challenged with sorafenib.[1] We further refined the functional roles of sorafenib in lung vascular remodeling by quantifying the levels of both apoptosis (anticleaved caspase 3 antibody) and proliferation (Ki-67 antibody) utilizing tissue microarrays (TMAs) and the Automated Cellular Imaging System (ACIS) apoptosis (Fig. 27.3a). PH-induced apoptosis is mainly localized to the endothelium and the adventitia in the lung vasculature (*arrows* in Fig. 27.3a). Increased cell proliferation was observed in hypoxia-Sugen-challenged rats compared to normoxia and was evident in all vessel layers with sorafenib-treated rats, demonstrating attenuation of both of these two cellular processes in both PH models[1] (Fig. 27.3b).

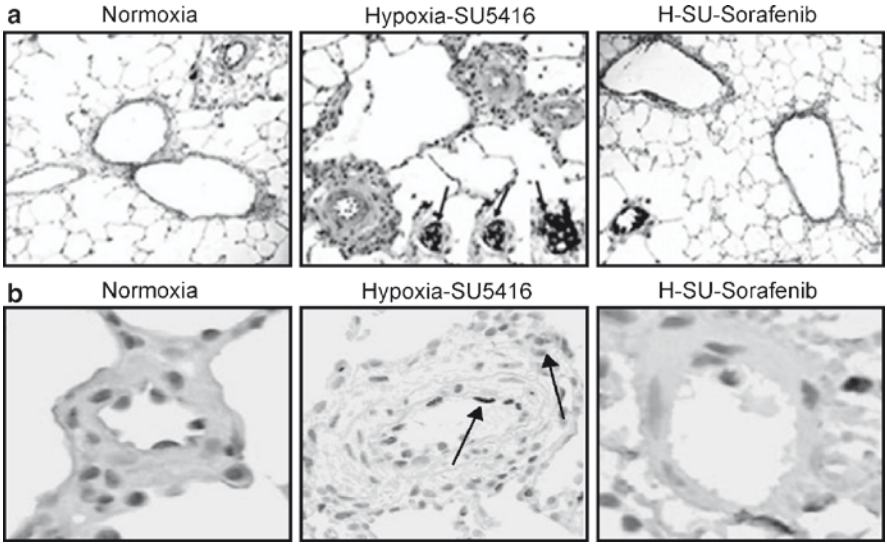


Fig. 27.3 Effect of sorafenib on the development of PH histopathology. Representative images for each group (hematoxylin and eosin staining) with *inset* (anti-vWF staining) demonstrate that compared to normoxic rats (**a**), rats exposed to hypoxia/SU5416 showed marked vascular remodeling with medial wall thickening, endothelial cell hyperproliferation, and formation of plexiform lesions with exuberant vWF-positive endothelial cell proliferation (three representative *inset arrows*). Sorafenib completely prevented the chronic hypoxia-SU5416-induced vascular remodeling. Differences in the level of apoptosis detected by cleaved caspase 3 staining and the level of cell proliferation using Ki67 staining were quantified as brown IOD per 10 μM^2 using ACIS. (**b**) Representative images for apoptosis from each condition. A stepwise increase in staining for apoptosis was demonstrated with hypoxia/SU5416 exposure compared to normoxia. Sorafenib produced dramatic inhibition of apoptosis compared to the hypoxia/SU5416 model, with less attenuation compared to hypoxia. $^{\#}p < 0.05$

6 Role of MAPK Pathway Components in Rodent Pulmonary Hypertension

The MAPK pathway exhibits ubiquitous involvement in cell proliferation and in responses to hemodynamic and environmental stress. Given the putative role of MAPK elements in the development of PH and pharmacodynamics of sorafenib, the posttranslational modification and protein expression levels of phosphorylated and unphosphorylated MEK-1/2 (p-MEK-1/2), ERK, and p38 MAPK (in addition to VEGFR-2) in PH lung homogenates has been studied.[1] Levels of p-ERK and p-MEK 1/2 were increased incrementally from hypoxia to hypoxia/SU5416-exposed rats compared to normoxia, while p-p38 showed a similar level of increased posttranslational modification in the two groups. The total amount of MAPK components was not altered compared to normoxia for all three proteins, again consistent with a posttranslational observation. Finally, the reduction in levels of p-VEGFR-2 with hypoxia/SU5416 has been confirmed by the inhibition of VEGFR-2 kinase activity by SU5416. Interestingly, levels of p-VEGFR-2 were increased in hypoxia compared to normoxia but remained downregulated by hypoxia/SU5416/sorafenib exposure, while total VEGFR-2 was not changed significantly throughout the various conditions.

7 Effect of Sorafenib on Lung Gene Expression Profiles in Rodent Pulmonary Hypertension

To study potential mechanisms of sorafenib effects on PH, we integrated bioinformatic analyses of expression profiles that revealed 1,019 transcripts differentially regulated by hypoxia. A comparison between the hypoxia/SU5416 and normoxia groups revealed an additional 465 differentially regulated transcript sets likely involved in the development of the severe PH phenotype. The subsequent comparison of sorafenib treatment in hypoxia/SU5416-exposed rats yielded 38 additional differentially regulated transcripts (Fig. 27.4a) potentially involved in the pathway of sorafenib-mediated PH attenuation. The decrease in the observed list of differentially regulated genes with addition of SU5416 to hypoxia may be explained by a potential drug effect. As a VEGFR inhibitor that disrupts downstream signaling, SU5416 may reduce the expression of numerous genes that are otherwise involved in lung responses to hypoxia and may also explain the potential for an augmented PH phenotype.[1]

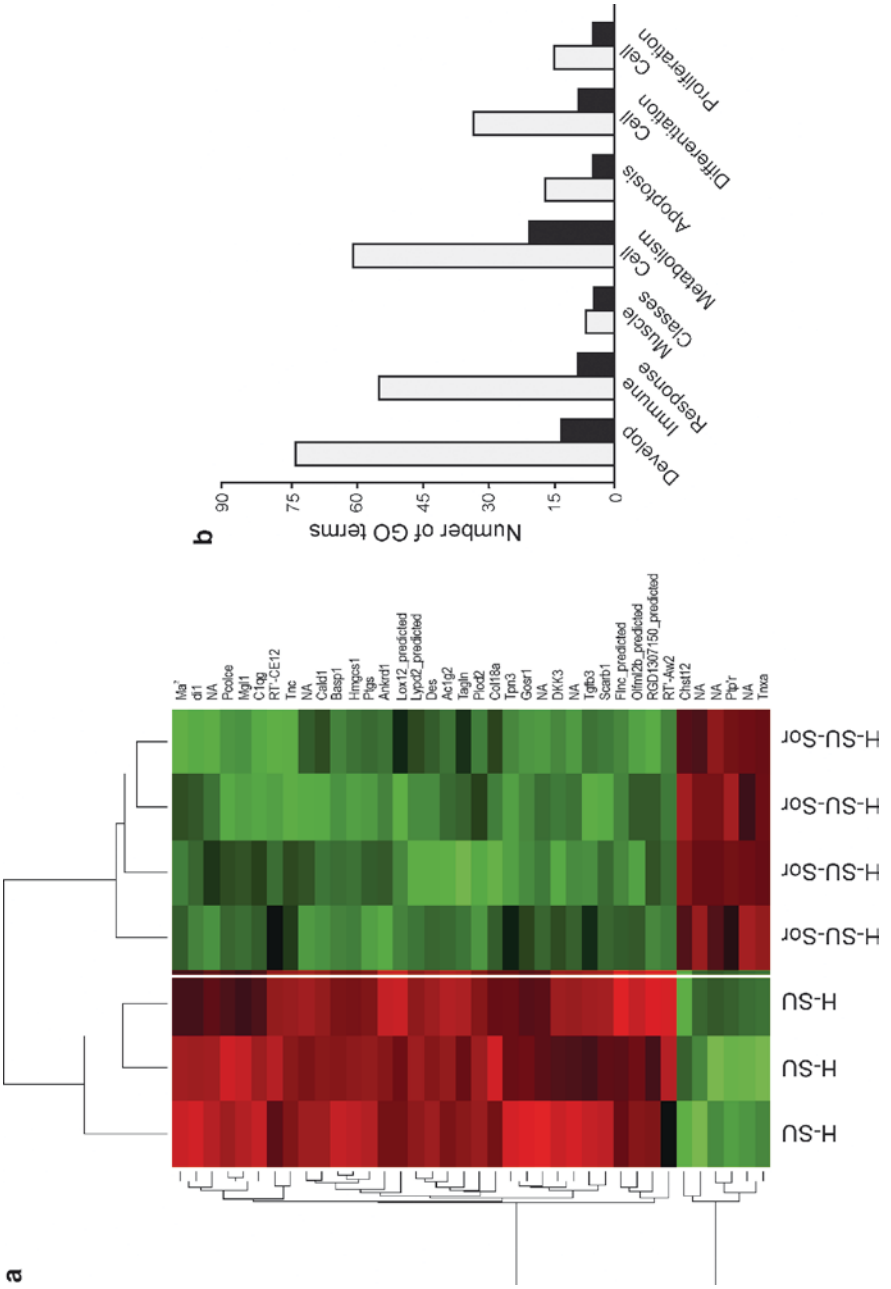
To derive the potential biological significance of sorafenib-driven genes, we first identified the significantly overrepresented biological processes in gene ontologies utilizing OntoExpress. While the sorafenib-driven gene profile derived from a comparison between the hypoxia/SU5416 group and hypoxia/SU5416/sorafenib group identified a number of biological process GO terms, a manual examination of these terms revealed six repeatedly represented overarching functional categories: cellular metabolic processes/metabolism (ten genes), developmental processes (seven genes), muscle development and regulation of muscle contraction (six genes),

defense response and immune system (five genes), cell differentiation (four genes), and cell proliferation (two genes). These genes are highlighted in the heat map of 38 differentially expressed transcripts between hypoxia/SU5416 and hypoxia/SU5416/sorafenib[1] (Fig. 27.4b).

For each of these overarching functional classes observed in the sorafenib-driven genes, we next compared the number of distinct significant biological processes (represented as GO terms) across the two data sets (normoxia vs. hypoxia/SU5416 and hypoxia/SU5416 vs. hypoxia/SU5416/sorafenib). Significance was based on the frequency of these biological process terms representing the differentially expressed genes as compared to those observed for all genes on the microarray chip (adjusted $p < 0.05$). To compare the relative enrichment of the biological process terms across the interventions, we analyzed the number of GO terms that were *identical*, *related*, or *unrelated* to each other between the different experimental conditions (Fig. 27.4b). The majority of muscle-related and cell proliferation GO terms involved in normoxia versus hypoxia or normoxia versus hypoxia/SU5416 were either related or identical to those affected by the hypoxia/SU5416 versus hypoxia/SU5416/sorafenib set. In addition, for the other four functional GO categories, nearly half of the GO terms could be explained by similar relationships to hypoxia/SU5416 versus hypoxia/SU5416/sorafenib, indicating that sorafenib regulates the majority of biological processes implicated in the hypoxia/SU5416 model of PH. Hierarchical relationships between the significant GO terms, defined as identical or related across all the two group conditions, within the example illustrate how the majority of these terms are related to cell development, immune response, and proliferation of (but not limited to) B lymphocytes, fibroblasts, neuroblasts, or epithelial cells.[1]

To complement gene ontology (GO) analysis, PubMatrix evaluation (which enumerates associated PubMed citations) was made of a selection of ten microarray-derived sorafenib candidate genes with a pair of genes representative of the most frequently appearing ontologies in our study (Table 27.1). This approach revealed a single previously identified PH candidate gene (Tgfβ3) as well as several potentially novel candidates as defined by the limited or absent number of PubMed citations. Several genes, however, have been reported to be involved in biological pathways that have been linked to PH, as evidenced by the number of citations in search terms such as cell proliferation, cancer, and angiogenesis.

Given both the ontology and PubMatrix analyses, we next sought to identify a subset of genes that have an increased likelihood of involvement in PH. Each of the 38 transcripts differentially regulated between hypoxia/SU5416 and hypoxia/SU5416/sorafenib groups intersected with those SAM-derived transcripts differentially regulated between normoxia and hypoxia/SU5416 as well as between normoxia and hypoxia. Therefore, the expression of particular genes arising from these three SAM data sets represents a dual screen for PH candidate genes: first in terms of their universal presence in all condition-based comparisons and then as strong statistical significance in expression levels given their SAM-based selection. A directional expression pattern for six genes across all four conditions with the values obtained from the original GCRMA-normalized data for each chip. This pattern is shown in Fig. 27.4a and clearly mirrors both the exacerbating effect of SU5416 on the hypoxic PH phenotype as well as the potent mitigating effect of sorafenib.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Basp1
Hmgcs1
Ptgs
Ankrd1
Lox12_predicted
Lypd2_predicted
Des
Ac1g2
Tagln
Plod2
Col18a
Tpm3
Goar1
NA
DKK3
NA
Tgfb3
Scarb1
Fhc_predicted
Olfml2b_predicted
RGD1307150_predicted
RT-Aw2
Chst12
NA
NA
Plp1r
NA
Tnxa
b
Number of GO terms
0
15
30
45
60
75
90
Develop
Immune
Response
Muscle
Classes
Cell
Metabolism
Apoptosis
Cell
Differentiation
Cell
Proliferation

Fig. 27.4 Heat map and gene ontology (GO) depiction of sorafenib-driven differentially regulated transcripts in the hypoxia-SU5416 pulmonary hypertension rodent model. (**a**) Microarray analysis of Dahl SS rat lungs (n = 4 chips/group) at 3.5 weeks revealed 38 differentially regulated transcripts between the hypoxia/SU5416 groups with and without sorafenib intervention. These 38 transcripts were assessed for significant directional expression pattern, yielding six genes that appeared across all four conditions. These six genes (*Cald1, Pcolce, C1qg, Idi1, Tgfβ3, Ptgis*) consistently displayed an increase in expression levels in hypoxia compared to normoxia, with greater expression level in the hypoxia/SU5416 model of PH. In each case, a reduction in gene expression was observed following sorafenib to levels comparable to normoxia. These transcripts were then clustered with a conventional heat map analysis with *red blocks* representing upregulation and *green blocks* representing downregulated expression of the relative transcript. (**b**) The transcript names were manually highlighted to show the significantly enriched GO classes that fell into the six most highly represented general categories and GO superclasses: "Developmental processes," "muscle development and regulation of muscle contraction," "defense response/immune system," "cell proliferation," "cell differentiation," and "cellular metabolic processes/metabolism" and apoptosis

Table 27.1 PubMatrix evaluation of selected sorafenib-driven genes across PH-related search terms

Gene name	*Gene symbol*	Ontology	PH	Proliferation	Hypoxia	Vascular remodeling	Angiogenesis	Cancer
Actin γ2	*Actg2*	Muscle	3	131	3	7	12	180
Tropomyosin 3, γ	*Tpm3*	Muscle	0	7	1	0	1	33
Complement component 1, q subcomponent, γ polypeptide	*C1qg*	Defense and immune	0	0	0	0	0	1
RT1 class1, CE12	*Rt1-CE12*	Defense and immune	0	0	0	0	0	0
Caldesmon 1	*Cald1*	Cell proliferation	1	73	7	7	9	212
V-maf musculoaponeurotic fibrosarcoma oncogene homolog	*Maf*	Cell proliferation	0	95	4	0	8	278
TGF-β3	*Tgfb3*	Cell differentiation	61	3,463	133	129	371	3,173
Protein tyrosine phosphatase, receptor type R	*Ptprr*	Cell differentiation	0	13	0	0	2	22
3-Hydroxy-3-methylglutaryl-coenzyme A synthase 1	*Hmgcs1*	Metabolism	7	28	8	6	13	32
Isopentenyl-diphosphate δ isomerase	*Idi1*	Metabolism	0	3	0	0	0	6

8 Comparison of Sorafenib-Modulated Microarray Data Sets with Prior PH Studies

Intersected microarray data with existing previously reported microarray studies of PH evidence the total number of common genes between the two comparison sets derived from our two models of PH and those of four previous published PH studies.[9,11,23,24] We observed a total of 57 overlapping hypoxia-driven *genes* with the Malek et al. report,[24] 35 common differentially expressed *genes* with hypoxia/SU5416, and 47 common differentially expressed hypoxia-driven *genes* in the Gharib et al.[9] report, as well as 26 common *genes* when compared with differentially expressed hypoxia/SU5416-driven rat *genes* from our study. Comparison with the Geraci et al. human PH report[9] revealed five common overlapping *genes* (Slc25a1, Shmt2, Plcb4, Pts, Fyn) in the hypoxia/SU5416 group. In addition, we also identified two microarray studies[11,23] in which we failed to identify any common genes after intersecting the orthologous rat *genes* and hypoxia/reoxygenation challenge in mice.[23] Similarly, we found no overlapping *genes* with the prior hypoxia versus hypoxia/simvastatin PH rats we previously reported.[11] Thus, ortholog approaches are helpful to identify *genes* that are shared by a significant number of rodent *genes* differentially expressed in mouse, rat, and human microarray experiments in PH, providing strong validation of the experimental model of our study. Examples of the various comparisons to establish hypoxia and PH candidate *genes* include bone morphogenetic protein 6, tissue plasminogen activator, and members of the prostaglandin receptor family. Solute carrier family 28 (sodium-coupled nucleoside transporter), member 2 (Slc28a2) was especially unique (identified in three of the five studies), and members of this transporter family were found in all but two total comparisons. Two of the *genes* highlighted in Fig. 27.4a, a member of the C1q family and isopentenyl-diphosphate delta isomerase (Idi1), are also commonly present in already published multiple comparison sets as well as a *gene* encoding the cytoskeletal protein caldesmon (Cald1).

9 Caldesmon Studies in Rodent Pulmonary Hypertension (Hypoxia/SU5416-Treated Rats)

Our reports indicated that p38 MAPK-mediated caldesmon (CaD) phosphorylation is involved in endothelial cytoskeleton remodeling and motility.[25] In our rodent samples with PH, CaD expression was low in normoxia but significantly elevated in hypoxia-SU5416-treated rats and was reduced by sorafenib treatment.[1] Figure 27.5 depicts myosin light chain (MLC)-dependent and -independent pathways in endothelium, where disruption of this vascular barrier integrity (i.e., by thrombin, a known vascular barrier-disruptive agent) activates the endothelial cytoskeleton in dynamic modulation of vascular barrier function. Whereas the EC cytoskeleton is composed of three primary components (actin filaments, intermediate filaments,

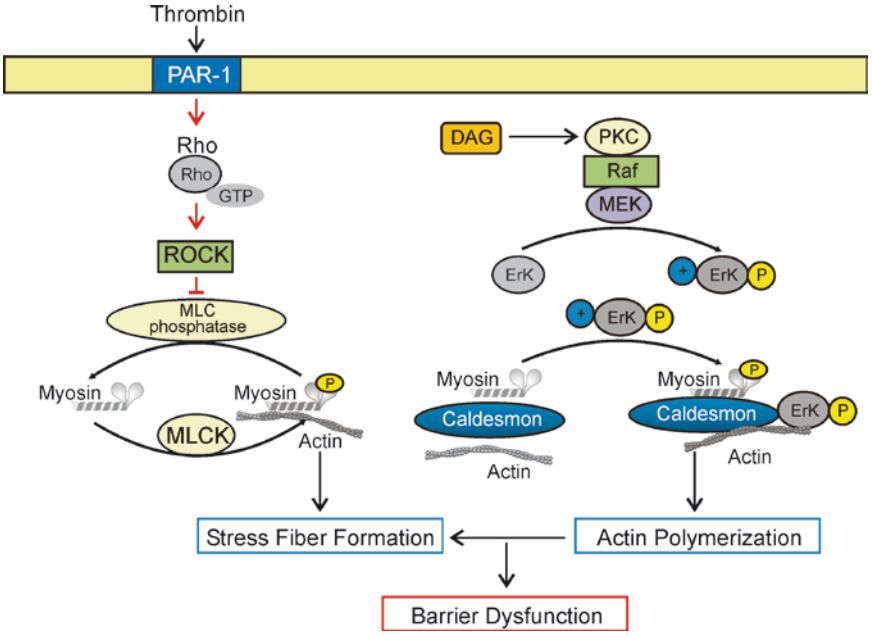

Fig. 27.5 Endothelial barrier-regulatory mechanisms: role of MLC and caldesmon. Pulmonary endothelial cells (ECs) are characterized by a thin cortical actin ring with associated cell–cell (adherens junctions and cell–matrix or focal adhesions) connections that provide the structural framework for barrier integrity. Thrombin, a known barrier-disruptive agent, results in rapid recruitment of its receptor (PAR-1) into membrane lipid rafts. For activation of PAR-1 receptor, activation is coupled to G_i signaling and subsequent activation of the small guanosine triphosphatase (GTPase) Rho. Rho stimulates rapid translocation of the actin-binding protein cortactin to the cell periphery, where it interacts with the barrier regulatory enzyme, myosin light chain kinase (MLCK). Phosphorylation of either of these proteins increases their interaction to actin in favor of stress fiber formation. Diacylglycerol (DAG) activates PKC, which activates Raf/MEK/ERK kinase activity. Erk phosphorylation subsequently facilitates the cytoskeletal caldesmon activation and phosphorylation, a key event for actin binding, polymerization, and fiber formation. This sequence of events occurs within minutes of stimulation and results in dramatically increased endothelial dysfunction. These pathways warrant evaluation of a potential therapeutic intervention with PTK such as sorafenib or statins for pulmonary hypertension

microtubules),[26] the actin cytoskeleton exhibits a primary role in EC barrier regulation. Early observations demonstrated that cytochalasin D, which disrupts the actin cytoskeleton, increased EC permeability, while phallacidin, an actin stabilizer, prevented barrier disruption by various agonists.[27,28] Actin filaments interact with myosin to generate EC tensile force that drives cell shape changes and barrier regulation. When this cellular contraction occurs along actin stress fibers that span the cell, gaps form between adjacent cells, and increased paracellular permeability ensues. Thrombin, a vascular barrier-disruptive agent, binds to its receptor PAR-1, triggering the Rho-GTP (guanosine triphosphate) activation and Rho kinase activity, which prevents MLC activation and phosphorylation and actin binding. EC activation

activates ERK, which promotes CaD activation via phosphorylation. Thus, controlling the functional role of CaD in binding to actin heads, actin polymerization, and stress fiber formation leads to EC barrier regulation and potentially vascular remodeling characteristic of PH. Our studies indicated that actomyosin cross-linking ability of CaD is essential for binding to microfilaments in ECs, and that this cytoskeletal rearrangement is present in rodent PH remodeling of the vascular endothelium, likely involving CaD phosphorylation (Fig. 27.5).

10 New Directions and Conclusions

In summary, we described the successful and novel prophylactic application of the multikinase inhibitor sorafenib in rodent models of PH with improved hemodynamic parameters and attenuation of significant structural PH changes[1] consistent with other reports that sorafenib improves hemodynamic parameters in other rodent models of PH.[17] Our investigation revealed biochemical and genomic evidence supporting the potential involvement of the MAPK cascade system and transforming growth factor β3 (TGFβ3) in PH development and the response to therapy. Integration of expression genomic analyses coupled with intense bioinformatics identified gene expression and ontology signatures in the development of PH and in the attenuation by sorafenib. Studies of sorafenib as a novel therapy in patients suffering from PH is already a well-tolerated, FDA-approved therapy in cancer; the clinical application of this novel therapy for PH can represent an excellent example of bedside-to-bench-to-bedside translation. Moreover, initial studies implicated the role of cytoskeletal protein such as CaD and MLCK (myosin light chain kinase) as key participants in the cytoskeletal rearrangement present during PH-induced remodeling and proliferation in endothelium. These represent potentially novel molecular targets for altering the devastating clinical course of patients with PH.

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References

1. Moreno-Vinasco L, Gomberg-Maitland M, Maitland ML et al (2008) Genomic assessment of a multikinase inhibitor, sorafenib, in a rodent model of pulmonary hypertension. Physiol Genomics 33:278–291
2. Higenbottam T, Cremona G (1993) Acute and chronic hypoxic pulmonary hypertension. Eur Respir J 6:1207–1212
3. Moudgil R, Michelakis ED, Archer SL (2005) Hypoxic pulmonary vasoconstriction. J Appl Physiol 98:390–403
4. Taraseviciene-Stewart L, Kasahara Y, Alger L et al (2001) Inhibition of the VEGF receptor 2 combined with chronic hypoxia causes cell death-dependent pulmonary endothelial cell proliferation and severe pulmonary hypertension. FASEB J 15:427–438
5. Raiesdana A, Loscalzo J (2006) Pulmonary arterial hypertension. Ann Med 38:95–110

6. Rhodes CJ, Davidson A, Gibbs JS, Wharton J, Wilkins MR (2009) Therapeutic targets in pulmonary arterial hypertension. Pharmacol Ther 121:69–88
7. Adatia I (2002) Recent advances in pulmonary vascular disease. Curr Opin Pediatr 14:292–297
8. Bull TM, Coldren CD, Nana-Sinkam P et al (2005) Microarray analysis of peripheral blood cells in pulmonary arterial hypertension, surrogate to biopsy. Chest 128:584S
9. Geraci MW, Gao B, Hoshikawa Y, Yeager ME, Tuder RM, Voelkel NF (2001) Genomic approaches to research in pulmonary hypertension. Respir Res 2:210–215
10. Girgis RE, Li D, Zhan X et al (2003) Attenuation of chronic hypoxic pulmonary hypertension by simvastatin. Am J Physiol Heart Circ Physiol 285:H938–H945
11. Girgis RE, Ma SF, Ye S et al (2005) Differential gene expression in chronic hypoxic pulmonary hypertension: effect of simvastatin treatment. Chest 128:579S
12. Bull TM, Coldren CD, Moore M et al (2004) Gene microarray analysis of peripheral blood cells in pulmonary arterial hypertension. Am J Respir Crit Care Med 170:911–919
13. Budhiraja R, Tuder RM, Hassoun PM (2004) Endothelial dysfunction in pulmonary hypertension. Circulation 109:159–165
14. Archer SL, Michelakis ED (2006) An evidence-based approach to the management of pulmonary arterial hypertension. Curr Opin Cardiol 21:385–392
15. Grant SK (2009) Therapeutic protein kinase inhibitors. Cell Mol Life Sci 66:1163–1177
16. McLaughlin VV, McGoon MD (2006) Pulmonary arterial hypertension. Circulation 114:1417–1431
17. Klein M, Schermuly RT, Ellinghaus P et al (2008) Combined tyrosine and serine/threonine kinase inhibition by sorafenib prevents progression of experimental pulmonary hypertension and myocardial remodeling. Circulation 118:2081–2090
18. Wilhelm SM, Carter C, Tang L et al (2004) BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. Cancer Res 64:7099–7109
19. Rahmani M, Davis EM, Bauer C, Dent P, Grant S (2005) Apoptosis induced by the kinase inhibitor BAY 43-9006 in human leukemia cells involves down-regulation of Mcl-1 through inhibition of translation. J Biol Chem 280:35217–35227
20. Rahmani M, Davis EM, Crabtree TR et al (2007) The kinase inhibitor sorafenib induces cell death through a process involving induction of endoplasmic reticulum stress. Mol Cell Biol 27:5499–5513
21. Rahmani M, Nguyen TK, Dent P, Grant S (2007) The multikinase inhibitor sorafenib induces apoptosis in highly imatinib mesylate-resistant bcr/abl$^+$ human leukemia cells in association with signal transducer and activator of transcription 5 inhibition and myeloid cell leukemia-1 down-regulation. Mol Pharmacol 72:788–795
22. Grant S (2008) Cotargeting survival signaling pathways in cancer. J Clin Invest 118: 3003–3006
23. Gharib SA, Luchtel DL, Madtes DK, Glenny RW (2005) Global gene annotation analysis and transcriptional profiling identify key biological modules in hypoxic pulmonary hypertension. Physiol Genomics 22:14–23
24. Malek RL, Wang HY, Kwitek AE et al (2006) Physiogenomic resources for rat models of heart, lung and blood disorders. Nat Genet 38:234–239
25. Mirzapoiazova T, Kolosova IA, Romer L, Garcia JG, Verin AD (2005) The role of caldesmon in the regulation of endothelial cytoskeleton and migration. J Cell Physiol 203:520–528
26. Dudek SM, Garcia JG (2001) Cytoskeletal regulation of pulmonary vascular permeability. J Appl Physiol 91:1487–1500
27. Phillips PG (1994) Thrombin-induced alterations in endothelial cell cytoarchitectural and functional properties. Semin Thromb Hemost 20:417–425
28. Phillips PG, Lum H, Malik AB, Tsan MF (1989) Phallacidin prevents thrombin-induced increases in endothelial permeability to albumin. Am J Physiol 257:C562–C567

PDGF Receptor and its Antagonists: Role in Treatment of PAH

Friedrich Grimminger and Ralph Theo Schermuly

Abstract Pulmonary Hypertension is a severe lung disease, which is characterized by vasoconstriction and remodelling of the vessel wall. Mostly addressing the increased vascular tone, prostacyclin and its analogues, endothelin-receptor antagonists and phosphodiesterase type 5 inhibitors have been approved for treatment of PAH and represent the current therapeutic options. Mechanistically, these vasodilators decrease pulmonary vascular resistance and reduce thereby shear stress, which is a strong proliferative stimulus *per se*. Beside the development of new vasodilators, current research focuses on the development of causal treatment regimens aiming a normalization of the vessel structure. Mechanistically, increased proliferation, migration and a resistance to apoptosis of vascular cells represent key events in disease progression. In this context, tyrosine kinase inhibitors like imatinib have been shown to possess reverse remodelling potential in preclinical models of pulmonary hypertension by inducing apoptosis and blocking proliferation. This book chapter describes the role of the platelet derived growth factor (PDGF) receptor and its antagonists for treatment of pulmonary hypertension.

Keywords Pulmonary hypertension • tyrosine kinase • PDGF • proliferation • growth factor

1 Introduction

Growth factors regulate diverse physiological responses, including proliferation, survival, migration, differentiation, morphogenesis, and metabolism. The vast majority of them, such as platelet-derived growth factor (PDGF), epidermal

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growth factor (EGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and others, exert their effects by binding to specific receptors with tyrosine kinase activity (RTKs).[1,2] Among these, the PDGFs and their tyrosine kinase receptors, PDGF receptor alpha (PDGFR-α) and PDGF receptor beta (PDGFR-β), are one of the best-studied systems.

PDGF, identified 30 years ago as a constituent of whole-blood serum that was absent in cell-free plasma-derived serum,[3] was initially purified from human platelets.[4] Although identified as a major mitogen for many cell types of mesenchymal origin (e.g., fibroblasts and smooth muscle cells [SMCs]), it has now been shown to be expressed and functionally relevant in a wide range of tissues.[5]

2 PDGF Ligands and Receptors

The PDGF family consists of four ligands (PDGF-A–D) and two receptors (PDGFR-α and PDGFR-β). PDGFs are disulfide-linked covalent peptides that make up five homo- and heterodimeric PDGF isoforms: PDGF-AA, -AB, -BB, -CC, and -DD.[6] PDGF-A and -B are secreted as active ligands, while C and D ligands, produced as latent factors, require cleavage of N-terminal CUB (for complement C1r/C1s, Uegf, Bmp1) domains to become active. The in vivo proteases responsible for activating C and D ligands have not been definitively identified; however, tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) have been proposed to activate PDGF-CC dimers and PDGF-DD dimers, respectively, in vivo.[7] Although not needing proteolytic activation, PDGF-A ligands can be processed at their carboxy termini, removing a retention motif that limits the ligands range of action through binding to heparan sulfate proteoglycans.[8]

PDGF dimers exert their cellular effects by activating two structurally related cell surface receptor tyrosine kinases (PDGFR-α and PDGFR-β).[5] However, each PDGF dimer differs in its interactions with the PDGFRs. Both PDGFRs contain five extracellular immunoglobulin-like domains, a transmembrane domain, a juxtamembrane domain, split kinase domains, a kinase insert domain, and a cytoplasmic tail. The receptors α and β can homo- and heterodimerize into αα, αβ, and ββ combinations. Since these two receptors share 31% identity in the ligand-binding domain, ligands have varying affinities for these different receptor combinations.[9] PDGF-AA effectively activates only PDGFR-αα, PDGF-AB can activate either PDGFR-αα or PDGFR-αβ, while PDGF-BB activates all three dimeric PDGF receptors (PDGFR-αα, PDGFR-αβ, PDGFR-ββ). The growth factor domain of PDGF-CC activates both PDGFR-αα and PDGFR-αβ, and the growth factor domain of PDGF-DD activates only the PDGFR- ββ. At present, it is unclear whether the PDGF-DD growth factor domain can activate PDGFR- αβ.[9]

3 PDGF-PDGFR-Mediated Intracellular Signal Transduction

The PDGFR possesses tyrosine kinase activity and is autophosphorylated on ligand binding. This autophosphorylation serves two important functions. On one hand, phosphorylation of a conserved tyrosine residue inside the kinase domains (Tyr849 in PDGFR-α and Tyr857 in PDGFR-β) leads to an increase in the catalytic efficiencies of the kinases for the PDGFR. On the other hand, autophosphorylation of tyrosine residues located outside the kinase domain creates docking sites for signal transduction molecules containing SH2 domains. In addition to SH2 domains, PTB (phosphotyrosine-binding domain) domains recognize phosphorylated tyrosine residues.[10] A large number of SH2 domain proteins have been shown to bind to both PDGFR-α and PDGFR-β receptors. These include some of the enzymes, such as phosphatidylinositol 3 kinase (PI3-kinase), phospholipase C (PLC) γ, the Src family of tyrosine kinases, the tyrosine phosphatase SHP-2 (SH2 domain containing tyrosine phosphatase 2), and a guanosine triphosphatase (GTPase)-activating protein for Ras (Ras-GAP). Other molecules, such as growth factor receptor-bound protein 2 (Grb2), growth factor receptor-bound protein 7 (Grb7), tyrosine-specific phosphatase (Syp), Src homology and collagen protein (Shc), and Crk (a group of adaptor proteins), are devoid of enzymatic activity and have adaptor functions, linking the receptor with downstream catalytic molecules. Also, members of the STAT (signal transducers and activators of transcription) family bind to the PDGFRs. They are transcription factors that after phosphorylation on tyrosine dimerize and translocate into the nucleus, where they affect the transcription of specific genes. Each SH2 domain molecule that binds to the PDGFRs initiates signal transduction pathways by activating downstream signaling molecules such as mitogen-activated protein (MAP) kinase family members (extracellular-regulated kinases [ERKs], JNKs (c-Jun N-terminal kinase), p38) and focal adhesion kinase (FAK; a mediator of the integrin-signaling pathway), among others.[11] These signals enter the nucleus and stimulate expression of a set of immediate-early-response genes (*c-fos, JunB, c-myc, egr-1*) that mediate PDGF-induced cellular processes, including cell cycle, cell migration, apoptosis, and transformation.[3,12,13]

Notably, the three dimeric PDGFR combinations transduce overlapping, but not identical, cellular signals, which can be explained by differential interactions with various SH2 domain proteins. The finding that while both α and β receptors effectively activate ERKs, PDGFR-αα, but not PDGFR-ββ, activates stress-activated protein kinase 1/c-Jun NH2-terminal kinase 1 (SAPK1/JNK-1) suggests the complex and unique nature of PDGFR signaling. In addition, autophosphorylation on different tyrosine residues might explain the unique properties of the heterodimeric PDGFR-αβ receptor complex in comparison with homodimeric receptors.[14]

4 Control of PDGF Signaling

Several mechanisms for modulation of signaling via PDGFRs have been elucidated. For instance, MAP kinase, which is activated by Ras, phosphorylates and inactivates Sos, which thereby leads to decreased Ras activation.[15] Another negative-feedback mechanism involves cyclic adenosine monophosphate (cAMP)-dependent protein kinase, which is activated by PDGF through induction of prostaglandin synthesis and activation of adenylyl cyclase[16]; the cAMP-dependent protein kinase inhibits several of the pathways that are activated in PDGF-stimulated cells through phosphorylation of components in these pathways. Finally, PDGF signaling can also be modulated in the extracellular milieu by matrix molecules. Such contacts are mediated by integrins, which are transmembrane receptors for matrix molecules. For example, integrins, the transmembrane receptors for matrix molecules, have been shown to enhance growth factor-mediated cell proliferation and cell migration and are necessary to prevent apoptosis. On the other hand, fibrillar collagen suppresses PDGF-induced DNA synthesis in arterial SMCs. This effect is likely to be mediated by an integrin-dependent suppression of cyclin E-Cdk2 activity.[17]

5 Expression and Functions of PDGF in the Vascular System

PDGF and its receptors are detected in many different vascular cells and in arteries following injury in vivo. However, PDGF-A and PDGF-B are expressed at very low or undetectable levels in normal vessels, whereas PDGF-C predominantly occurs in medial SMCs and PDGF-D in fibroblastic adventitial cells. PDGFR-α and PDGFR-β are present in SMCs of normal vessels at the messenger RNA (mRNA) level but very low levels of protein.[5,18] The synthesis is often increased in response to external stimuli, such as exposure to low oxygen tension; thrombin; or stimulation with various growth factors and cytokines.[19] PDGF is a mitogen and chemoattractant for vascular SMCs in vitro, but its activities in vivo remain largely undefined. Most importantly, infusion of recombinant PDGF-BB into rats subjected to carotid injury produced a small increase (two- to threefold) in medial SMC proliferation. More importantly, PDGF-BB greatly increased (20-fold) the intimal thickening and the migration of SMCs from the media to the intima during the first 7 days after injury, suggesting in vivo activities of PDGF.[20]

In vivo, PDGF has also been implicated in the contraction and maintenance of tone, the major physiologic functions of blood vessels. On one hand, PDGF polypeptides (PDGF-AA, PDGF-AB, and PDGF-BB) induced constriction of different types of blood vessels. On the other hand, PDGF-BB stimulation of endothelial cells induced a nitric oxide (NO)-mediated relaxation of rat aorta via PDGFR-β.[21] A more direct test of the effect of PDGF on vascular tone involved intravenous administration of PDGF-BB, but not PDGF-AB or PDGF-AA, in rats was found to

decrease systolic blood pressure through an increase in macrovascular compliance that was NO dependent.[22]

Another effect of PDGF that is of importance in the vascular system is its feedback control effect on platelet aggregation. Human platelets, which are a rich source of PDGF, contain functionally active PDGFR-α, but not PDGFR-β, and importantly, PDGF stimulation leads to decreased platelet aggregation.[23] Thrombin stimulation of platelets induces PDGF release, which results in phosphorylation of the PDGFR-α, and preincubation of platelets with PDGF inhibits thrombin-induced platelet activation, indicating that the PDGF released from platelets serves an autocrine feedback role in control of platelet aggregation.[24]

Furthermore, a determinant role of PDGF in innate and adaptive immune responses was demonstrated by numerous reports. Macrophages in atherosclerosis lesions are known to express PDGF-A and PDGF-B and have been shown to stimulate SMC accumulation. On the other hand, monocyte expression of PDGFR-β has been shown to increase with differentiation into macrophages at the mRNA and protein levels.[25] In addition, numerous reports have suggested PDGF-mediated regulation of angiogenic activity. Increasing evidence suggests that PDGF-B and paracrine PDGFR-β signaling, instead of having a direct effect on endothelial cells, was shown to maintain mural cells that cover all capillaries.[26]

6 PDGF Signaling in Vascular Disorders

The PDGF system has been implicated in a broad range of diseases, such as cancer, vascular diseases, and fibrosis. Atherosclerosis, restenosis, transplant arteriosclerosis, retinal vascular diseases, and pulmonary hypertension are the major ones among the vascular disorders for which PDGF signaling has been incriminated in the pathogenesis.

6.1 Atherosclerosis, Restenosis, and Transplant Arteriosclerosis

Atherosclerosis is a complex inflammatory response in large and medium arteries to a number of different risk factors. The atherosclerotic lesion is characterized by an accumulation of cells and extracellular matrix (ECM) in the intimal space, and the eccentric lesion forms focally at sites of low shear stress, turbulence, and oscillating flow in arteries. The early "fatty streak" lesions consist primarily of macrophages and T lymphocytes, and the lesion progresses by accumulation of the emigrating medial SMCs and their deposition of connective tissue. More advanced fibrous plaques are usually covered by a dense cap of fibrous connective tissue with embedded SMCs that usually overlay a core of lipid and necrotic debris.[27] Consequently, the lumen of the affected arteries becomes narrow. The surgical

approaches of percutaneous transluminal coronary angioplasty (PCTA) and stenting are being practiced to successfully open the narrowed arteries. However, restenosis, the renarrowing of the arterial lumen, remains as a common problem.[28] Arteriosclerosis also results following organ or tissue transplantation. Unlike atherosclerosis, transplant arteriosclerosis gives rise to diffuse intimal thickening that results in a concentric lesion, often involving long segments of affected arteries. Transplant vasculopathy, characterized by intimal accumulation of SMCs, infiltrating mononuclear cells, and ECM in the arteries of transplanted solid organs, including heart, kidney, liver, and lung, is the most common cause of late graft failure and death in transplant recipients.[29]

In all these diseases, the PDGF-PDGFR system is shown to be highly altered. In particular, PDGF-AA and -BB are upregulated in atherosclerotic lesions compared to the normal vessel wall.[30] Moreover, an increased level of PDGFR-β is present in infiltrating macrophages and SMCs of atherosclerotic vessel walls.[31] As in the case of atherosclerosis, both PDGF-AA and PDGF-BB are expressed at sites of transluminal coronary angioplasty in addition to PDGFR-β expression in SMCs and in macrophages.[32] Taken together, the data suggest that PDGFR-β signaling plays a major role in the lesion development and progression in atherosclerosis and restenosis. Transplant arteriosclerosis is also associated with an increased PDGFR-β expression in vascular SMCs of transplanted human organs. However, the marked increase in PDGFR-α expression in renal transplant and in cardiac allograft shows a notable difference between transplant arteriosclerosis and atherosclerosis.[30] In summary, the expression and localization data clearly suggest a role for the PDGF system in vascular pathology/remodeling.

Similar findings are also observed in animal models of neointimal lesions. In agreement with the expression data, the interventional studies in a rat carotid artery balloon injury model, a commonly used model of neointimal lesions, demonstrated that inhibition of PDGF signaling by antisense oligonucleotides to the PDGFR-β,[33] PDGF-BB aptamers,[34] PDGFR tyrosine kinase inhibitors,[35] and antibodies against PDGFR-β[36] reduce accumulation of SMCs in intimal lesions, attributing an important role of PDGF-BB signaling in disease pathogenesis. Similarly, an increasing body of literature attributes a role for PDGFR-β signaling in atherosclerotic lesion progression and specifically SMC accumulation in both hypercholesterolemic rabbits and apolipoprotein E (ApoE)-deficient mice.[37,38]

In addition, interventional studies clearly suggest the involvement of the PDGF system in the pathogenesis of restenosis. Bilder et al. evaluated angiographically the effect of PDGFR inhibitor in hypercholesterolemic pigs following coronary angioplasty.[39] Their findings suggest that the inhibition of PDGFR tyrosine kinase prevents angiographic loss of gain following PTCA and significantly reduced intimal hyperplasia.

6.2 Pulmonary Hypertension

Pulmonary arterial hypertension (PAH) a life-threatening disease characterized by a marked and sustained elevation of pulmonary artery pressure and is therefore

defined as a mean pulmonary arterial pressure greater than 25 mmHg at rest or greater than 30 mmHg during exercise.[40] PAH can be idiopathic or unexplained (formerly termed primary pulmonary hypertension); PAH can also occur in association with connective tissue diseases, HIV infection, congenital heart disease, portal hypertension, and appetite suppressant exposure. Idiopathic PAH occurs more often in women than in men, with a median survival of 2.8 years if untreated. The mean age at diagnosis is 35 years. The 5-year survival remains at 50% for this devastating disease, although current treatment options have markedly improved overall quality of life and survival in PAH.[41] We still do not know what initiates this disease with its subsequent progressive pulmonary vascular obstruction, even if we have learned a great deal about the pathobiology of PAH.

PAH has a multifactorial pathobiology: An inappropriate vasoconstriction, vascular remodeling, and in situ thrombosis contribute to this pathological process.[41] All layers of the vessel wall are involved in the process of vascular remodeling; each cell type (endothelial cells, SMCs, and fibroblasts) as well as inflammatory cells and platelets play a significant role in PAH.[42] The distal extension of pulmonary arterial smooth muscle cells (PASMCs) into small peripheral, normally nonmuscular, pulmonary arteries within the respiratory acinus is a feature common to all forms of PAH. In addition, a formation of a layer of myofibroblasts and ECM occurs between the endothelium and the internal elastic lamina, termed the *neointima*.[43] Disorganized endothelial cell proliferation leading to the formation of so termed plexiform lesions is described in many cases of PAH.[44] Endothelial dysfunction is considered a key element in the pathobiology in PAH, with increased levels of endothelin (ET) occurring concomitantly with decreased NO and prostacyclin levels.[45] Alterations of rates of both proliferation and apoptosis result in thickened, obstructive pulmonary arteries in PAH as evidence has shown. Taken together, the elevated pulmonary vascular resistance leads to an increased right ventricle afterload and, eventually, to the failure of the afterload-intolerant right ventricle.[46] Many of the perturbations associated with the endothelial dysfunction promote vascular remodeling in addition to increasing pulmonary vascular tone, although it remains unclear whether excessive vasoconstriction is associated with the endothelial dysfunction. Prostacyclin, NO, ET, angiopoietin I, serotonin, cytokines, chemokines, and members of the transforming growth factor β (TGF-β) superfamily have all been implicated in the pathobiology of PAH.[41]

The field of PAH has been advancing rapidly, and the pace continues to accelerate, resulting in three currently approved therapeutic modalities for the treatment of PAH – prostacyclins, phosphodiesterase (PDE) inhibitors, and ET receptor antagonists – and targeting prostacyclin and NO deficiencies and increased ET levels, respectively, in PAH patients.[47] Still, current treatment primarily attenuates vasoconstriction and provides only symptomatic relief as well as some improvement of prognosis. However, convincing evidence for direct antiproliferative effects of these approaches is largely missing. Due to the chronic proliferation of pulmonary vascular tissue, it is obvious that the efficiency of vasodilatory therapies is limited, especially in advanced stages of the disease. Some of the currently used therapeutic implications seem to have at least some secondary antiproliferative effects, which

are mostly due to the reduction of pulmonary vascular resistance. In 1998, the idea of monoclonal endothelial proliferation as the cause of the progressive vascular obstruction in PAH was introduced the working group of Voelkel and Tuder.[48] They found monoclonal endothelial cell conglomerates in plexiform lesions of patients with primary, but not secondary, pulmonary hypertension. Initially, this investigation led to the idea that cell proliferations in PAH are similar to those seen in various types of cancer and lately to the proof that molecular mechanisms, leading to cell proliferation in a tumor, play a central role in chronic vascular changes seen in PAH.[49] Taken together, this led to the concept that anticancer drugs might be effective in PAH. This was the beginning of the paradigm shift in the treatment of PAH – from vasodilation to "reverse remodeling."

Compelling evidence suggests that PDGF is closely involved in the pulmonary vascular pathology of PAH. PDGF isoforms exert their effects on target cells by activating two structurally related RTKs: PDGFR-α and -β.[5] In 1998, Humbert et al. found that PDGF expression is elevated in lung biopsies of HIV-patients displaying PAH.[50] Second, PDGF acts as a potent mitogen and chemoattractant for PASMCs.[51] Third, both PDGFR-α and PDGFR-β are upregulated in lambs with chronic intrauterine PAH, suggesting a role for PDGF in PASMC thickening in PAH.[52] The finding of this work group further supported the concept that treatment with a selective PDGF inhibitor reduces pulmonary arterial wall thickness and right ventricular hypertrophy in a model of perinatal PAH by partial ligation of the ductus arteriosus in the late fetal lamb. Fourth, Jankov et al. found that PDGFR-β and its ligands, particularly PDGF-BB, which binds to both PDGFR-α and -β, play a major role in the pathogenesis of vascular SMC proliferation in newborn rats with PAH.[53] In 2008, Perros et al. showed that in small remodeled pulmonary arteries, PDGF-A and PDGF-B are mainly localized to PASMCs and endothelial cells, whereas PDGFR-α and PDGFR-β mainly stained in PASMCs and to a lesser extent in endothelial cells.[54] It was shown that inhibition of PDGFRs by the tyrosine kinase inhibitor STI571 (imatinib, Gleevec® or Glivec) reverses pulmonary vascular remodeling in two different animal models of severe PAH. In lung biopsies from patients with severe PAH, PDGF-A chain and PDGFR-β expression was significantly increased.[49] Inhibition of PDGF-induced PASMC migration and proliferation with imatinib supports the possible therapeutic role of PDGF inhibition as a novel approach in PAH.

In a translational research approach, the hypothesis of tyrosine kinase inhibitors being effective in PAH has been supported by individual clinical case reports from patients with PAH indicating a beneficial effect of imatinib (Gleevec), which is an inhibitor of the tyrosine kinases PDGFR, BCR-Abl, and c-kit and is approved for treatment of chronic myeloid leukemia and gastrointestinal stromal tumor. The first patient ever reported was a 61-year-old man with rapidly progressing PAH, who received imatinib in addition to bosentan, iloprost, sildenafil, oral anticoagulants, and diuretics[55]; after 3 months, he had greatly improved exercise capacity, reduced pulmonary vascular resistance, decreased pulmonary artery pressure, increased cardiac index, and an improvement from functional class IV New York Heart Association (NYHA) to class II, with no apparent adverse effects. Similar improve-

ments in clinical condition were documented from other groups in a 52-year-old man with refractory idiopathic PAH[56] and in two patients with PAH who received imatinib for treatment of leukemia.[57] Based on the scientific rationale, the intriguing basic research findings, and the encouraging early clinical results, a multinational, multicenter, randomized, placebo-controlled, long-term clinical phase II trial with imatinib added to ongoing conventional PAH treatments has been initiated.

6.3 Cardiotoxicity of Imatinib

However, a matter of current controversy arises from anecdotal case reports about the potential cardiotoxicity of imatinib and other small-molecular tyrosine kinase inhibitors in patients receiving the drug long term in the original indication of cancer. It was supposed that some of these agents cause congestive heart failure (CHF) or asymptomatic left ventricular dysfunction.[58] In August 2006, Kerkelä et al. reported ten cases of heart failure after treatment with imatinib (Glivec or Gleevec) in patients suffering from chronic myelogenous leukemia (CML) without a prior history of heart disease.[59] Kerkelä and coworkers found that imatinib has deleterious effects on cardiomyocytes in culture and in vivo. Their studies in cultured cardiomyocytes and mice treated with imatinib showed that imatinib leads to significant mitochondrial dysfunction with loss of membrane potential, cytochrome *c* release into the cytosol, impaired energy generation with reduction of ATP content and, finally, cell death.[59] It was shown that the inhibition of one major target of imatinib, c-Abl, mediates this cardiotoxicity because cardiomyocytes expressing the imatinib-resistant mutant c-Abl (T3151) were partially rescued from imatinib-induced toxicity.[60] It was proposed that the triggering mechanism for imatinib-induced cardiomyocyte death is the activation of the endoplasmic reticulum (ER) stress response, although the pathway linking c-Abl inhibition with the induction of ER stress remains unclear.[61] In contrast to these findings, in a 5-year follow-up study of 553 patients receiving imatinib for CML therapy, published by Drucker et al. in December 2006, only one patient developed CHF reported as drug related.[62]

Taken together, further studies are needed to clarify the underlying mechanisms leading to cardiotoxicity of imatinib and to guide the development of strategies to prevent the development of heart failure resulting from these promising PAH therapies. Careful cardiovascular monitoring and cardiac treatment at the first signs of myocardial damage might be a solution to manage this problem.

References

1. Yarden Y, Ullrich A (1988) Growth factor receptor tyrosine kinases. Annu Rev Biochem 57:443–478
2. Ullrich A, Schlessinger J (1990) Signal transduction by receptors with tyrosine kinase activity. Cell 61:203–212

3. Ross R, Glomset J, Kariya B, Harker L (1974) A platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscle cells in vitro. Proc Natl Acad Sci U S A 71:1207–1210
4. Antoniades HN, Scher CD, Stiles CD (1979) Purification of human platelet-derived growth factor. Proc Natl Acad Sci U S A 76:1809–1813
5. Heldin CH, Westermark B (1999) Mechanism of action and in vivo role of platelet-derived growth factor. Physiol Rev 79:1283–1316
6. Fredriksson L, Li H, Eriksson U (2004) The PDGF family: four gene products form five dimeric isoforms. Cytokine Growth Factor Rev 15:197–204
7. Fredriksson L, Li H, Fieber C, Li X, Eriksson U (2004) Tissue plasminogen activator is a potent activator of PDGF-CC. EMBO J 23:3793–3802
8. Feyzi E, Lustig F, Fager G, Spillmann D, Lindahl U, Salmivirta M (1997) Characterization of heparin and heparan sulfate domains binding to the long splice variant of platelet-derived growth factor A chain. J Biol Chem 272:5518–5524
9. Heidaran MA, Beeler JF, Yu JC et al (1993) Differences in substrate specificities of alpha and beta platelet-derived growth factor (PDGF) receptors. Correlation with their ability to mediate PDGF transforming functions. J Biol Chem 268:9287–9295
10. Claesson-Welsh L (1994) Platelet-derived growth factor receptor signals. J Biol Chem 269:32023–32026
11. Stiles CD (1983) The molecular biology of platelet-derived growth factor. Cell 33:653–655
12. Grotendorst GR, Chang T, Seppa HE, Kleinman HK, Martin GR (1982) Platelet-derived growth factor is a chemoattractant for vascular smooth muscle cells. J Cell Physiol 113:261–266
13. Okura T, Igase M, Kitami Y et al (1998) Platelet-derived growth factor induces apoptosis in vascular smooth muscle cells: roles of the Bcl-2 family. Biochim Biophys Acta 1403:245–253
14. Rosenkranz S, Kazlauskas A (1999) Evidence for distinct signaling properties and biological responses induced by the PDGF receptor alpha and beta subtypes. Growth Factors 16:201–216
15. Porfiri E, McCormick F (1996) Regulation of epidermal growth factor receptor signaling by phosphorylation of the ras exchange factor hSOS1. J Biol Chem 271:5871–5877
16. Graves LM, Bornfeldt KE, Sidhu JS et al (1996) Platelet-derived growth factor stimulates protein kinase A through a mitogen-activated protein kinase-dependent pathway in human arterial smooth muscle cells. J Biol Chem 271:505–511
17. Assoian RK (1997) Anchorage-dependent cell cycle progression. J Cell Biol 136:1–4
18. Uutela M, Lauren J, Bergsten E et al (2001) Chromosomal location, exon structure, and vascular expression patterns of the human PDGFC and PDGFD genes. Circulation 103:2242–2247
19. Betsholtz C, Lindblom P, Bjarnegard M, Enge M, Gerhardt H, Lindahl P (2004) Role of platelet-derived growth factor in mesangium development and vasculopathies: lessons from platelet-derived growth factor and platelet-derived growth factor receptor mutations in mice. Curr Opin Nephrol Hypertens 13:45–52
20. Jawien A, Bowen-Pope DF, Lindner V, Schwartz SM, Clowes AW (1992) Platelet-derived growth factor promotes smooth muscle migration and intimal thickening in a rat model of balloon angioplasty. J Clin Invest 89:507–511
21. Berk BC, Alexander RW, Brock TA, Gimbrone MA Jr, Webb RC (1986) Vasoconstriction: a new activity for platelet-derived growth factor. Science 232:87–90
22. Ikeda M, Morita C, Mizuno M, Sada T, Koike H, Kurokawa K (1997) PDGF-BB decreases systolic blood pressure through an increase in macrovascular compliance in rats. Am J Physiol 273:H1719–H1726
23. Vassbotn FS, Havnen OK, Heldin CH, Holmsen H (1994) Negative feedback regulation of human platelets via autocrine activation of the platelet-derived growth factor α-receptor. J Biol Chem 269:13874–13879
24. Bryckaert MC, Rendu F, Tobelem G, Wasteson A (1989) Collagen-induced binding to human platelets of platelet-derived growth factor leading to inhibition of P43 and P20 phosphorylation. J Biol Chem 264:4336–4341

25. Tzeng DY, Deuel TF, Huang JS, Baehner RL (1985) Platelet-derived growth factor promotes human peripheral monocyte activation. Blood 66:179–183
26. Lindblom P, Gerhardt H, Liebner S et al (2003) Endothelial PDGF-B retention is required for proper investment of pericytes in the microvessel wall. Genes Dev 17:1835–1840
27. Insull W Jr (2009) The pathology of atherosclerosis: plaque development and plaque responses to medical treatment. Am J Med 122:S3–S14
28. Min SK, Kenagy RD, Clowes AW (2008) Induction of vascular atrophy as a novel approach to treating restenosis. A review. J Vasc Surg 47:662–670
29. Belperio JA, Ardehali A (2008) Chemokines and transplant vasculopathy. Circ Res 103:454–466
30. Raines EW (2004) PDGF and cardiovascular disease. Cytokine Growth Factor Rev 15:237–254
31. Wilcox JN, Smith KM, Williams LT, Schwartz SM, Gordon D (1988) Platelet-derived growth factor mRNA detection in human atherosclerotic plaques by in situ hybridization. J Clin Invest 82:1134–1143
32. Tanizawa S, Ueda M, van der Loos CM, van der Wal AC, Becker AE (1996) Expression of platelet derived growth factor B chain and beta receptor in human coronary arteries after percutaneous transluminal coronary angioplasty: an immunohistochemical study. Heart 75:549–556
33. Noiseux N, Boucher CH, Cartier R, Sirois MG (2000) Bolus endovascular PDGFR-β antisense treatment suppressed intimal hyperplasia in a rat carotid injury model. Circulation 102:1330–1336
34. Leppänen O, Janjic N, Carlsson MA et al (2000) Intimal hyperplasia recurs after removal of PDGF-AB and -BB inhibition in the rat carotid artery injury model. Arterioscler Thromb Vasc Biol 20:E89–E95
35. Yu JC, Lokker NA, Hollenbach S et al (2001) Efficacy of the novel selective platelet-derived growth factor receptor antagonist CT52923 on cellular proliferation, migration, and suppression of neointima following vascular injury. J Pharmacol Exp Ther 298:1172–1178
36. Hart CE, Kraiss LW, Vergel S et al (1999) PDGFβ receptor blockade inhibits intimal hyperplasia in the baboon. Circulation 99:564–569
37. Rutherford C, Martin W, Carrier M, Anggard EE, Ferns GA (1997) Endogenously elicited antibodies to platelet derived growth factor-BB and platelet cytosolic protein inhibit aortic lesion development in the cholesterol-fed rabbit. Int J Exp Pathol 78:21–32
38. Sano H, Sudo T, Yokode M et al (2001) Functional blockade of platelet-derived growth factor receptor-β but not of receptor-α prevents vascular smooth muscle cell accumulation in fibrous cap lesions in apolipoprotein E-deficient mice. Circulation 103:2955–2960
39. Bilder G, Wentz T, Leadley R et al (1999) Restenosis following angioplasty in the swine coronary artery is inhibited by an orally active PDGF-receptor tyrosine kinase inhibitor, RPR101511A. Circulation 99:3292–3299
40. Rubin LJ (2006) Pulmonary arterial hypertension. Proc Am Thorac Soc 3:111–115
41. Barst RJ (2005) PDGF signaling in pulmonary arterial hypertension. J Clin Invest 115:2691–2694
42. Humbert M, Morrell NW, Archer SL et al (2004) Cellular and molecular pathobiology of pulmonary arterial hypertension. J Am Coll Cardiol 43:13S–24S
43. Jeffery TK, Morrell NW (2002) Molecular and cellular basis of pulmonary vascular remodeling in pulmonary hypertension. Prog Cardiovasc Dis 45:173–202
44. Cool CD, Stewart JS, Werahera P et al (1999) Three-dimensional reconstruction of pulmonary arteries in plexiform pulmonary hypertension using cell-specific markers. Evidence for a dynamic and heterogeneous process of pulmonary endothelial cell growth. Am J Pathol 155:411–419
45. Humbert M, Montani D, Perros F, Dorfmüller P, Adnot S, Eddahibi S (2008) Endothelial cell dysfunction and cross talk between endothelium and smooth muscle cells in pulmonary arterial hypertension. Vascul Pharmacol 49:113–118

46. Olsson KM, Hoeper MM (2009) Novel approaches to the pharmacotherapy of pulmonary arterial hypertension. Drug Discov Today 14:284–290
47. Rhodes CJ, Davidson A, Gibbs JS, Wharton J, Wilkins MR (2009) Therapeutic targets in pulmonary arterial hypertension. Pharmacol Ther 121:69–88
48. Lee SD, Shroyer KR, Markham NE, Cool CD, Voelkel NF, Tuder RM (1998) Monoclonal endothelial cell proliferation is present in primary but not secondary pulmonary hypertension. J Clin Invest 101:927–934
49. Schermuly RT, Dony E, Ghofrani HA et al (2005) Reversal of experimental pulmonary hypertension by PDGF inhibition. J Clin Invest 115:2811–2821
50. Humbert M, Monti G, Fartoukh M et al (1998) Platelet-derived growth factor expression in primary pulmonary hypertension: comparison of HIV seropositive and HIV seronegative patients. Eur Respir J 11:554–559
51. Yu Y, Sweeney M, Zhang S et al (2003) PDGF stimulates pulmonary vascular smooth muscle cell proliferation by upregulating TRPC6 expression. Am J Physiol Cell Physiol 284:C316–C330
52. Balasubramaniam V, Le Cras TD, Ivy DD, Grover TR, Kinsella JP, Abman SH (2003) Role of platelet-derived growth factor in vascular remodeling during pulmonary hypertension in the ovine fetus. Am J Physiol Lung Cell Mol Physiol 284:L826–L833
53. Jankov RP, Kantores C, Belcastro R et al (2005) A role for platelet-derived growth factor beta-receptor in a newborn rat model of endothelin-mediated pulmonary vascular remodeling. Am J Physiol Lung Cell Mol Physiol 288:L1162–L1170
54. Perros F, Montani D, Dorfmuller P et al (2008) Platelet-derived growth factor expression and function in idiopathic pulmonary arterial hypertension. Am J Respir Crit Care Med 178:81–88
55. Ghofrani HA, Seeger W, Grimminger F (2005) Imatinib for the treatment of pulmonary arterial hypertension. N Engl J Med 353:1412–1413
56. Patterson KC, Weissmann A, Ahmadi T, Farber HW (2006) Imatinib mesylate in the treatment of refractory idiopathic pulmonary arterial hypertension. Ann Intern Med 145:152–153
57. Souza R, Sitbon O, Parent F, Simonneau G, Humbert M (2006) Long term imatinib treatment in pulmonary arterial hypertension. Thorax 61:736
58. Force T, Krause DS, Van Etten RA (2007) Molecular mechanisms of cardiotoxicity of tyrosine kinase inhibition. Nat Rev Cancer 7:332–344
59. Kerkelä R, Grazette L, Yacobi R et al (2006) Cardiotoxicity of the cancer therapeutic agent imatinib mesylate. Nat Med 12:908–916
60. Gorre ME, Mohammed M, Ellwood K et al (2001) Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. Science 293:876–880
61. Will Y, Dykens JA, Nadanaciva S et al (2008) Effect of the multitargeted tyrosine kinase inhibitors imatinib, dasatinib, sunitinib, and sorafenib on mitochondrial function in isolated rat heart mitochondria and H9c2 cells. Toxicol Sci 106:153–161
62. Druker BJ, Guilhot F, O'Brien SG et al (2006) Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. N Engl J Med 355:2408–2417

PPARγ and the Pathobiology of Pulmonary Arterial Hypertension

Marlene Rabinovitch

Abstract Peroxisome proliferator-activated receptor γ (PPARγ) is a nuclear receptor that functions as a transcription factor to regulate adipogenesis and metabolism by binding to PPAR response elements (PPAREs) in the promoter region of various target genes. Activation of PPARγ suppresses smooth muscle cell proliferation and migration. This chapter discusses the potential protective role of PPARγ and its downstream signaling cascades in the development of pulmonary arterial hypertension. Furthermore, the chapter also provides an overview on the cellular and molecular mechanisms involved in PPARγ-mediated inhibitory effect on pulmonary vascular remodeling, a major contributor to the elevated pulmonary vascular resistance in patients with pulmonary arterial hypertension.

Keywords Peroxisome proliferator-activated receptor γ (PPARγ) • nuclear receptor • pulmonary vascular remodeling • cell proliferation and migration

1 Introduction

Peroxisome proliferator-activated receptor γ (PPARγ) is one of a family of three nuclear receptors (PPARγ, -α, and -δ) that can function as transcription factors to regulate adipogenesis and glucose metabolism.[1–3] On activation by mechanisms not well understood, PPARs heterodimerize with the retinoid X receptor (RXR) and bind to PPAR response elements (PPREs) in regulatory promoter regions of their target genes.[4] Many of these are implicated in suppressing smooth muscle cell (SMC) proliferation and migration.[4] For example, PPARγ activation blocks platelet-derived growth factor (PDGF) gene expression[5] and induces the expression of

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lipoprotein-like receptor protein 1 (LRP1),[6] the receptor necessary for apolipoprotein (Apo) E-mediated suppression of PDGF-BB signaling[7,8] as discussed in this chapter. Moreover, PPARγ activation can induce apoptosis of SMCs by phosphorylation of the retinoblastoma (RB) gene[9] and by increasing the proapoptotic protein Gadd 45.[10] PPARs can also interact with signaling molecules to regulate gene expression independent of DNA binding. For example, PPARγ impairs phosphorylation (i.e., activation) of extracellular-regulated kinase (ERK),[11,12] a mitogen-activated protein kinase (MAPK), downstream of PDGF-BB/PDGFR-β signaling. Moreover, activated PPARγ stabilizes the cyclin-dependent kinase inhibitor p27KIP1[9] and inhibits telomerase activity,[13] retinoblastoma protein phosphorylation,[9] and cell cycle progression associated with vascular SMC proliferation.[9] By blocking important pathways downstream of activated PDGFR-β (i.e., phosphoinositol-3-kinase (PI3K))[14] PPARγ agonists can also cause apoptosis of proliferating vascular cells.[4,15] In addition, it is known that PPARγ ligands impair production of matrix metalloproteinases[16] that can be activated by elastase.[17] Our group has shown that inhibition of this proteolytic cascade not only prevents but also reverses advanced fatal pulmonary artery hypertension (PAH) in rats.[18]

In endothelial cells (ECs), PPARγ activation reduces levels of endothelin 1 (ET-1)[19] and the endogenous nitric oxide synthase inhibitor asymmetric dimethylarginine (ADMA),[20,21] factors that are implicated in both insulin resistance and in the pathobiology of PAH.[21] PPARγ has anti-inflammatory properties that include suppression of factors implicated in PAH, such as vascular cell adhesion molecule (VCAM), interleukin 6,[22,23] fractalkine,[24,25] and monocyte chemoattractant protein 1.[26] PPARγ also protects ECs against apoptosis[27,28] and may also promote EC proliferation and migration through production of endothelial nitric oxide synthase (eNOS)[29] as well as hemoxygenase (HO) 1.[30] In contrast, HO-1 represses SMC proliferation,[30] consistent with PPARγ-mediated repression of proliferation and migration of arterial SMCs in culture[31,32] and in animal models.[33]

Thus, extrapolating from data in systemic vascular disease[2] suggests that impaired activation of PPARγ transcriptional targets could lead to the pathology of PAH. This is reinforced by studies showing that the levels of PPARγ and its putative transcriptional target ApoE are reduced on complementary DNA (cDNA) microarrays from lung tissues and vessels in patients with PAH.[34,35] There is supporting evidence that links PPARγ with transcription of ApoE. A functional PPRE is present in the ApoE promoter,[36] conditional disruption of the PPARγ gene in mice results in decreased ApoE expression in macrophages,[37] and PPARγ activation leads to ApoE messenger RNA (mRNA) expression and protein secretion in an adipocyte cell line.[38]

2 Insulin Resistance, Pulmonary Hypertension, and PPARγ Agonist Treatment

Mice that are null for ApoE are made insulin resistant by being fed a high-fat diet. We showed that these mice have PAH in association with abnormal muscularization of distal arteries, and that the muscularization could be reversed following treat-

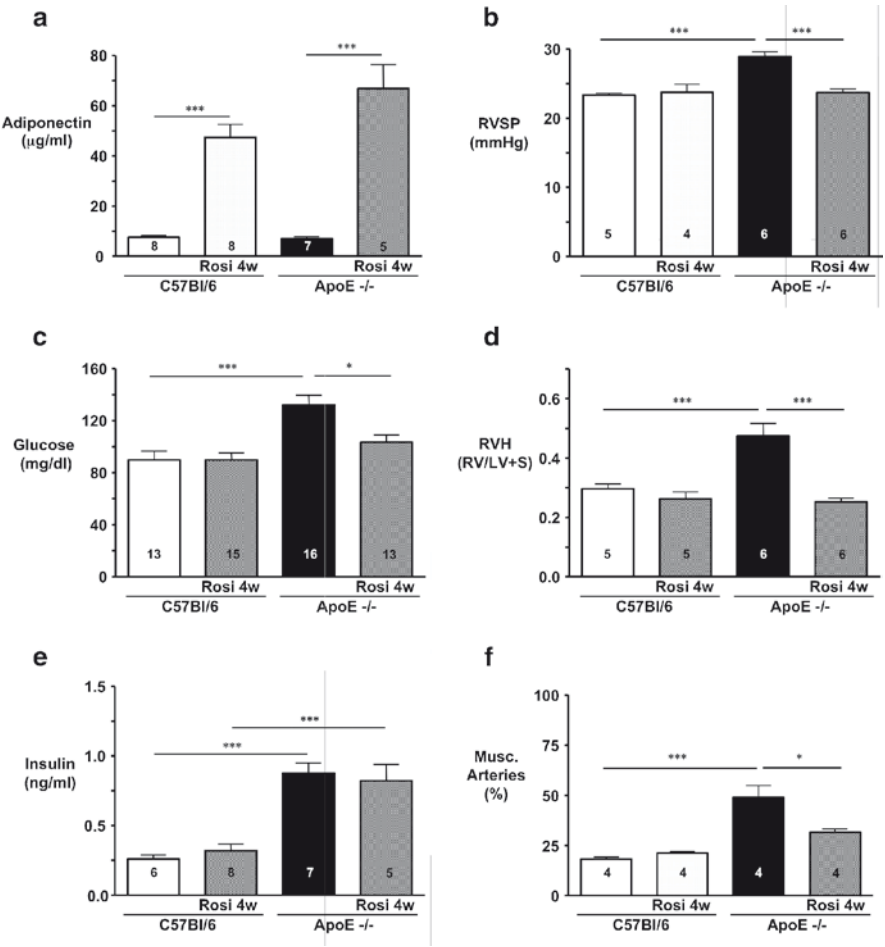

Fig. 29.1 Four-week treatment with the PPARγ agonist rosiglitazone reverses PAH, increases plasma adiponectin, and induces insulin sensitivity. Measurements of plasma adiponectin (**a**), right ventricular systolic pressure (RVSP) (**b**), blood glucose (**c**), right ventricular hypertrophy (RVH) (**d**), plasma insulin (**e**), and muscularization of alveolar wall arteries (**f**). Nineteen-week-old male C57Bl/6 and ApoE$^{-/-}$ mice, all on high fat (HF) diet for 15 weeks, were used. Bars represent mean ± standard error (SEM) (n = 4–16 as indicated in column graphs). *$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$. Reproduced with permission[39]

ment with rosiglitazone, a PPARγ agonist[39] (Fig. 29.1). It was interesting that the female cohort of ApoE$^{-/-}$ mice had less-severe PAH as judged by lower levels of right ventricular systolic pressure, right ventricular hypertrophy, and muscularized distal arteries. We attributed this phenotype to the fact that the females had higher levels of adiponectin. The mechanism appears to be related to the fact that testosterone inhibits secretion of adiponectin,[40] and adiponectin can sequester PDGF.[41] We subsequently showed that adiponectin, a target of PPARγ-mediated transcriptional activity in adipocytes can repress pulmonary artery SMC (PASMC) proliferation in response to growth factors as does ApoE (Fig. 29.2).

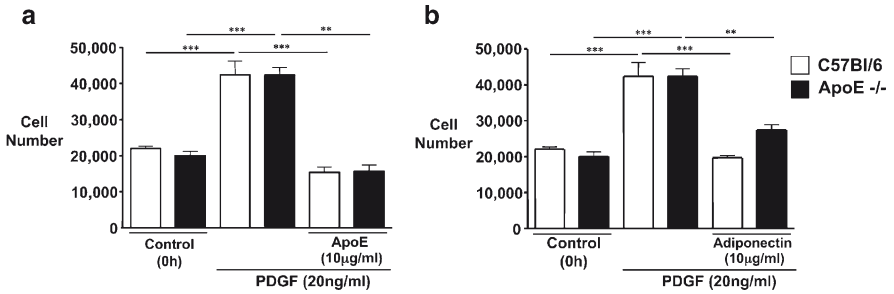

Fig. 29.2 Recombinant ApoE (**a**) and adiponectin (**b**) inhibit PDGF-BB-induced (20 ng/ml) proliferation of murine PASMCs harvested from both C57Bl/6 and ApoE$^{-/-}$ mice. Bars represent mean ± SEM ($n = 3$). *$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$. Reproduced with permission[39]

In unpublished data from our group, we have found that older mice that are null for ApoE also develop PAH in the absence of a high-fat diet or insulin resistance. We speculate that this is related to the loss of the protective effect of ApoE in suppressing episodic PDGF-BB-mediated SMC proliferation over time. It has been shown that ApoE can bind to low-density LRP1, also a target of PPARγ-mediated gene transcription.[6] When this occurs, LRP1 targets the PDGFRβ for endocytosis,[42–44] repressing its function as an SMC mitogen. However, in contrast to its adverse effects as a smooth muscle mitogen, PDGF is also important in normal cell viability, in maintaining pericytes, and in the prevention of vascular endothelial growth factor (VEGF) overexpression and aberrant angiogenesis.[45] So, it could be proposed that PPARγ agonists may "fine-tune," allowing PDGF-mediated EC survival and pericyte recruitment while repressing aberrant angiogenesis and abnormal muscularization of distal vessels as well as proliferation of SMCs.

In light of these experimental studies and in keeping with clinical observations related to obesity in the population of patients with PAH, we investigated whether insulin resistance was prevalent in patients with PAH. Studies reported by Zamanian et al.[46] indicated a significantly higher proportion of female patients with PAH and insulin resistance when compared with the general population (45.7 vs. 21.5%), as judged by an abnormal elevation in the ratio of triglycerides to high-density lipoproteins.[46] It is of further interest that this high evidence of insulin resistance did not correlate with obesity as judged by the body mass index (BMI) or relate to the hemodynamic severity of the disease but was associated with a poorer 6-month event-free survival (58 vs. 79%) (Fig. 29.3).

3 PPARγ and the Bone Morphogenetic Protein Pathway

Mutations in bone morphogenetic protein receptor (BMPR) II that cause loss of function of the receptor are associated with familial and sporadic idiopathic PAH[47–49] and reduced expression of BMPR-II has been related to PAH regardless of etiology.[50] It was therefore of interest that in studies investigating transcription factors that are

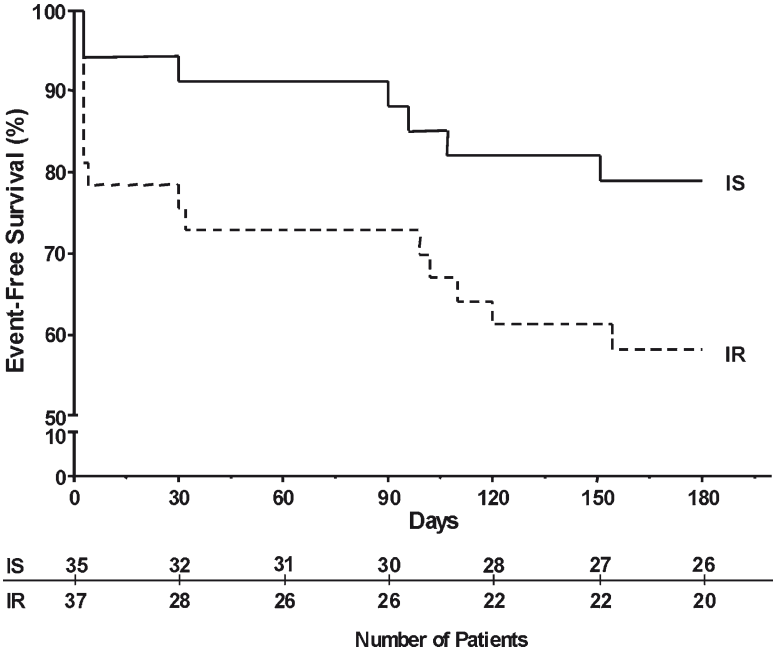

Fig. 29.3 Kaplan-Meier 6-month event-free survival curve in pulmonary arterial hypertension (PAH) females. Insulin-sensitive (*solid line*) PAH females had significantly better outcome compared with their insulin-resistant (*dashed line*) counterparts (79% vs. 58%; $P < 0.05$). Events were defined as death, transplantation, or acute hospitalization due to PAH exacerbation or right heart failure. Reproduced with permission[46]

regulated by signaling via BMPR-II, we identified PPARγ as a target.[51] We showed enhanced DNA binding of PPARγ following stimulation of PASMCs with bone morphogenetic protein (BMP) 2. We then showed that the ability of BMP2 to repress PDGF-BB-mediated PASMC proliferation depended on PPARγ (Fig. 29.4). Most interesting was the observation that loss of the function of BMPR-II to repress PDGF-BB-mediated proliferation could be rescued by a PPARγ agonist (Fig. 29.4). We then showed that BMP2-mediated inhibition of PDGF-BB-induced SMC proliferation requires not only activation of PPARγ but also that of its target of transcription, ApoE. We showed that both PPARγ and ApoE act downstream of BMP2/BMPR-II in human and murine PASMCs and prevent their proliferation in response to PDGF-BB. Bone morphogenetic protein-2 (BMP2)-mediated PPARγ activation occurs earlier than Smad1/5/8 phosphorylation and therefore appears to be independent of this established signaling axis downstream of BMPR-II. The BMPR-II ligand BMP2 induces a decrease in nuclear phospho-ERK, and rapid nuclear shuttling and DNA binding of PPARγ, whereas PDGF-BB has the opposite effects. Both BMP2 and the PPARγ agonist rosiglitazone stimulate production and secretion of ApoE in PASMCs (Fig. 29.5). Moreover, BMP2-mediated suppression of PDGF-BB-induced proliferation was absent in SMCs from a patient with a BMPR-II mutation and PAH, but this inhibition could be restored with a rosiglitazone (Fig. 29.6).

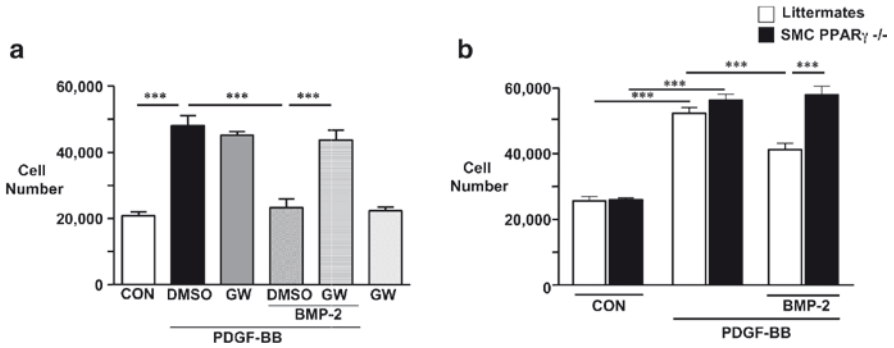

Fig. 29.4 (**a**) PASMCs were seeded at 2.5×10^4 cells per well of a 24-well plate in 500 μl of growth medium and allowed to adhere overnight. The cells were washed with phosphate-buffered saline (PBS) prior to the addition of starvation media (0.1% fetal bovine serum [FBS]) and incubated for 24 h (murine pulmonary artery smooth muscle cells (PASMCs)) or 48 h human PASMC (HPASMCs) and then stimulated with PDGF-BB (20 ng/ml) for 72 h. BMP-2 (10 ng/ml) was added to quiescent cells 30 min prior to PDGF-BB stimulation. The PPARγ antagonist GW9662 (GW; 1 μ*M*) was added 24 h prior to the addition of BMP2. Cells were finally washed twice with PBS, trypsinized, and counted in a hemocytometer (4 counts per well). Cell numbers in controls at time points 0 (CON) and 72 h were not significantly different. (**b**) Littermates, littermate control PASMCs; SMC PPAR$\gamma^{-/-}$, PASMCs isolated from *SM22α Cre PPARγflox/flox* mice. Bars represent mean ± SEM, $n = 4$ in (**a**) and 3 in (**b**). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ as indicated; analysis of variance (ANOVA) with Bonferroni's multiple-comparison test. Reproduced with permission[51]

Fig. 29.5 (continued) (**a**) BMP2 inhibits SMC proliferation via PPARγ and ApoE. ApoE impairs PDGF-BB/MAPK signaling by binding to low-density lipoprotein (LDL) receptor-related protein (LRP), thereby initiating endocytosis and degradation of the LRP/PDGFR-β/PDGF-B complex. PPARγ induces LRP and other growth-inhibitory/proapoptotic genes in SMCs and inhibits cell cycle and other growth-promoting genes, such as telomerase, cyclin D1, and retinoblastoma protein. Moreover, PPARγ induces phosphatases that can directly inactivate phospho-ERK. (**b**) BMPR-II dysfunction promotes SMC proliferation and survival in PAH. Heightened PDGF-BB signaling leading to SMC proliferation is a key clinical feature of PAH. Deficiency of both ApoE and LRP enhances mitogenic PDGF-BB/MAPK signaling. Loss-of-function mutations in the BMPR-II gene will decrease endogenous PPARγ activity, leading to unopposed MAPK signaling, SMC proliferation and survival, and ultimately development of PAH. *TF* transcription factor. (**c**) PPARγ agonists can rescue BMPR-II dysfunction and reverse PAH. PPARγ agonists such as rosiglitazone or pioglitazone might reverse SMC proliferation and vascular remodeling in PAH patients with or without BMPR-II dysfunction via induction of ApoE and other growth-inhibitory/proapoptotic genes (as indicated) and through repression of growth-promoting genes (not shown). Reproduced with permission[51]

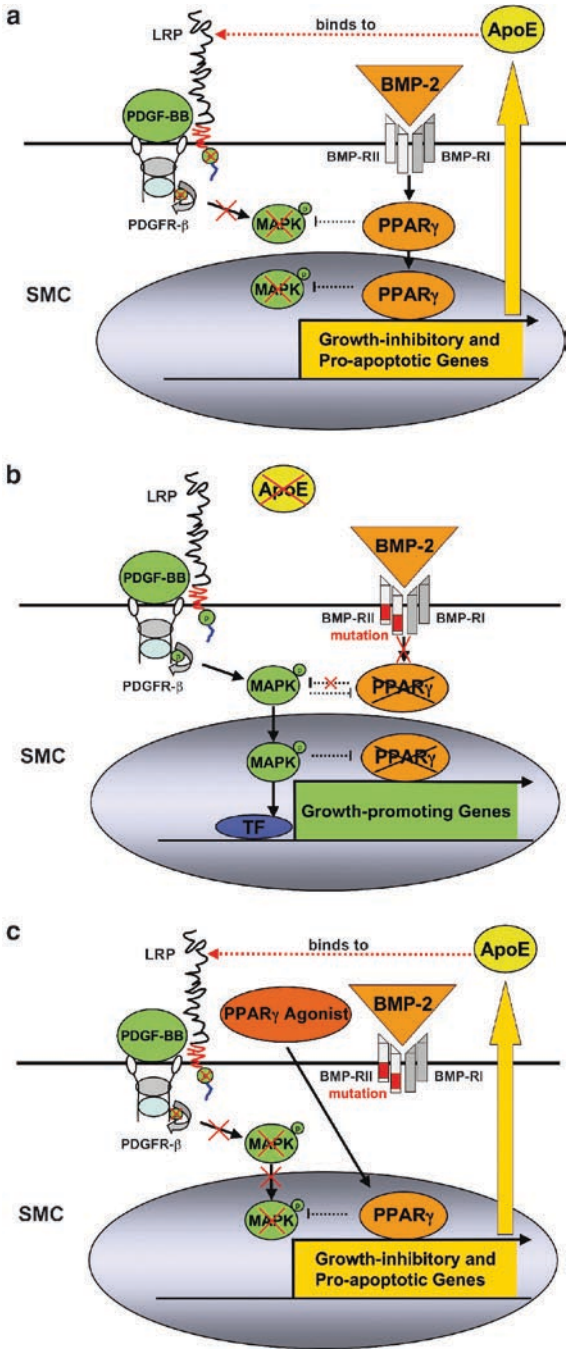

Fig. 29.5 Model: A novel antiproliferative BMP2/PPARγ/ApoE axis protects against PAH. This schema incorporates the findings described in our chapter and the literature to date as discussed.

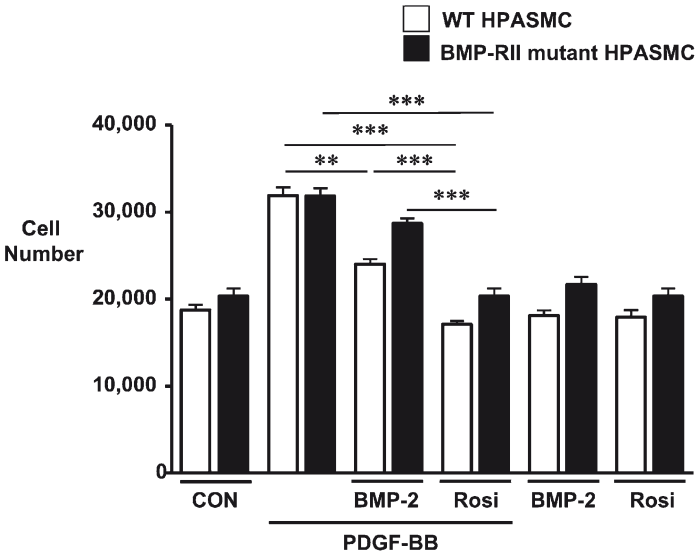

Fig. 29.6 Antiproliferative effects of BMP2 and the PPARγ agonist rosiglitazone on PDGF-BB-induced proliferation of human wild-type and BMPR-II mutant PASMCs. Control PASMCs were isolated from surgical resection specimens derived from patients undergoing lobectomy or pneumonectomy for suspected lung tumor. Additional peripheral pulmonary arteries (<1- to 2-mm external diameter) were obtained from a patient undergoing heart-lung transplantation for familial PAH (FPAH) and known to harbor a mutation (W9X) in BMPR-II. The nature of the BMPR-II mutation, cell isolation, culture techniques, and cell counts is the same as shown in Fig. 29.1. HPASMCs were incubated for 48 h in starvation media (0.1% FBS) and then stimulated with PDGF-BB (20 ng/ml) for 72 h. BMP2 (10 ng/ml) or rosiglitazone (1 μ*M*) were added to quiescent cells 30 min prior to PDGF-BB stimulation. Bars represent mean ± SEM ($n = 3$). $^{**}P < 0.01$; $^{***}P < 0.001$ as indicated; ANOVA with Bonferroni's multiple-comparison test. The number of PDGF-BB-stimulated cells was significantly higher than that of untreated control cells ($P < 0.001$). Reproduced with permission[51]

To determine whether, in addition to ApoE, other targets of PPARγ-mediated transcription in PASMCs were essential to inhibit the development of PAH, we made a mouse in which SM22-driven Cre was used to delete critical exons of a floxed PPARγ. This mouse had spontaneous PAH in the absence of a high-fat diet in association with muscularized distal arteries and right ventricular hypertrophy. Moreover, the pulmonary hypertensive response to chronic hypoxia is exaggerated in this mouse (Fig. 29.7).

We have bred the Tie2-expressing Cre mouse with the mouse in which PPAR is floxed. Our unpublished studies revealed that this mouse has a mild form of pulmonary hypertension under room air conditions but fails to show a heightened response to hypoxia. The mechanism does not appear to be related to ApoE but is associated with heightened signaling through PDGFRβ. While chronic hypoxia does not result in an exaggeration of PAH in these mice with EC deletion of PPARγ, reversal of PAH following exposure to chronic hypoxia is impaired.

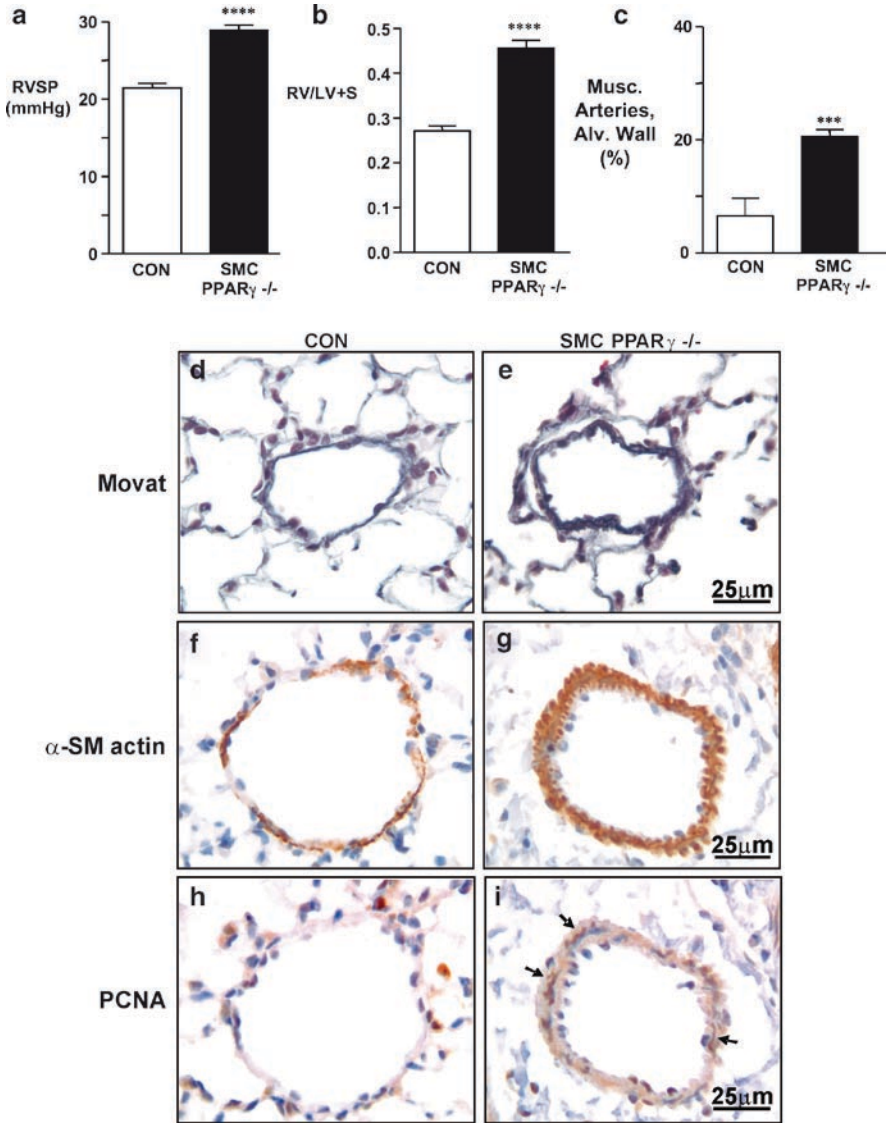

Fig. 29.7 PAH in mice with targeted deletion of PPARγ in SMCs. Thirteen- to 15-week-old mice underwent right ventricular (RV) catheterization, followed by organ harvest. (**a**) RVSP measurements. (**b**) Right ventricular hypertrophy (RVH), measured as ratio of the weight of the RV to that of the left ventricle (LV) plus septum (RV/LV + S). (**c**) Muscularization of alveolar wall arteries (Musc. Arteries Alv. Wall). (**d**) Representative photomicrographs of lung tissue (stained by Movat pentachrome) of 15-week-old mice showing a typical nonmuscular peripheral alveolar artery in a littermate control mouse. (**e**) A similar section in the *SM22α Cre PPARγflox/flox* (SMC PPARγ$^{-/-}$) mouse shows an alveolar wall artery surrounded by a rim of muscle. (**f–i**) Immunohistochemistry in serial lung tissue sections from littermate control (CON) and SMC PPARγ$^{-/-}$ mice stained for smooth muscle α-actin (α-SMA) (**f**, **g**) and proliferating cell nuclear antigen (PCNA; **h** and **i**). *Arrows* in (**i**) indicate enhanced PCNA staining in PASMCs. Bars represent mean ± SEM ($n = 5$). ***$P < 0.001$ vs. control; unpaired two-tailed t test. Reproduced with permission[51]

Studies have been carried out in which PPARγ agonists have been used to inhibit and reverse hypoxia-induced PAH. If is of interest that PPARγ agonists can completely reverse structural changes in response to chronic hypoxia but not the elevation in pulmonary arterial pressure.[52]

PPARγ agonists appear to act favorably to facilitate suppression of proliferation in PASMCs, but it will be important to determine whether they also support endothelial growth and stability because our studies in cultured cells suggest that certain agents could impede PPARγ-mediated endothelial gene regulation.

References

1. He W, Barak Y, Hevener A et al (2003) Adipose-specific peroxisome proliferator-activated receptor γ knockout causes insulin resistance in fat and liver but not in muscle. Proc Natl Acad Sci U S A 100:15712–15717
2. Hevener AL, He W, Barak Y et al (2003) Muscle-specific PPARγ deletion causes insulin resistance. Nat Med 9:1491–1497
3. Lehrke M, Lazar MA (2005) The many faces of PPARγ. Cell 123:993–999
4. Marx N, Duez H, Fruchart JC, Staels B (2004) Peroxisome proliferator-activated receptors and atherogenesis: regulators of gene expression in vascular cells. Circ Res 94:1168–1178
5. Zhang J, Fu M, Zhao L, Chen YE 2002) 15-Deoxy-prostaglandin J_2 inhibits PDGF-A and -B chain expression in human vascular endothelial cells independent of PPARγ. Biochem Biophys Res Commun 298:128–132
6. Gauthier A, Vassiliou G, Benoist F, McPherson R (2003) Adipocyte low density lipoprotein receptor-related protein gene expression and function is regulated by peroxisome proliferator-activated receptor γ. J Biol Chem 278:11945–11953
7. Boucher P, Gotthardt M, Li WP, Anderson RG, Herz J (2003) LRP: role in vascular wall integrity and protection from atherosclerosis. Science 300:329–332
8. Newton CS, Loukinova E, Mikhailenko I et al (2005) Platelet-derived growth factor receptor-β (PDGFR-β) activation promotes its association with the low density lipoprotein receptor-related protein (LRP). Evidence for co-receptor function. J Biol Chem 280: 27872–27878
9. Wakino S, Kintscher U, Kim S, Yin F, Hsueh WA, Law RE (2000) Peroxisome proliferator-activated receptor γ ligands inhibit retinoblastoma phosphorylation and $G_1 \rightarrow S$ transition in vascular smooth muscle cells. J Biol Chem 275:22435–22441
10. Bruemmer D, Blaschke F, Law RE (2005) New targets for PPARγ in the vessel wall: implications for restenosis. Int J Obes Relat Metab Disord 29:S26–S30
11. Benkirane K, Amiri F, Diep QN, El Mabrouk M, Schiffrin EL (2006) PPAR-γ inhibits ANG II-induced cell growth via SHIP2 and 4E-BP1. Am J Physiol Heart Circ Physiol 290:H390–H397
12. Wakino S, Kintscher U, Liu Z et al (2001) Peroxisome proliferator-activated receptor γ ligands inhibit mitogenic induction of $p21^{Cip1}$ by modulating the protein kinase Cδ pathway in vascular smooth muscle cells. J Biol Chem 276:47650–47657
13. Ogawa D, Nomiyama T, Nakamachi T et al (2006) Activation of peroxisome proliferator-activated receptor γ suppresses telomerase activity in vascular smooth muscle cells. Circ Res 98:e50–e9
14. Vantler M, Caglayan E, Zimmermann WH, Baumer AT, Rosenkranz S (2005) Systematic evaluation of anti-apoptotic growth factor signaling in vascular smooth muscle cells. Only phosphatidylinositol 3′-kinase is important. J Biol Chem 280:14168–14176
15. Bruemmer D, Yin F, Liu J et al (2003) Regulation of the growth arrest and DNA damage-inducible gene 45 (GADD45) by peroxisome proliferator-activated receptor γ in vascular smooth muscle cells. Circ Res 93:e38–e47

16. Worley JR, Baugh MD, Hughes DA et al (2003) Metalloproteinase expression in PMA-stimulated THP-1 cells. Effects of peroxisome proliferator-activated receptor-γ (PPARγ) agonists and 9-cis-retinoic acid. J Biol Chem 278:51340–51346
17. Nagase H, Enghild J, Suzuki K, Salvesen G (1990) Stepwise activation mechanisms of the precursor of matrix metalloproteinase 3 (stromelysin) by proteinases and (4-aminophenyl) mercuric acetate. Biochemistry 29:5783–5789
18. Cowan KN, Heilbut A, Humpl T, Lam C, Ito S, Rabinovitch M (2000) Complete reversal of fatal pulmonary hypertension in rats by a serine elastase inhibitor. Nat Med 6:698–702
19. Martin-Nizard F, Furman C, Delerive P et al (2002) Peroxisome proliferator-activated receptor activators inhibit oxidized low-density lipoprotein-induced endothelin-1 secretion in endothelial cells. J Cardiovasc Pharmacol 40:822–831
20. Wakino S, Hayashi K, Tatematsu S et al (2005) Pioglitazone lowers systemic asymmetric dimethylarginine by inducing dimethylarginine dimethylaminohydrolase in rats. Hypertens Res 28:255–262
21. Kielstein JT, Bode-Boger SM, Hesse G et al (2005) Asymmetrical dimethylarginine in idiopathic pulmonary arterial hypertension. Arterioscler Thromb Vasc Biol 25:1414–1418
22. Combs CK, Johnson DE, Karlo JC, Cannady SB, Landreth GE (2000) Inflammatory mechanisms in Alzheimer's disease: inhibition of β-amyloid-stimulated proinflammatory responses and neurotoxicity by PPARγ agonists. J Neurosci 20:558–567
23. Humbert M, Monti G, Brenot F et al (1995) Increased interleukin-1 and interleukin-6 serum concentrations in severe primary pulmonary hypertension. Am J Respir Crit Care Med 151:1628–1631
24. Imaizumi T, Matsumiya T, Tamo W et al (2002) 15-Deoxy-D12,14-prostaglandin J_2 inhibits CX3CL1/fractalkine expression in human endothelial cells. Immunol Cell Biol 80:531–536
25. Balabanian K, Foussat A, Dorfmüller P et al (2002) CX_3C chemokine fractalkine in pulmonary arterial hypertension. Am J Respir Crit Care Med 165:1419–1425
26. Ikeda Y, Yonemitsu Y, Kataoka C et al (2002) Anti-monocyte chemoattractant protein-1 gene therapy attenuates pulmonary hypertension in rats. Am J Physiol Heart Circ Physiol 283:H2021–H2028
27. Gensch C, Clever YP, Werner C, Hanhoun M, Böhm M, Laufs U (2007) The PPAR-γ agonist pioglitazone increases neoangiogenesis and prevents apoptosis of endothelial progenitor cells. Atherosclerosis 192:67–74
28. Levonen AL, Dickinson DA, Moellering DR, Mulcahy RT, Forman HJ, Darley-Usmar VM (2001) Biphasic effects of 15-deoxy-$\delta^{12,14}$-prostaglandin J_2 on glutathione induction and apoptosis in human endothelial cells. Arterioscler Thromb Vasc Biol 21:1846–1851
29. Cho DH, Choi YJ, Jo SA, Jo I (2004) Nitric oxide production and regulation of endothelial nitric-oxide synthase phosphorylation by prolonged treatment with troglitazone: evidence for involvement of peroxisome proliferator-activated receptor (PPAR) γ-dependent and PPAR γ-independent signaling pathways. J Biol Chem 279:2499–2506
30. Kronke G, Kadl A, Ikonomu E et al (2007) Expression of heme oxygenase-1 in human vascular cells is regulated by peroxisome proliferator-activated receptors. Arterioscler Thromb Vasc Biol 27:1276–1282
31. Goetze S, Xi XP, Kawano H et al (1999) PPARγ-ligands inhibit migration mediated by multiple chemoattractants in vascular smooth muscle cells. J Cardiovasc Pharmacol 33:798–806
32. Benson S, Wu J, Padmanabhan S, Kurtz TW, Pershadsingh HA (2000) Peroxisome proliferator-activated receptor (PPAR)-γ expression in human vascular smooth muscle cells: inhibition of growth, migration, and c-fos expression by the peroxisome proliferator-activated receptor (PPAR)-γ activator troglitazone. Am J Hypertens 13:74–82
33. Law RE, Goetze S, Xi XP et al (2000) Expression and function of PPARγ in rat and human vascular smooth muscle cells. Circulation 101:1311–1318
34. Ameshima S, Golpon H, Cool CD et al (2003) Peroxisome proliferator-activated receptor γ (PPARγ) expression is decreased in pulmonary hypertension and affects endothelial cell growth. Circ Res 92:1162–1169
35. Geraci MW, Moore M, Gesell T et al (2001) Gene expression patterns in the lungs of patients with primary pulmonary hypertension: a gene microarray analysis. Circ Res 88:555–562

36. Galetto R, Albajar M, Polanco JI, Zakin MM, Rodriguez-Rey JC (2001) Identification of a peroxisome-proliferator-activated-receptor response element in the apolipoprotein E gene control region. Biochem J 357:521–527
37. Akiyama TE, Sakai S, Lambert G et al (2002) Conditional disruption of the peroxisome proliferator-activated receptor γ gene in mice results in lowered expression of ABCA1, ABCG1, and ApoE in macrophages and reduced cholesterol efflux. Mol Cell Biol 22:2607–2619
38. Yue L, Rasouli N, Ranganathan G, Kern PA, Mazzone T (2004) Divergent effects of peroxisome proliferator-activated receptor γ agonists and tumor necrosis factor α on adipocyte ApoE expression. J Biol Chem 279:47626–47632
39. Hansmann G, Wagner RA, Schellong S et al (2007) Pulmonary arterial hypertension is linked to insulin resistance and reversed by peroxisome proliferator-activated receptor-γ activation. Circulation 115:1275–1284
40. Xu A, Chan KW, Hoo RL et al (2005) Testosterone selectively reduces the high molecular weight form of adiponectin by inhibiting its secretion from adipocytes. J Biol Chem 280:18073–18080
41. Wang Y, Lam KS, Xu JY et al (2005) Adiponectin inhibits cell proliferation by interacting with several growth factors in an oligomerization-dependent manner. J Biol Chem 280:18341–18347
42. Swertfeger DK, Bu G, Hui DY (2002) Low density lipoprotein receptor-related protein mediates apolipoprotein E inhibition of smooth muscle cell migration. J Biol Chem 277:4141–4146
43. Boucher P, Gotthardt M (2004) LRP and PDGF signaling: a pathway to atherosclerosis. Trends Cardiovasc Med 14:55–60
44. Boucher P, Liu P, Gotthardt M, Hiesberger T, Anderson RG, Herz J (2002) Platelet-derived growth factor mediates tyrosine phosphorylation of the cytoplasmic domain of the low density lipoprotein receptor-related protein in caveolae. J Biol Chem 277:15507–15513
45. Wilkinson-Berka JL, Babic S, De Gooyer T et al (2004) Inhibition of platelet-derived growth factor promotes pericyte loss and angiogenesis in ischemic retinopathy. Am J Pathol 164:1263–1273
46. Zamanian RT, Hansmann G, Snook S et al (2009) Insulin resistance in pulmonary arterial hypertension. Eur Respir J 33:318–324
47. Lane KB, Machado RD, Pauciulo MW et al (2000) Heterozygous germline mutations in BMPR2, encoding a TGF-β receptor, cause familial primary pulmonary hypertension. Nat Genet 26:81–84
48. Deng Z, Morse JH, Slager SL et al (2000) Familial primary pulmonary hypertension (gene PPH1) is caused by mutations in the bone morphogenetic protein receptor-II gene. Am J Hum Genet 67:737–744
49. Machado RD, Aldred MA, James V et al (2006) Mutations of the TGF-β type II receptor BMPR2 in pulmonary arterial hypertension. Hum Mutat 27:121–132
50. Atkinson C, Stewart S, Upton PD et al (2002) Primary pulmonary hypertension is associated with reduced pulmonary vascular expression of type II bone morphogenetic protein receptor. Circulation 105:1672–1678
51. Hansmann G, de Jesus Perez VA, Alastalo TP et al (2008) An antiproliferative BMP-2/PPARγ/ApoE axis in human and murine SMCs and its role in pulmonary hypertension. J Clin Invest 118:1846–1857
52. Crossno JT Jr, Garat CV, Reusch JE et al (2007) Rosiglitazone attenuates hypoxia-induced pulmonary arterial remodeling. Am J Physiol Lung Cell Mol Physiol 292:L885–L897

Targeting TASK-1 Channels as a Therapeutic Approach

Andrea Olschewski

Abstract The voltage-independent background two-pore domain K^+ channel TASK-1 sets the resting membrane potential in excitable cells and renders these cells sensitive to a variety of vasoactive factors. There is clear evidence for TASK-1 in human pulmonary artery smooth muscle cells and TASK-1 channels are likely to regulate the pulmonary vascular tone through their regulation by hypoxia, pH, inhaled anesthetics, and G protein-coupled pathways. Furthermore, TASK-1 is a strong candidate to play a role in hypoxic pulmonary vasoconstriction. On the other hand, consistent with the activation of TASK-1 channels by volatile anesthetics, TASK-1 contributes to the anesthetic-induced pulmonary vasodilation. TASK-1 channels are unique among K^+ channels because they are regulated by both, increases and decreases from physiological pH, thus contributing to their protective effect on the pulmonary arteries. Moreover, TASK-1 may also have a critical role in mediating the vasoactive response of G protein-coupled pathways in resistance arteries which can offer promising therapeutic solutions to target diseases of the pulmonary circulation.

Keywords TASK-1 • pulmonary artery • smooth muscle • hypoxic pulmonary vasoconstriction • inhaled anesthetics • endothelin-1 • prostacyclin

1 The TASK Background Two-Pore-Domain Potassium Channels

Background or leak K^+-selective channels, as defined by a lack of time and voltage dependency, play an essential role in setting the resting membrane potential and input resistance in excitable cells. Alteration of K^+ conductance in these cells

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influences cellular activity via membrane potential changes. Two-pore-domain potassium (K_{2P}) channels have been shown to conduct several leak K^+ currents. This new gene family of K^+ channels has been progressively identified over the last few years (reviewed in Ref. [1]). Members of the KCNK family present structural features that suggest a dimeric arrangement, with each subunit comprising four transmembrane segments and two pore-forming regions (Fig. 30.1). The channels formed by members of this family are distinct from voltage-dependent

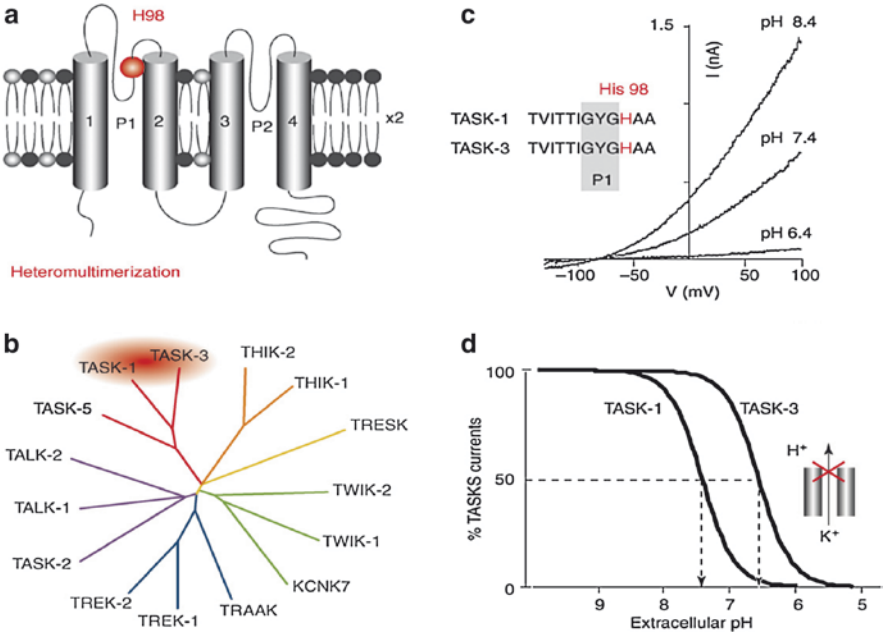

Fig. 30.1 The TASK K_{2P} channels. (**a**) The subunits of dimeric K^+ channels each contain four transmembrane segments (4-TMs) and two conserved motifs called the P (pore-forming) domain that forms part of the wall of the channel through which K^+ ions are conducted; these are known as K_{2P} channels. His98 in the P1 domain is involved in sensing pH. (**b**) Fifteen human K_{2P} channel subunits have been identified and are classified into six different structural and functional subgroups. The TASK-1 and TASK-3 subunits share a background K^+ channel activity that is inhibited at extracellular acidic pH. Heteromultimerization, which contributes to the increase in the functional diversity of these channels, has been demonstrated for TASK-1 and TASK-3. TASK-5 is structurally related to TASK-1 and TASK-3 but is electrically silent. (**c**) Effect of extracellular pH on TASK-1 recorded in a transfected COS cell. TASK channel activity monitored between -120 and 100 mV using a voltage ramp is increased by alkaline extracellular pH (pH 8.4) but inhibited by extracellular acidosis (pH 6.4). This *curve* represents the whole-cell current amplitude (in nA) as a function of the membrane voltage (in mV). The pH sensitivity of the TASK-1 and TASK-3 channels is dependent on the protonation of His98 in the first pore domain (P1) as shown in (**a**). (**d**) TASK-1 and TASK-3 show different pH sensitivities with a pK of about 7.3 and 6.7 for TASK-1 and TASK-3, respectively. Reproduced with permission[61]

or inwardly rectifying K^+ channels inasmuch as most are constitutively active at resting membrane potentials, and they generate currents that rectify only very weakly. They can be grouped into six subfamilies based on their structural and functional properties in mammals, the TWIK (two-pore domain weakly inward rectifying K^+; TWIK-1, TWIK-2, KCNK-7); TASK (TWIK-related, acid-sensitive K^+; TASK-1, TASK-3, TASK-5); TREK (TWIK-related K^+; TREK-1, TREK-2, TWICK-related arachidonic acid-activated K^+ channel (TRAAK)); TALK (TWIK-related alkaline activated K^+; TALK-1, TALK-2, TASK-2); THIK (tandem pore domain halothane-inhibited K^+; THIK-1, THIK-2); and TRESK (TWIK-related spinal cord K^+) subfamilies.[2,3]

TASK-1 and TASK-3, along with the silent subunit TASK-5, form a distinct structural and functional subgroup. The TASK-1 amino acid sequence shares 54% identity with TASK-3, and heteromultimerization between both subunits has been demonstrated.[4,5] TASK-1 is a two-pore-domain K^+ channel that generates a pH-sensitive, weakly rectifying K^+ current[6–8] and is called an "open rectifier" because it exhibits no time dependence or extremely fast kinetics.[9] The current-to-voltage relationship of TASK channels becomes linear in a symmetrical K^+ gradient (reversing at 0 mV). This typical channel behavior is expected of a K^+-selective leak channel, as predicted by the Goldman Hodgkin Katz formulation (open rectifier). The homomultimers TASK-1 and TASK-3 and the heteromultimer TASK-1-TASK-3 have single-channel conductances (determined in a symmetrical K^+ gradient) of 14, 37, and 38 pS, respectively, and are characterized by a typical flickery gating.[5,10]

K_{2P} channels are found in neuronal and nonneuronal tissues and provide a wide variety of important functions, including the sensing of oxygen and pH,[11] the setting of resting membrane potential,[8,12] the sensing of changes in $[K^+]$, the responses to agonists,[13] neuroprotection,[14] and mechanosensitivity.[13,15,16] These channels are also candidates for the action of volatile anesthetics on neural excitability.[17] Selective pharmacology for the K_{2P} channels is lacking, and most detailed information about the function of these channels comes from studies on cloned channels expressed heterologously in model cells.

Several studies have inferred that K_{2P} channels may be involved in important cerebellar functions, such as spatial determination and accuracy, motor coordination, balance, muscle tone, and learning of motor skills. In other regions of the brain, K_{2P} channels, such as TASK-1 and TASK-3, are expressed and may function in behavior such as sleep and wakefulness, contribute to background conductances, and provide acid and volatile anesthetic sensitivity.[18] In contrast, there have been limited reports of K_{2P} channels in cardiac or smooth muscle tissues. Cardiac myocytes display a background K^+ current, due to TASK-1,[8] that influences the amplitude and duration of action potentials.[19] Smooth muscle cells in the carotid body express TASK-3. Another group has reported expression of TASK and TREK isoforms in human myometrium with reverse-transcription polymerase chain reaction (RT-PCR), and Western analysis showed expression of TASK-1 and TREK-1.[20] Subsequently, Sanders and Koh found expression and function of TASK and TREK channels in gastrointestinal smooth

muscle cells.[21] Their findings support the role of TASK-1 in regulating responses of gastrointestinal smooth muscles. In addition, TASK-1 channels appear to participate in establishing the moment-to-moment state of excitability in gastrointestinal muscles. Finally, there is evidence that TASK-1 maintains the resting potential in bladder smooth muscle.[22] The relevance of TASK-1 in vascular smooth muscle cells with special focus on pulmonary arteries is described in the next section.

2 TASK-1 Channels in Pulmonary Artery Smooth Muscle Cells

Macroscopic currents that occur due to TASK-1 channels display kinetic and voltage-dependent properties very similar to TASK-3 (i.e., TASK-3 is also an open rectifier),[23,24] but the two channels can be distinguished by the pH range over which their activity is modulated (Fig. 30.1). The p*K* of TASK-1 channels is 7.4, squarely in the physiological range, whereas the p*K* for TASK-3 currents is shifted to a more acidic level (6.7).[6–8,24] The TASK-2 channel sequence shares less homology with the other TASK channels, and it generates outwardly rectifying pH-sensitive currents with a p*K* of 8.4.[25]

Gurney and colleagues showed the TASK-1 expression in smooth muscle cells of the pulmonary artery in a rabbit.[26] The authors also demonstrated functional expression of a conductance in these cells that is inhibited by extracellular pH and Zn^{2+} and by the endocannabinoid anandamide, but the conductance was insensitive to intracellular Ca^{2+}, 4-aminopyridine, and quinine. In addition, the noninactivating K^+ current was facilitated by halothane, causing hyperpolarization of pulmonary artery smooth muscle cells (PASMCs). These are properties that closely mimic the pharmacology of heterologously expressed TASK-1 channels. To investigate the expression of TASK channels in human pulmonary arteries, we developed the preparation of primary human pulmonary artery smooth muscle cells (hPASMCs).[27] In this study, we demonstrated the expression of TASK-1 messenger RNAs (mRNAs) and proteins in hPASMCs through the simultaneous absence of TASK-2 and TASK-3 expression (Fig. 30.2). In addition, we found an anandamide-sensitive conductance in both primary and cultured hPASMCs that had properties similar to TASK-1 channels. The native conductance showed an outward rectification in low external K^+ solution, was instantaneous and noninactivating, was activated by alkalotic pH, and was blocked by anandamide. Transfection of TASK-1 small-interfering RNA (siRNA) into hPASMCs significantly depolarized the resting membrane potential and abolished the effect of pH or anandamide. We concluded that TASK-1 channels are responsible for the pH-sensitive, voltage-independent background conductance that sets the resting membrane potential in human pulmonary artery smooth muscle.

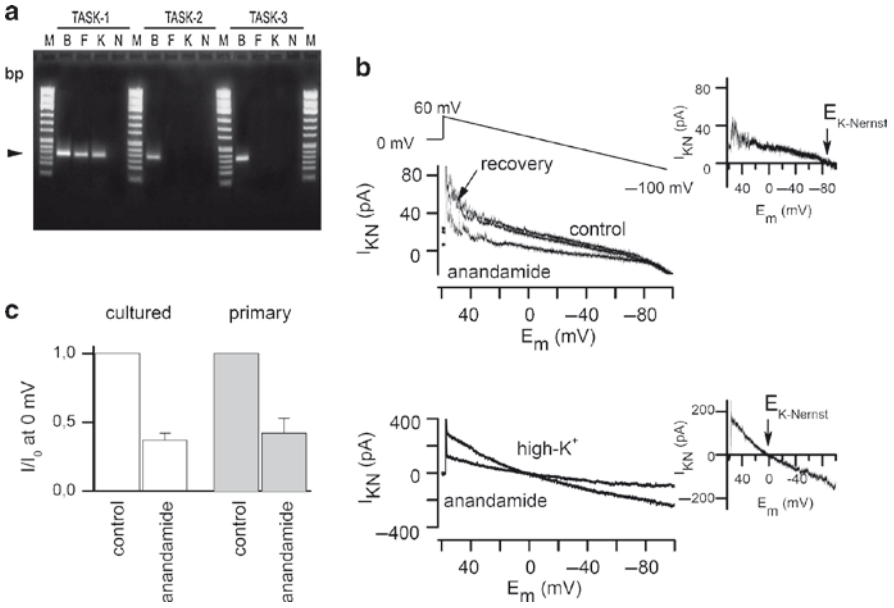

Fig. 30.2 TASK-1 in primary PASMC. (**a**) Representative gel shows mRNA expression of TASK-1 (91 bp), TASK-1 (94 bp), and TASK-3 (104 bp) in human brain (B), primary hPASMCs (F), and cultured hPASMCs (K). Only TASK-1 is expressed in primary hPASMCs (F) and cultured hPASMCs (K). *Arrow* indicates 100 bp; *M* the molecular weight marker used to indicate the size of the PCR fragments; *N* no template control. Identical results were obtained with at least three additional preparations of RNA. (**b**) *Top* Effect of 10 μmol/L anandamide on I_{KN} recorded during ramp, voltage protocol inset (*left*) and difference current trace, obtained by subtracting current amplitudes in the presence of TASK-1 blocker from those obtained under control conditions (*right*). Difference current reversed close to –84 mV, as expected for a K^+-selective conductance under these conditions. *Bottom* I_{KN} evoked under symmetrical (155 K^+) conditions by ramp in the same cell (voltage protocol above), before (high-K^+) and during application of 10 μmol/L anandamide (*left*) and the subtracted trace (*right*). Difference current was voltage independent and reversed close to 0 mV, as expected for a K^+-selective conductance under these conditions. (**c**) Histogram summarizing the effect of 10 μmol/L anandamide on I_{KN} calculated at 0 mV in cultured and primary hPASMCs ($n = 5$ each group). I/I_0 is the current in the presence of anandamide expressed as a fraction of the current before anandamide application. $E_{K\text{-Nernst}}$ indicates Nernst equilibrium potential. Reproduced with permission[27]

3 TASK-1 Is Regulated by Extracellular pH and Possibly Involved in Hypoxic Pulmonary Vasoconstriction

The pulmonary vascular bed is unique compared with most studied systemic vascular beds. During normoxic conditions, the pulmonary circulation is at low pressure – that is, vasodilated – compared with the high-pressure systemic circulation. In the systemic circulation, hypoxemia elicits vasodilation that increases O_2 delivery to

the tissues. In contrast, small resistance arteries in the pulmonary circulation constrict in response to hypoxia. This physiological response of small pulmonary arteries, known as *hypoxic pulmonary vasoconstriction* (HPV) or von Euler–Liljestrand mechanism, diverts mixed venous blood away from hypoxic alveoli, thus optimizing the matching of perfusion and ventilation and preventing arterial hypoxemia. When only a small region of the lung is hypoxic, HPV can occur without significant effect on pulmonary arterial pressure.[28] However, when hypoxia is generalized, as seen with many lung diseases and in high-altitude exposure, the subsequent pulmonary vasoconstriction contributes to pulmonary hypertension, heart failure, and death.

Hypoxia appears to activate mechanisms intrinsic to the pulmonary vasculature that are independent of blood-borne factors or influences requiring the central nervous system as HPV can be demonstrated in isolated perfused lungs and isolated pulmonary arteries.[29,30] Acute hypoxic inhibition of K^+ channels is a critical step in regulatory processes designed to link the lowering of O_2 levels to cellular responses. It is often questioned whether the K^+ channels are active at sufficiently negative potentials to set the resting membrane potential of PASMCs and whether K^+ channels could mediate HPV. TASK-1 with the biophysical profile of a background K^+ channel could be the perfect candidate for the initiation of hypoxia-induced depolarization in PASMCs. We were able to show the hypoxia sensitivity of the TASK-1 current in primary hPASMCs (Fig. 30.3). Although the mechanisms involved in the hypoxia-induced inhibition of TASK-1 are still not clearly understood, TASK-1 channels may play an important role in regulating resting membrane potential in human pulmonary arteries and eliciting vasoconstriction responses during hypoxia.

The mechanisms of the alkalosis- and hypocarbia-dependent vasodilatory responses of the pulmonary circulation are not yet fully understood. Hyperventilation has been demonstrated to reduce elevated pulmonary arterial pressure in different animal models and is employed during anesthesia for treatment of children with pulmonary hypertensive disorders or for adults with elevated pulmonary arterial pressure intra- or postoperatively. The induction of hypocapnic alkalosis is believed to play an important role in these vasodilatory effects,[31,32] with alkalosis but not hypocapnia the crucial factor.[33] Although the results generally indicate that extracellular alkalosis inhibits and hypercarbia or acidosis potentiates the hypoxic pressor response, the main mechanism responsible for the beneficial effect of hypocapnic alkalosis is not known. TASK-1 is unique among ion channels cloned to date in generating an open-rectifier "leak" K^+ current that is regulated by both increases and decreases from physiological extracellular pH. Thus, alkalosis would lead to a facilitation of TASK-1, resulting in hyperpolarization of the pulmonary arterial smooth muscle membrane and consequently in pulmonary vasodilation. The molecular site underlying the pH sensitivity of TASK-1 was investigated extensively by Morton et al.[34] Mutation of H98 reduced the pH sensitivity of TASK-1, implying its involvement in pH sensing. However, the reduction in sensitivity was not complete with H98N or H98D channels. This implies the presence of a second pH sensory mechanism in TASK-1.

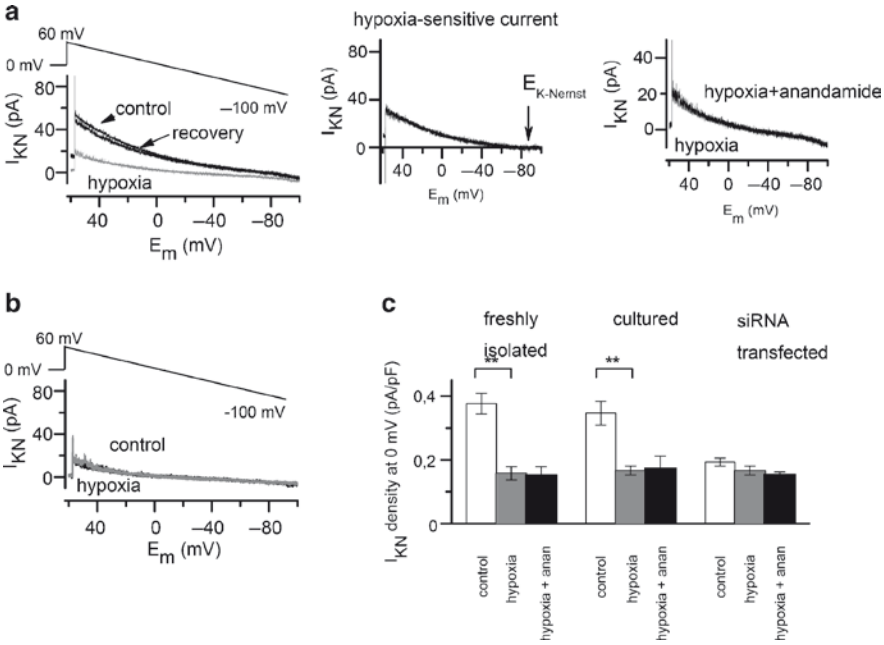

Fig. 30.3 Hypoxia blocks TASK-1 in primary and cultured hPASMCs. (**a**) Effect of hypoxia on I_{KN} recorded by ramp in primary hPASMCs (*left*) and difference currents trace, obtained by subtracting current amplitudes in the presence of hypoxia from those obtained under control conditions (*middle*). Difference current reversed close to –84 mV, as expected for a K^+-selective conductance under these conditions. Application of 10 μmol/L anandamide under hypoxic conditions did not cause a further change in the current (*right*), suggesting that the hypoxia-sensitive current is carried by TASK-1. (**b**) Lack of hypoxia effect on I_{KN} in siRNA-transfected hPASMCs. (**c**) Histogram summarizing the effect of hypoxia and anandamide (anan) under hypoxic conditions on I_{KN} calculated at 0 mV in primary, cultured, and siRNA-transfected hPASMCs (n = 5 each group; $^{**}p < 0.01$). $E_{K\text{-Nernst}}$ indicates Nernst equilibrium potential. Reproduced with permission[27]

4 Hypoxic Pulmonary Vasoconstriction Is Attenuated by Inhaled Anesthetics Due to Activation of TASK-1 Channels

Nowhere is the clinical relevance of the HPV more evident than during single-lung anesthesia. In thoracic surgery, for almost all lung surgery procedures, single-lung ventilation is needed because the operative lung must be collapsed (as it cannot be ventilated) to permit access. Under anesthesia, the systemic PaO_2 is supported by HPV. During single-lung anesthesia, when one lung is made hypoxic, HPV doubles mean pulmonary artery pressure and triples pulmonary vascular resistance, resulting in a reduction of flow to the hypoxic lung.[35]

During the last two decades, the attenuation of HPV by inhaled anesthetics has been shown by several groups. Johnson et al. studied the interactions of atelectasis and halothane on HPV using an isolated canine lobe and found that halothane

prevented the increases in pulmonary vascular resistance observed with either atelectasis or hypoxic ventilation dose dependently.[36] Marshall et al. investigated the effects of halothane, isoflurane, and enflurane on HPV in isolated rat lungs and showed that halogenated volatile anesthetics inhibit HPV with almost the same potency.[37] In contrast, in a comparative human study, isoflurane was found to be more effective in increasing PaO_2 during one-lung ventilation in comparison to enflurane.[38] Finally, Loer et al. provided support for the role of the new volatile agent desfluran in inhibition of HPV within the therapeutic range.[39]

While the exact identity of the structures targeted by inhaled anesthetics in pulmonary arteries has not yet been identified, there is accumulating evidence that TASK-1 plays a major role in the volatile anesthetic-induced pulmonary vasodilation. In addition to its pH sensitivity, another hallmark of the TASK-1 channels is their sensitivity to clinical concentrations of inhalation anesthetics.[26,27,40] The role of TASK-1 in halothane-induced pulmonary vasorelaxation has been reported. Gurney et al. showed that acidosis inhibited the facilitation of the noninactivating K^+ current caused by halothane in rabbit PASMC.[26] We found in human PASMCs that isoflurane activated the noninactivating K^+ current.[41] Because TASK-2 and TASK-3 are not expressed in hPASMC,[27] this finding suggests that isoflurane acts on a TASK-1 current. To further confirm the role of TASK-1 in the isoflurane pathway in hPASMCs, we knocked down TASK-1 expression using TASK-1 siRNA. In the siRNA-transfected cells, no facilitation of the remaining current by isoflurane or halothane was detected in comparison to primary control hPASMCs or to scrambled-siRNA-transfected hPASMCs. Because activation of TASK-1 at clinically relevant concentrations has been postulated to play a role in the mechanism of action of volatile anesthetics, their effects on TASK-1 have been extensively investigated in in vivo models.[40,42] When human TASK-1 was expressed in *Xenopus* oocytes, volatile but not intravenous anesthetics activated the two-pore-domain channel.[43]

If the decreased acute HPV found in human and animal models reported is due to the facilitation of TASK-1, what is the mechanism of that loss? For TASK-1, a molecular basis has been established by Talley and Bayliss foramodulathon by i haled a.esthetias.[44] Mutations in a six-residue sequence at the beginning of the cytoplasmic C terminus virtually abolished anesthetic activation. Talley and Bayliss interpreted their results to suggest that the anesthetic effect requires a region at the interface between the final transmembrane domain and the cytoplasmic C terminus that has not been previously associated with receptor signal transduction. Finally, the modulation of inhaled anesthetic effect was characterized using TASK-1 knockout mice and tested their sensitivity to the volatile anesthetics halothane and isoflurane. Unfortunately, only behavioral and physiological characterization of the phenotype was performed.[45] It is striking that TASK-1 knockout mice displayed a largely normal behavioral and physiological phenotype. It is also remarkable that TASK-1 knockout mice showed only a slightly reduced sensitivity to halothane and isoflurane, reflected as a rightward shift in the concentration-response plots in the auditory and nociceptive responses. It has been postulated that the combined activation of multiple K_{2P} channels is crucial for the analgesic effects of inhalation anesthetics.

However, it is also possible that the TASK-1 knockout mice may have been functionally compensated by the expression of TASK-3 or other gene products. Therefore, further studies investigating HPV and the expression of K_{2P} in the pulmonary arteries in this model are required to elucidate the relevance of TASK-1 in pulmonary circulation.

5 TASK-1 Is Modulated by G Protein-Coupled Receptor-Activated Pathways

A number of G protein-coupled pathways have been shown to regulate the activity of K_{2P} channels. The most commonly occurring regulation seems to be Gαq-mediated inhibition, which is seen for both TASK and TREK channels. It seems increasingly likely that a number of different potential regulatory pathways are stimulated following Gαq activation. It is possible that these may act in parallel to ensure robust channel regulation. Alternatively, the dominant mechanism may depend on the particular receptor stimulated, the relative expression levels of the different proteins involved, or the cell type studied (reviewed in Ref. [46]).

Endothelin 1 (ET-1) is considered to be a major player within the pathologic mechanisms involved in pulmonary arterial hypertension (PAH),[47,48] and specific antagonists of ET-1 receptors represent an important pillar of modern therapy of this devastating disease.[49,50] In addition, a correlation between an increased ET-1 expression in the lung of patients with pulmonary hypertension and the severity of the disease was demonstrated.[48] Although these evidences indicate that the ET system plays a key role in the pathogenesis of pulmonary hypertension, the molecular targets of ET-1 have not been characterized in detail. To date, the involvement of voltage-activated (K_V)[51] or adenosine triphosphate (ATP)-dependent K channels (K_{ATP})[52] has been established. Because TASK-1 is active at rest in hPASMCs compared to the K^+ channels indicated, its inhibition would lead to cell depolarization that enhances the open probability of L-type Ca^{2+} channels in smooth muscle cells, causing periodic Ca^{2+} entry and vasoconstriction. We have now identified TASK-1 channels as an important target for ET-1 at clinically relevant concentrations in primary hPASMCs[41] (Fig. 30.4). We found that antagonists of the G protein-coupled receptor ET_A fully abolished the TASK-1 inhibition in response to ET-1. These data may further confirm that ET antagonism is an effective treatment option for patients with symptomatic PAH. Furthermore, we showed that antagonists of protein kinase C (PKC) but not antagonists of protein kinase A (PKA) inhibited the ET-1 effect on TASK-1, suggesting that PKC mediates the agonist-induced inhibition. Our data indicate that ET-1 can stimulate the serine or threonine phosphorylation of TASK-1, and they are consistent with the detection of phosphoserine in TASK-1[53] as well as with the presence of consensus serine and threonine PKA and PKC phosphorylation sites in the TASK-1 peptide.[6] While a tyrosine kinase consensus site is also present in the TASK-1 peptide, ET-1 did not effect tyrosine phosphorylation of TASK-1.

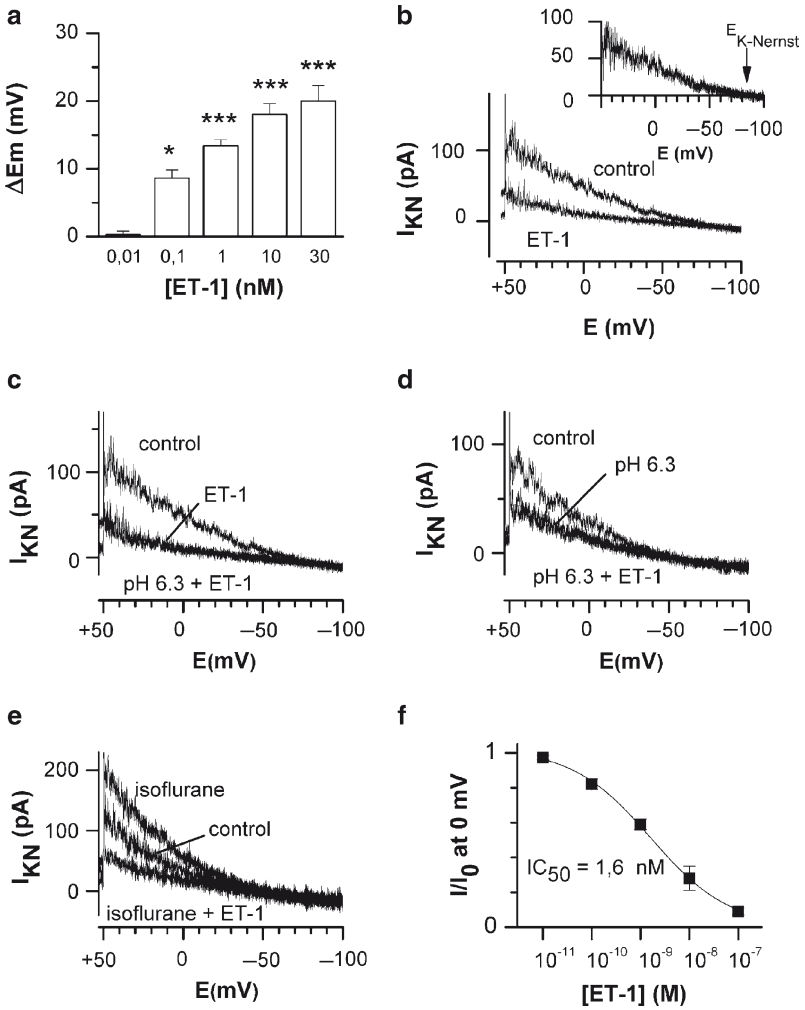

Fig. 30.4 ET-1 blocks TASK-1 in primary human PASMCs at clinically relevant concentrations. (**a**) ET-1 significantly depolarized primary human PASMCs ($n = 5$ each group; $^{*}p < 0.05$, $^{***}p < 0.001$). (**b**) Effect of 10 n*M* ET-1 on I_{KN} recorded during ramp, voltage protocol inset (*left*), and "difference" currents trace, obtained by subtracting current amplitudes in the presence of ET-1 from those obtained under control conditions (*right*). Difference current reversed close to –84 mV, as expected for a K^+-selective conductance under these conditions. (**c**) Representative recordings for TASK-1 current after applying ET-1 (10 n*M*) and when ET-1 was applied at pH 6.3. (**d**) TASK-1 recorded in control, following acidification to pH 6.3, and after application of ET-1 (10 n*M*) under acidification. (**e**) ET-1 reverses I_{KN} opening by isoflurane (1 m*M*). (**f**) Concentration-response curve for ET-1. I/I_0 is the current in the presence of ET-1 expressed as a fraction of the current prior to ET-1 application. The line is the best fit to the Hill equation using an IC_{50} of 1.6 ± 0.3 n*M*. Reproduced with permission[41]

Several studies have examined the mechanism by which a G protein-coupled receptor agonist may inhibit K_{2P} channels via PKC. These studies suggested that agonist-induced inhibition of the K_{2P} channels was due to ATP-dependent pathways,[54] by depletion of phosphatidylinositol 4,5-bisphosphate (PIP_2) levels,[55] by a direct action of diacylglycerol (DAG) and phosphatidic acid that are generated via phospholipase C (PLC),[56] or by elevated intracellular Ca^{2+} levels.[54] Moreover, a recent study suggested that there may be a direct interaction of Gαq with TASK-1 in a mammalian heterologous expression system.[57] The results obtained from different laboratories suggest the possibility that two-pore-domain channels may be modulated not by a single mechanism but via distinct pathways in different cell types. We found that inhibition of the PLC abolished the ET-1 effect on TASK-1, indicating that PLC is required, as is also shown in *Xenopus laevis* oocytes,[58] in contrast to the report in a mammalian heterologous expression system.[44] Additional evidence comes from the use of COS-7 cells expressing TREK-2 and muscarinic receptor M_3, for which the same PLC inhibitor was applied to prevent acetylcholine-induced inhibition of TREK-2.[59] The inhibitory effect of the PIP_2 scavenger on TASK-1 observed in this study confirmed previously reported results obtained with different K_{2P} channels expressed in *Xenopus* oocytes[55] and strongly suggests that PIP_2 hydrolysis by PLC indeed affects channel activity. Moreover, we showed that DAG, the downstream product of PIP_2 hydrolysis, underlies the agonist-induced inhibition of TASK-1 in hPASMCs. These results were further supported by our experiments showing that the DAG kinase inhibitor abolished the ET-1 effect on TASK-1 (Fig. 30.5).

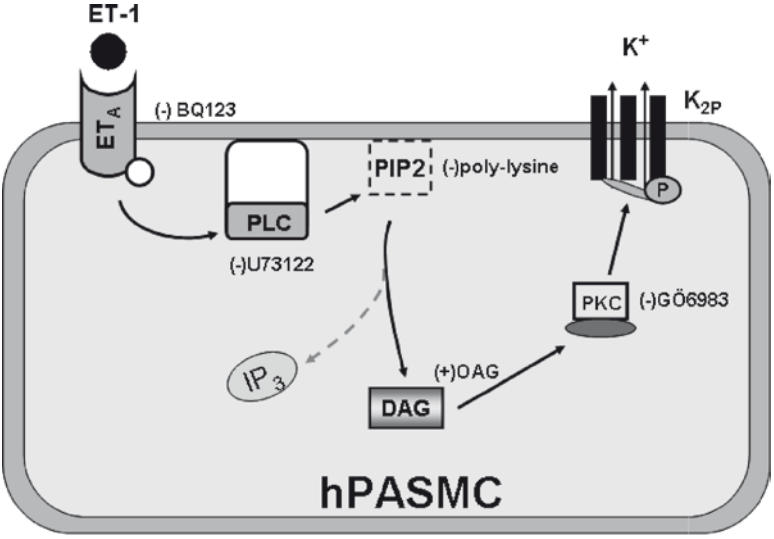

Fig. 30.5 Schematic presentation of the ET-1 signaling pathway in hPASMCs. ET-1 binds to the G protein-coupled receptor (Gαq) ET_A, leading to the protein kinase C (PKC)-induced phosphorylation of TASK-1 channels through phospholipase C (PLC), phosphatidylinositol 4,5-biphosphate (PIP_2), and diacylglycerol (DAG). (+) indicate agonist and (–) indicate antagonist effects. Reproduced with permission[41]

There is accumulating evidence that intracellular protein kinases downstream from G protein-coupled receptors undertake important modulation of K_{2P} channels. Cyclic adenosine monophosphate (cAMP)-dependent protein kinases are activated by the signal chain coming from the G protein via adenylate cyclase and cAMP. In human primary PASMCs, treprostinil, a stable analogue of prostacyclin, enhances TASK-1 at clinically relevant concentrations (IC_{50} = 1.2 n*M*).[27] Targeting TASK-1 may explain the potent pulmonary vasodilator effect of treprostinil in PAH. A membrane-permeable analogue of cAMP (8-br-cAMP) exhibits similar effects. The activation of TASK-1 was still detected during coapplication of treprostinil with ITX (iberiotoxin), a selective blocker of Ca^{2+}-activated K^+ channels, but treprostinil did not show any effect after pretreatment with a specific TASK-1 inhibitor anandamide. The activation of TASK-1 by treprostinil was abolished after preincubation with KT5720, an inhibitor of cAMP-dependent protein kinase. Moreover, our data indicate that treprostinil can stimulate the serine or threonine phosphorylation of TASK-1 and are consistent with the detection of phosphoserine in TASK-1 and the presence of consensus serine and threonine PKA and PKC phosphorylation sites in the TASK-1 peptide.[6] Whereas a tyrosine kinase consensus site is also present in the TASK-1 peptide,[6] treprostinil did not affect tyrosine phosphorylation of TASK-1. Members of the K_{2P} superfamily such as TWIK-1 currents are potentiated by activators of PKC, whereas TREK-1 or TREK-2 currents are inhibited.[60] When human TASK-1 was expressed in *Xenopus* oocytes, the current was insensitive to activation of adenyl cyclase by forskolin or IBMX.[6] In another study, the activation of PKA (Proteinkinase A) inhibited TASK-1 cloned from rat cerebellum,[7] whereas we observed PKA-mediated activation of TASK-1 by treprostinil and by 8-br-cAMP in hPASMCs. This disparity in effects between these results could be related to clone specificity (human vs. rat) or to preparations (oocytes vs. native PASMCs) with possible differences between cAMP levels and PKA activity.

Taken together, it is apparent that data in support of an involvement of the G protein-coupled pathways mediated in the regulation of TASK channels are contradictory and inconclusive. It is possible that at least some of these differences may be attributed to differences between cell types and channel and regulatory protein expression levels; nevertheless, the regulation of TASK channels by G protein-coupled pathways represents an important therapeutic approach and needs further investigation.

References

1. Bayliss DA, Talley EM, Sirois JE, Lei Q (2001) TASK-1 is a highly modulated pH-sensitive 'leak' K^+ channel expressed in brainstem respiratory neurons. Respir Physiol 129:159–174
2. Goldstein SA, Wang KW, Ilan N, Pausch MH (1998) Sequence and function of the two P domain potassium channels: implications of an emerging superfamily. J Mol Med 76:13–20
3. O'Connell AD, Morton MJ, Hunter M (2002) Two-pore domain K^+ channels-molecular sensors. Biochim Biophys Acta 1566:152–161

4. Kang D, Han J, Talley EM, Bayliss DA, Kim K (2004) Functional expression of TASK-1/TASK-3 heteromers in cerebellar granule cells. J Physiol 554:64–77
5. Czirjak G, Enyedi P (2002) Formation of functional heterodimers between the TASK-1 and TASK-3 two-pore domain potassium channel subunits. J Biol Chem 277:5426–5432
6. Duprat F, Lesage F, Fink M, Reyes R, Heurteaux C, Lazdunski M (1997) TASK, a human background K^+ channel to sense external pH variations near physiological pH. EMBO J 16: 5464–5471
7. Leonoudakis D, Gray AT, Winegar BD et al (1998) An open rectifier potassium channel with two pore domains in tandem cloned from rat cerebellum. J Neurosci 18:868–877
8. Kim D, Fujita A, Horio Y, Kurachi Y (1998) Cloning and functional expression of a novel cardiac two-pore background K^+ channel (cTBAK-1). Circ Res 82:513–518
9. Lopes CM, Gallagher PG, Buck ME, Butler MH, Goldstein SA (2000) Proton block and voltage gating are potassium-dependent in the cardiac leak channel Kcnk3. J Biol Chem 275:16969–16978
10. Han J, Truell J, Gnatenco C, Kim D (2002) Characterization of four types of background potassium channels in rat cerebellar granule neurons. J Physiol 542:431–444
11. Patel AJ, Honoré E (2001) Molecular physiology of oxygen-sensitive potassium channels. Eur Respir J 18:221–227
12. Millar JA, Barratt L, Southan AP et al (2000) A functional role for the two-pore domain potassium channel TASK-1 in cerebellar granule neurons. Proc Natl Acad Sci U S A 97:3614–3618
13. Kim D (2003) Fatty acid-sensitive two-pore domain K^+ channels. Trends Pharmacol Sci 24:648–654
14. Buckler KJ, Honoré E (2005) The lipid-activated two-pore domain K^+ channel TREK-1 is resistant to hypoxia: implication for ischaemic neuroprotection. J Physiol 562:213–222
15. Bang H, Kim Y, Kim D (2000) TREK-2, a new member of the mechanosensitive tandem-pore K^+ channel family. J Biol Chem 275:17412–17419
16. Chemin J, Patel AJ, Duprat F, Lauritzen I, Lazdunski M, Honoré E (2005) A phospholipid sensor controls mechanogating of the K^+ channel TREK-1. EMBO J 24:44–53
17. Buckingham SD, Kidd JF, Law RJ, Franks CJ, Sattelle DB (2005) Structure and function of two-pore-domain K^+ channels: contributions from genetic model organisms. Trends Pharmacol Sci 26:361–367
18. Meuth SG, Budde T, Kanyshkova T, Broicher T, Munsch T, Pape HC (2003) Contribution of TWIK-related acid-sensitive K^+ channel 1 (TASK1) and TASK3 channels to the control of activity modes in thalamocortical neurons. J Neurosci 23:6460–6469
19. Backx PH, Marban E (1993) Background potassium current active during the plateau of the action potential in guinea pig ventricular myocytes. Circ Res 72:890–900
20. Bai X, Bugg GJ, Greenwood SL et al (2005) Expression of TASK and TREK, two-pore domain K^+ channels, in human myometrium. Reproduction 129:525–530
21. Sanders KM, Koh SD (2006) Two-pore-domain potassium channels in smooth muscles: new components of myogenic regulation. J Physiol 570:37–43
22. Beckett EA, Han I, Baker SA, Han J, Britton FC, Koh SD (2008) Functional and molecular identification of pH-sensitive K^+ channels in murine urinary bladder smooth muscle. BJU Int 102:113–124
23. Kim Y, Bang H, Kim D (2000) TASK-3, a new member of the tandem pore K^+ channel family. J Biol Chem 275:9340–9347
24. Rajan S, Wischmeyer E, Xin LG et al (2000) TASK-3, a novel tandem pore domain acid-sensitive K^+ channel. An extracellular histiding as pH sensor. J Biol Chem 275:16650–16657
25. Reyes R, Duprat F, Lesage F et al (1998) Cloning and expression of a novel pH-sensitive two pore domain K^+ channel from human kidney. J Biol Chem 273:30863–30869
26. Gurney AM, Osipenko ON, MacMillan D, McFarlane KM, Tate RJ, Kempsill FE (2003) Two-pore domain K channel, TASK-1, in pulmonary artery smooth muscle cells. Circ Res 93:957–964
27. Olschewski A, Li Y, Tang B et al (2006) Impact of TASK-1 in human pulmonary artery smooth muscle cells. Circ Res 98:1072–1080

28. Nakanishi K, Tajima F, Osada H et al (1996) Pulmonary, vascular responses in rats exposed to chronic hypobaric hypoxia at two different altitude levels. Pathol Res Pract 192:1057–1067
29. Weir EK, Archer SL (1995) The mechanism of acute hypoxic pulmonary vasoconstriction: the tale of two channels. FASEB J 9:183–189
30. Weissmann N, Grimminger F, Walmrath D, Seeger W (1995) Hypoxic vasoconstriction in buffer-perfused rabbit lungs. Respir Physiol 100:159–169
31. Fineman JR, Wong J, Soifer SJ (1993) Hyperoxia and alkalosis produce pulmonary vasodilation independent of endothelium-derived nitric oxide in newborn lambs. Pediatr Res 33:341–346
32. Yamaguchi K, Takasugi T, Fujita H et al (1996) Endothelial modulation of pH-dependent pressor response in isolated perfused rabbit lungs. Am J Physiol 270:H252–H258
33. Schreiber MD, Heymann MA, Soifer SJ (1986) Increased arterial pH, not decreased $PaCO_2$, attenuates hypoxia-induced pulmonary vasoconstriction in newborn lambs. Pediatr Res 20:113–117
34. Morton MJ, O'Connell AD, Sivaprasadarao A et al (2003) Determinants of pH sensing in the two-pore domain K^+ channels TASK-1 and -2. Pflügers Arch 445:577–583
35. Bindslev L, Jolin A, Hedenstierna G, Baehrendtz S, Santesson J (1985) Hypoxic pulmonary vasoconstriction in the human lung: effect of repeated hypoxic challenges during anesthesia. Anesthesiology 62:621–625
36. Johnson DH, Hurst TS, Mayers I (1991) Effects of halothane on hypoxic pulmonary vasoconstriction in canine atelectasis. Anesth Analg 72:440–448
37. Marshall C, Lindgren L, Marshall BE (1984) Effects of halothane, enflurane, and isoflurane on hypoxic pulmonary vasoconstriction in rat lungs in vitro. Anesthesiology 60:304–308
38. Slinger P, Scott WA (1995) Arterial oxygenation during one-lung ventilation. A comparison of enflurane and isoflurane. Anesthesiology 82:940–946
39. Loer SA, Scheeren TW, Tarnow J (1995) Desflurane inhibits hypoxic pulmonary vasoconstriction in isolated rabbit lungs. Anesthesiology 83:552–556
40. Patel AJ, Honoré E, Lesage F, Fink M, Romey G, Lazdunski M (1999) Inhalational anesthetics activate two-pore-domain background K^+ channels. Nat Neurosci 2:422–426
41. Tang B, Li Y, Nagaraj C et al (2009) Endothelin-1 inhibits background two-pore domain channel TASK-1 in primary human pulmonary artery smooth muscle cells. Am J Respir Cell Mol Biol 41(4):476-83
42. Sirois JE, Lei Q, Talley EM, Lynch C III, Bayliss DA (2000) The TASK-1 two-pore domain K^+ channel is a molecular substrate for neuronal effects of inhalation anesthetics. J Neurosci 20:6347–6354
43. Putzke C, Hanley PJ, Schlichthörl G et al (2007) Differential effects of volatile and intravenous anesthetics on the activity of human TASK-1. Am J Physiol Cell Physiol 293:C1319–C1326
44. Talley EM, Bayliss DA (2002) Modulation of TASK-1 (Kcnk3) and TASK-3 (Kcnk9) potassium channels: volatile anesthetics and neurotransmitters share a molecular site of action. J Biol Chem 277:17733–17742
45. Linden AM, Aller MI, Leppä E et al (2006) The in vivo contributions of TASK-1-containing channels to the actions of inhalation anesthetics, the α_2 adrenergic sedative dexmedetomidine, and cannabinoid agonists. J Pharmacol Exp Ther 317:615–626
46. Mathie A (2007) Neuronal two-pore-domain potassium channels and their regulation by G protein-coupled receptors. J Physiol 578:377–385
47. Stewart DJ, Levy RD, Cernacek P, Langleben D (1991) Increased plasma endothelin-1 in pulmonary hypertension: marker or mediator of disease? Ann Intern Med 114:464–469
48. Giaid A, Yanagisawa M, Langleben D et al (1993) Expression of endothelin-1 in the lungs of patients with pulmonary hypertension. N Engl J Med 328:1732–1739
49. Rubin LJ, Badesch DB, Barst RJ et al (2002) Bosentan therapy for pulmonary arterial hypertension. N Engl J Med 346:896–903
50. Olschewski H, Olschewski A, Rose F et al (2001) Physiologic basis for the treatment of pulmonary hypertension. J Lab Clin Med 138:287–297

51. Shimoda LA, Sylvester JT, Booth GM et al (2001) Inhibition of voltage-gated K^+ currents by endothelin-1 in human pulmonary arterial myocytes. Am J Physiol Lung Cell Mol Physiol 281:L1115–L1122
52. Park WS, Ko EA, Han J, Kim N, Earm YE (2005) Endothelin-1 acts via protein kinase C to block K_{ATP} channels in rabbit coronary and pulmonary arterial smooth muscle cells. J Cardiovasc Pharmacol 45:99–108
53. O'Kelly I, Butler MH, Zilberberg N, Goldstein SA (2002) Forward transport. 14-3-3 binding overcomes retention in endoplasmic reticulum by dibasic signals. Cell 111:577–588
54. Enyeart JJ, Danthi SJ, Liu H, Enyeart JA Angiotensin II (2005) inhibits bTREK-1 K^+ channels in adrenocortical cells by separate Ca^{2+}- and ATP hydrolysis-dependent mechanisms. J Biol Chem 280:30814–30828
55. Lopes CM, Rohács T, Czirják G, Balla T, Enyedi P, Logothetis DE (2005) PIP2 hydrolysis underlies agonist-induced inhibition and regulates voltage gating of two-pore domain K^+ channels. J Physiol 564:117–129
56. Chemin J, Girard C, Duprat F, Lesage F, Romey G, Lazdunski M (2003) Mechanisms underlying excitatory effects of group I metabotropic glutamate receptors via inhibition of 2P domain K^+ channels. EMBO J 22:5403–5411
57. Chen X, Talley EM, Patel N et al (2006) Inhibition of a background potassium channel by Gq protein α-subunits. Proc Natl Acad Sci U S A 103:3422–3427
58. Czirják G, Petheo GL, Spät A, Enyedi P (2001) Inhibition of TASK-1 potassium channel by phospholipase C. Am J Physiol Cell Physiol 281:C700–C708
59. Kang D, Han J, Kim D (2006) Mechanism of inhibition of TREK-2 (K2P10.1) by the Gq-coupled M3 muscarinic receptor. Am J Physiol Cell Physiol 291:C649–C656
60. Fink M, Duprat F, Lesage F et al (1996) Cloning, functional expression and brain localization of a novel unconventional outward rectifier K^+ channel. EMBO J 15:6854–6862
61. Duprat F, Lauritzen I, Patel A, Honoré E (2007) The TASK background K_{2P} channels: chemo- and nutrient sensors. Trends Neurosci 30:573–580

Pharmacological Targets for Pulmonary Vascular Disease: Vasodilation versus Anti-Remodelling

Matthew Thomas

Abstract Two gross mechanisms of pathology are central to pulmonary arterial hypertension - increased pulmonary vascular tone and remodelling of the pulmonary arteries. These pathologies can be caused by a variety of aberrant processes, and combine to cause an increase in pulmonary vascular resistance and consequent right ventricular hypertrophy, eventually leading to dysfunction and death. Current therapeutic strategies have focused on altering the vasoconstrictive elements of the disease. Whilst improvements in life expectancy have been observed, current therapies have not managed to halt or reverse progression of the disease. Here we discuss said unmet medical need and postulate as to the impact on disease anti-remodelling therapy might provide. The mechanisms of remodelling in pulmonary arterial hypertension are reviewed, and leading examples of potential targets within such mechanisms are discussed.

Keywords remodelling • pulmonary arterial hypertension • therapy

1 Current Therapeutic Options

As an aggressive and complex disease affecting small arteries of the lung, pulmonary arterial hypertension (PAH) is characterized by both increased vascular tone and remodelled vessels. Predominantly affecting young women, PAH is rapidly progressive, and if left untreated, the median time to death by right-sided heart failure is less than 3 years. The introduction of currently approved therapies has improved this survival rate by between 40 and 80%. However, most patients with PAH will eventually fail to respond to current available therapies and are placed on lung transplantation lists.

Current therapies for PAH are palliative and are dominated by vasodilatory approaches. Early non-selective vasodilators, such as angiotensin-converting enzyme

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inhibitors and hydralazine, had little or no effect on PAH pathologies without more severe effects on the systemic circulation.[1] High-dose calcium channel blockers, such as nifedipine, can be effective vasodilators of the pulmonary system, yet treatment is only possible in approximately 10% of PAH patients and can become refractory at any time. Several prostacyclin analogues are available, although only modest efficacy is generally observed, possibly due to tachyphylaxis, with other systemic side effects such as nausea, diarrhoea, flushing, rash, hypotension and jaw pain diminishing the therapeutic window. Inconvenient routes of administration (continuous intravenous infusion for epoprostenol) or frequent administration due to poor pharmacokinetic profile (six to nine times daily via nebulization for iloprost), together with associated costs make prostacyclin analogues an unattractive option.[2] Clinical trails investigating endothelin receptor antagonists such as bosentan have been shown to decrease right ventricular (RV) pressure, increase cardiac output and 6-min walk distance, as well as increase the time to clinical worsening. However, these observations were subtle, conferring a modest clinical improvement, and associated with safety concerns, including liver toxicity, haemorrhage and a potential for major birth defects.[3] Finally, phosphodiesterase 5 (PDE5) inhibitors such as sildenafil, although showing modest clinical improvement, have little impact on disease progression.[4] The bias of current therapies toward inhibiting the increased vascular tone associated with PAH, when viewed in the context of the modest clinical impact of said strategies, would suggest that a move toward inhibiting remodelling aspects of the disease may produce a more profound clinical outcome.

2 Mechanisms of Pulmonary Vessel Remodelling

Vascular remodelling in PAH is a multi-factorial, multi-cellular system of processes. Injury to endothelium in the context of an altered bone morphogenetic protein (BMP) axis or serotonergic signalling system, whether genetic or environmentally induced, leads to enhanced apoptosis of pulmonary artery endothelial cells (PAECs).[5] The loss of endothelial integrity would expose pulmonary artery smooth muscle cells (PASMCs) to various growth factors, such as platelet-derived growth factor (PDGF), thus inducing proliferation and reducing apoptosis.[6] The induction of the transcription factor NFAT (nuclear factor of activated T cells) may be key to the development of said phenotype via the inhibition of voltage-gated K^+ 1.5 (K_V1.5) channels, the upregulation of bcl-2 and mitochondrial potential, as demonstrated in PASMC studies from PAH patients – evidence of further mechanisms by which resistance to apoptosis may occur.[7] The ensuing disruption to the interstitial matrix will activate elastase and metalloproteinases and promote the release of other matrix-associated growth factors such as epidermal growth factor (EGF).[8] The resultant cocktail of secreted vascular effectors will not only enhance the pathogenesis of resident cell populations but also may act as a beacon for circulating inflammatory cells and the potential engraftment and differentiation of vascular progenitors. Following the initial loss of PAECs, survivin-expressing, apoptosis-resistant clones emerge that

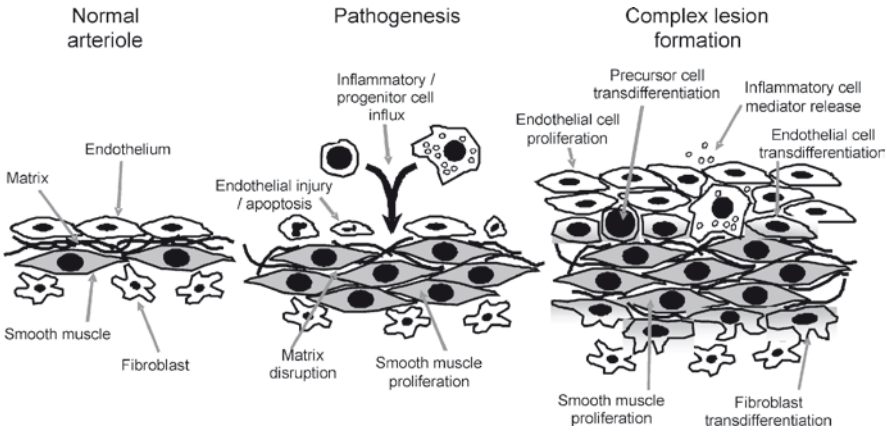

Fig. 31.1 Several mechanisms and cell populations are involved in the progressive remodelling pathologies of PAH. The normal small pulmonary arteriole is structured as single-cell thin layers of endothelium, matrix, smooth muscle and fibroblasts. Susceptibility to pathogenesis may be conferred by genetic or environmental changes in BMP or serotonin signalling that allow a second trigger, inflammation or progenitor mediated, to cause endothelial injury/apoptosis, matrix disruption and smooth muscle cell proliferation. An array of growth factors derived from precursor, inflammatory, endothelial or smooth muscle cells provides an environment promoting endothelial, precursor and fibroblast transdifferentiation and further smooth muscle and endothelial cell proliferation. The relative balance between these processes leads to medial hypertrophy, neointima formation and plexiform legion development

contribute to complex lesion formation in disregulated attempts to repair initial vessel loss.[9] It is also possible that endothelial cells transdifferentiate to a smooth muscle cell phenotype.[10] The remodelling aspects of PAH are illustrated in Fig. 31.1.

3 Novel and Emerging Targets for Anti-remodelling Therapies

It is unlikely that targeting a specific pathway relevant to PAH pathologies will selectively impact remodelling with no influence on vascular tone, just as it is unlikely that currently approved vasodilatory therapies have absolutely no effect on remodelling (e.g. endothelin signalling stimulates PASMC proliferation[3)]. However, the targets detailed next are either primarily anti-remodelling or at least provide a strong rationale for affecting both areas of PAH pathology.

3.1 Platelet-Derived Growth Factor

PDGF has been shown to induce both proliferation and migration of smooth muscle cells and fibroblasts and has thus been proposed as a key mediator of several fibroproliferative diseases.[11] Preclinical model studies of pulmonary hypertension in

foetal lambs demonstrated PDGF upregulation,[12] later also observed in rat models of monocrotaline treatment and chronic hypoxia. Concise evidence for the role PDGF in human PAH is more recent, with increased protein expression of PDGF-A and -B in PAH patient PAECs, PASMCs and perivascular inflammatory infiltrate demonstrated, together with increased expression and phosphorylation of PDGFR-β.[13] Further evidence for the central role of PDGF in PAH remodelling has come from rodent studies in which the PDGFR inhibitor imatinib mesylate (Gleevec) was demonstrated to reverse established vascular remodelling, RV pressure and RV hypertrophy in both monocrotaline and chronic hypoxia rat models.[14] The rationale for PDGFR inhibition as a PAH therapy has been further strengthened by promising results from a 59-patient, multi-centre phase II clinical trial in which a significant improvement in pulmonary vascular resistance and a numerical increase in cardiac output, key hemodynamic measures used to monitor the progression of the disease, were demonstrated. Improvements in the 6-min walk test, the primary endpoint of the study, approached, but did not reach, statistical significance (ERS presentation). However, Gleevec inhibits not only PDGF receptor (PDGFR) signalling, but also Abelson (abl) and c-kit – both of which have proposed roles in the signalling mechanisms or pathogenesis of PAH. Abl signalling is known to be downstream of TGFb, the signalling of which is altered by polymorphisms in bone morphogenic receptor 2 related familial forms of the disease (discussed in Chapter 16). Infiltrating c-kit+ cell precursors have also been implicated in the generation of complex lesions, and thus the inhibition of such signaling may impact later stage vessel remodelling.[15,16] Studies are currently ongoing comparing Gleevec and related compounds of varying selectivity to determine which elements of inhibition provide efficacy.

3.2 RhoA/ROK Pathway

Inhibition of the small guanosine triphosphatase (GTPase) RhoA (Ras homologous) or its downstream effector Rho-associated kinase (ROK) have been suggested as a new therapeutic option for PAH patients. RhoA/ROK inhibitors such as fasudil, simvastatin or Y-27632 have well-defined and dramatic vasodilatory effects by blocking the RhoA/ROK-dependent actions of G protein-coupled receptor (GPCR) agonists such as endothelin, serotonin (5-HT) or thromboxane.[17] However, RhoA/ROK has also been associated with neointimal cell proliferation and coordinate decreases in apoptosis, as well as increases in pro-inflammatory cytokines, such as monocyte chemoattractant protein (MCP) 1, leading to vascular accumulation of macrophages and angiotensin II/connective tissue growth factor (CTGF)-mediated perivascular fibrosis.[18,19]

Given these proposed mechanisms of action, it is perhaps unsurprising that RhoA/ROK inhibitors have demonstrated profound efficacy in rodent models of PAH. Acute dosing of fasudil or Y-27632 normalizes elevated RV pressures observed in either

chronic hypoxia or monocrotaline models of PAH.[20] However, in models in which complex lesion development is more profound (hypoxia/vascular endothelial growth factor receptor [VEGFR] inhibition or pneumonectomy/monocrotaline rat), acute RhoA/ROK inhibition is less efficacious.[21] Reversal of remodelling in monocrotaline-induced PAH requires chronic treatment – efficacy characterized by suppressed macrophage infiltration and PASMC proliferation and enhanced apoptosis, together with reduced matrix protein expression.[22] Anti-remodelling effects of fasudil were elegantly demonstrated by the improved efficacy observed when co-administered with the prostacyclin analogue beraprost to monocrotaline-treated rats.[23] Furthermore, a recent trial with simvastatin[24] indicated beneficial effects on RV pressure and 6-min walk, as well as a reduction in the required concurrent prostacyclin treatment; indeed, fasudil is licensed for use in Japan. However, there is evidence that levels of RhoA/ROK inhibition required to have an impact on PAH pathologies, when systemically exposed, cause peripheral hypotension – a liability observed with several chemotypes, thus suggesting said issues to be target, rather than compound class, related.

3.3 Activin-Like Kinase 5

Germline mutations in the transforming growth factor β (TGF-β) superfamily receptor bone morphogenetic protein receptor II (BMPR-II) gene are strongly linked to the development of familial and some sporadic forms of idiopathic pulmonary arterial hypertension (iPAH), the evidence for which is detailed in Chap. 16. In vitro studies have suggested that aberrant signalling via TGF-β receptor I/activin receptor-like kinase 5(ALK-5) may bd key to development of pulmonary hypertensive pathologies.

We have investigated the therapeutic potential of SB525334 (6-[2-tert-butyl-5-(6-methyl-pyridin-2-yl)-1H-imidazol-4-yl]-quinoxaline), a well-characterized and potent ALK-5 inhibitor, within in vitro and in vivo models of PAH. TGF-β treatment of PASMCs isolated from patients with iPAH promotes enhanced transcription of the TGF-β-responsive genes JunB, CCN1 and PAI-1 compared to the response observed in PASMCs from normotensive donor controls. In addition, iPAH PASMCs demonstrated markedly enhanced cellular proliferation. Furthermore, administration of SB525334 inhibited TGFβ-mediated proliferation of iPAH PASMCs. Consistent with the role of TGF-β in the development of iPAH, we demonstrated SB525334 significantly reversed pulmonary arterial pressure and inhibited RV hypertrophy in rats in a monocrotaline model of established PAH. Immunohistochemistry studies confirmed a significant reduction in pulmonary arteriole muscularisation induced by monocrotaline following treatment of rats with SB525334. Collectively, these data are consistent with a role for ALK-5 in the progression of PAH pathology and imply that strategies to inhibit ALK-5 signalling may have therapeutic benefit in the treatment and reversal of this disease.

3.4 *Anti-inflammatory Targets*

Non-specific anti-inflammatory strategies for the treatment of PAH are largely ineffectual; indeed, historical mis-diagnosis of PAH has often been discovered on the basis of non-responsiveness to steroids. However, various inflammatory stimuli have been proposed as key to either the initiation of pathology (second trigger) or progression of the disease via recruitment and differentiation of circulating leukocyte-derived mesenchymal precursors to the pulmonary, but not systemic, arteries.[25] Studies in human tissue and rodent models have proposed a wide array of potential inflammatory targets associated with these processes, including: TGF-β, interleukin (IL)-6, IL-1β, tumour necrosis factor α (TNF-α), IL-8, Monocyte chemotactic protein (MCP)-1, complement C5, vascular endothelial growth factor (VEGF)-A, RANTES, Macrophage inflammatory protein (MIP)-1β, stromal cell-derived factor (SDF)-1, Intercellular Cell *Adhesion* Molecule (ICAM)-1 and vascular Cell *Adhesion* Molecule (VCAM)-1.[26–29] The relative importance of a single inflammatory mediator can be assessed by exposing a genetic knockout (KO) to chronic hypoxia and determining effects on RV pressure and hypertrophy vs. wild-type (WT) controls. Such studies often reveal that although a mediator may be up-regulated in disease-relevant tissues, it may not represent a viable therapeutic target. Such a strategy is demonstrated by fractalkine, a CX(3)C chemokine produced by endothelial cells which can capture leukocytes rapidly and firmly in an integrin-independent manner under high blood flow. Fractalkine and fractalkine receptor (CX(3)CR1) was increased in patients with PAH, potentially contributing to the influx of inflammatory cells to lesion sites.[30] However, the degree of chronic hypoxia-induced PAH pathology in CX(3)CR1 KO mice was unaffected, therefore throwing some doubt on the validity of fractalkine as a therapeutic target (Fig. 31.2).

Many studies investigating the inflammatory elements of PAH are conflicting. Characterisation of the cytokine levels in the serum from patients with PAH suggests that the immune system is in a state of chronic inflammation, potentially T-helper 2 biassed.[31] Elevated $CD4^+$ CD25 high regulatory T cells have also been identified in patients with iPAH, although whether linked directly with disease or present as a consequence of/to control inflammation is controversial.[32] Hypoxia studies in athymic rats demonstrated enhanced pathology development associated with higher B cell numbers (reversible by WT splenocyte adoptive transfer).[33] As PAH is often associated with circulating auto-antibodies, it is possible that T-cell depletion/inactivation may lead to a loss of B-cell regulation – a phenomenon seen in phosphoinositide 3 kinase (PI3K) delta knockout mice.[34] The evidence for inflammatory mediators as second triggers to induce pathology has been strengthened by several studies using adenoviral delivery of a mediator into genetically susceptible mice. For transgenic mice expressing an inducible dominant negative BMPR2 in smooth muscle, injection with an IL6-expressing virus caused a twofold increase in expression of the BMP signalling target Id1.[35] Comparable studies using a 5-lipoxygenase-expressing virus delivered to a BMPR2 heterozygous mouse resulted in leukotriene and subsequent cytokine release, enhanced RV pressure, hypertrophy and vascular remodelling.[36] The impact of these data on drug discovery

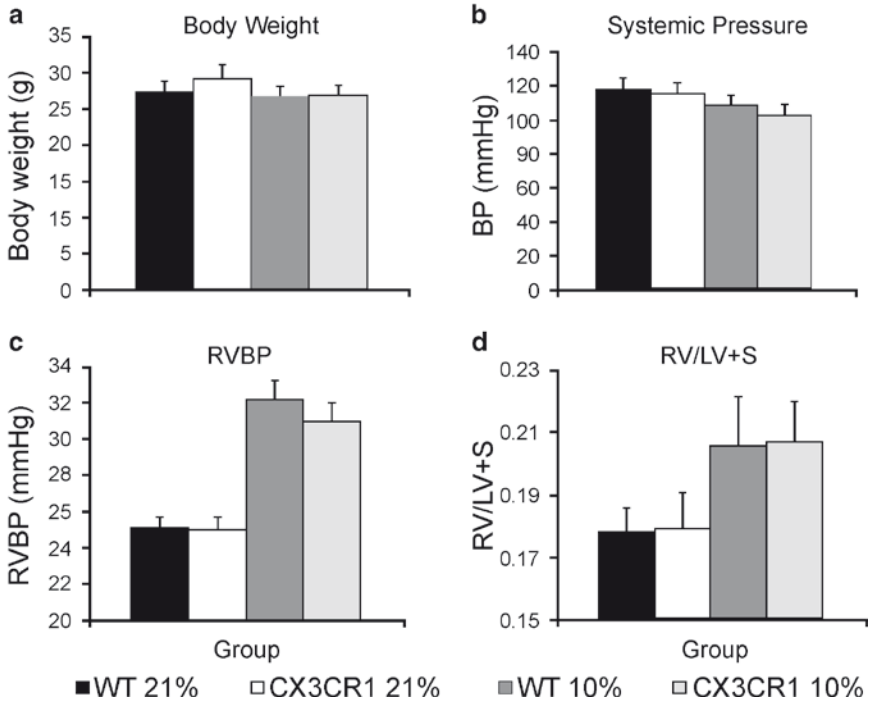

Fig. 31.2 Haemodynamic measures of wild-type (WT) and CX3CR1 knockout (KO) mice following chronic hypoxia. KO and C57Bl/6 WT mice (groups of 12) were placed in a normobaric chamber containing 10% O_2, flushed with N_2, for 3 weeks. Temperature, humidity and CO_2 levels were controlled to match the normoxic environment of WT and KO control animals. After 3 weeks, changes in body weight were determined (**a**), and the systemic pressure was measured via a tail cuff (**b**), with no differences observed between normoxic WT (*black*) and KO (*white*) controls or hypoxic WT (*dark grey*) and KO (*light grey*) test groups. Right ventricular blood pressure was measured under terminal anaesthesia via catheterization of the jugular vein and progression into the right ventricle with a Milar PV catheter (**c**). A profound rise in RV blood pressure was observed following hypoxia, although no significant changes in responsiveness were observed between WT and KO test groups. A similar pattern of response was seen when hearts were dissected and the Fulton index (RV/LV + S – a measure of right ventricular hypertrophy) was calculated, with no apparent differences in response between WT and KO animals

efforts is difficult to conclude; it can be argued that the area of inflammation and iPAH is still extremely complex and poorly understood and may be better investigated opportunistically with specific inhibitors for each of the proposed cytokine/receptor targets currently developed for alternative disease indications.

3.5 Potassium Channels

The relationship between potassium channel inhibition and PAH pathologies has been investigated for several years. However, the mechanisms by which potassium

channel changes may influence mechanisms of remodelling have only become apparent in more recent experiments. Early studies demonstrated that potassium channel activation leads to membrane hyper-polarization, whereas inhibition causes depolarization, the activation of voltage-operated calcium channels (VOCCs), an increase in intracellular calcium $[Ca^{2+}]_i$ and subsequent vasoconstriction.[37]

Of the many types of potassium channel, voltage-operated (K_V) channels have been demonstrated as essential in regulating resting membrane potential, $[Ca^{2+}]_i$ and contraction of vascular smooth muscle.[38] Indeed, K_V channels in PASMCs are inhibited by hypoxia, endothelin, thromboxane A_2 and anorectic drugs, providing further links to mechanisms common to PAH.[39,40] Furthermore, decreased K_V channel expression or function has previously been shown to be a characteristic of anorexigen-induced PAH.[41] Altered expression or activity in the $K_V1.5$ isoform in particular was identified as relevant to PAH.[42] In vivo transfer of $K_V1.5$ was shown to reduce PAH and restore hypoxic pulmonary vasoconstriction,[43] and 5-HT (via $5\text{-}HT_{2A}$ receptor) signalling demonstrated inhibition of $K_V1.5$ current and induced internalization, leading to membrane depolarization and contraction of pulmonary arteries.[44]

In addition to both clinical and mechanistic links to vasoconstriction, decreased expression/function of K_V channels in PASMCs led to inhibition of apoptosis (via decreasing caspase activity), thus promoting pulmonary vascular medial hypertrophy.[45] Furthermore, upregulation of $K_V1.5$ correlated with an increase in apoptosis and a decrease in proliferation and was able to prevent and reverse MCT-induced PAH, using dichloroacetate (DCA) as a potassium channel corrector.

DCA functions by enhancing oxidative phosphorylation via inhibition of mitochondrial pyruvate dehydrogenase kinase, thus depolarizing mitochondria and causing release of H_2O_2 and cytochrome *c*. This in turn leads to reversal of $K_V1.5$ down-regulation and a tenfold increase in apoptosis and consequent reduction in PASMC proliferation.[46] DCA is widely used as a treatment for lactic acidosis and mitochondrial diseases; however, a clinical trail for mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) was stopped early due to onset or worsening of peripheral neuropathy in 17 of 19 patients,[47] therefore limiting the development of DCA as a viable therapy for PAH. Targeting alternative potassium channels, to act either as surrogates to restore decreased $K_V1.5$ expression/function or as genuine targets in their own right, continues to be the focus of considerable investigation. The viability of KCNQ channel agonists as potential novel therapies for PAH is discussed in Chap. 26.

3.6 Notch3

The Notch pathway is an evolutionary conserved cell–cell communication pathway that regulates differentiation and development. Signalling via Notch is initiated by binding of a transmembrane ligand on an opposing cell and leads to generation of

the Notch intracellular domain (NICD), which translocates to the nucleus to activate transcription of effector genes. There are four Notch receptors and five ligands: Delta-like 1, 3 and 4 and Jagged 1 and 2. The receptors and ligands are partially redundant in function, but differences in expression and mouse KO phenotypes suggest that each has distinct roles as well.[48] Despite the fundamental nature of Notch signalling, Notch3 isoform expression is restricted to vascular smooth muscle cells (VSMCs) in adults, possibly only small/medium penetrating arteries,[49] whereas the ligands are expressed on endothelial cells (indeed, Jagged expression is increased in PAECs of PAH patients; unpublished observation).

An expanding body of evidence links Notch 3 signalling to PAH in addition to appropriate expression profile. Notch3 signalling has been shown to promote resistance to VSMC apoptosis by increasing cFLIP expression (a competitive inhibitor of Fas/caspase apoptosis pathway) and induce proliferation, thereby contributing to abnormal accumulation of VSMCs in the neo-intima.[50] Increases in Notch3 expression have also been observed in both chronic hypoxia and monocrotaline models of PAH, the pathologies within which can be attenuated by either pharmacologic inhibition with DAPT (*N*-[*N*-(3,5-diflurophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester; γ-secretase inhibitor) or by using a Notch3 KO mouse, studies detailed by Patricia Thistlethwaite in Chap. 18. Notch3 signalling is also associated with a number of mediators linked with PAH pathologies, including HIF-1α (hypoxia-inducible factor 1α; in response to hypoxia), PDGFR and BMP.

The Notch pathway has been associated with vascular development, intimal lesion formation and endothelial-to-mesenchymal transition.[10] Indeed, a study by Lilly et al. used both DAPT and Notch3-specific small-interfering RNA (siRNA) to demonstrate a critical role in the gene regulation of fibroblasts from endothelial cell co-cultures under angiogenic conditions.[51] There is also evidence that Notch3 signalling may play a role in vascular tone as well as remodelling, for which KO mouse data demonstrate a dramatic reduction in pressure-induced myogenic tone via interaction with the RhoA/ROK pathway.[52]

The potential adverse consequences of targeting Notch3 must be considered. Mutations in the Notch3 gene cause a vascular degenerative disease, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL). However, the mechanisms by which said mutations cause this disease are unknown. Three hypotheses persist to suggest that CADASIL may be due to incorrect presentation of receptors on the cell membrane, which block the normal cascade of events; an irreversible accumulation of Notch3ECD (extra cellular domain) in the membrane that dominantly inhibits the normal signalling system by competitive inhibition or by sequestering the ligand or by a toxic effect caused by accumulation of the mutant receptor form, as observed in other neurodegenerative diseases reviewed.[53] Regardless of the mechanism, CADASIL is unlikely to be reproduced by pharmacologic inhibition and is also a late-onset disease (mirrored in Notch3 KO mice, which develop pathology only after 18 months) and therefore of little concern to patients suffering from an aggressive disease such as PAH.

3.7 Serotonin

The association between serotonin (5-HT) and PAH has been well established in many studies demonstrating genetic, molecular, functional and pathologic links in both patients and preclinical rodent models. A more detailed overview of the 5-HT pathway and PAH is discussed in Chap. 20. In brief, elevated circulating levels of 5-HT have been identified in patients with PAH, correlating with pathology development.[54] 5-HT has also been shown to promote PASMC proliferation, pulmonary arterial vasoconstriction and local microthrombosis.[55] Long allele polymorphisms in the 5-HT transporter (SERT), resulting in increased gene transcription, has been linked to PAH – at least with regard to early presentation of the disease.[56] Mice over-expressing SERT develop spontaneous PAH and show enhanced pathologies under chronic hypoxic condition, whereas mice lacking SERT are resistant.[57] Furthermore, the association between dieting drugs such as dexfenfluramine are thought to be due to their action as competitive SERT substrates. Previous attempts to target 5-HT function have been hindered by the complexity and compensatory nature of the receptors (particularly $5HT_{1B}$, $5HT_{2A}$ or $5HT_{2B}$) vs. the transporter (SERT), reviewed in Ref. 58.

Tryptophan hydroxylase (TPH)–1 is the rate-limiting step in peripheral 5-HT production and thus provides a novel opportunity to effectively inhibit the various elements or signalling pathways of 5-HT in PAH. TPH1 gene expression and 5-HT production are increased in the lungs and PAECs of patients with iPAH, which together with increased SERT expression in PASMCs contributes to the PASMC hyperplasia central to the disease.[59] In hypoxia studies comparing WT with TPH1 KO mice, rises in mean RV pressure were ablated in mice lacking TPH1. The percentage of remodelled vessels was also profoundly reduced.[60] Furthermore, the TPH1/2 inhibitor *p*-chloro-phenyl-alanine (pCPA) has demonstrated efficacy in the monocrotaline preclinical model of PAH.[61] TPH1-mediated inhibition of the 5-HT pathway represents a mechanistic node central to both vasoconstrictor and remodelling aspects of PAH pathology. The action of 5-HT via $5\text{-}HT_{1B}$, $5\text{-}HT_{2A}$, $5\text{-}HT_{2B}$ or SERT has direct effects on PASMC proliferation, PAEC proliferation and transdifferentiation and thrombosis. 5-HT also interacts with ROK, thereby influencing inflammatory elements associated with the disease, as well as ROK-mediated vasoconstriction. 5-HT also affects vasoconstriction via decreasing adenyl cyclase, which raises intracellular Ca^{2+} and actin polymerisation in PASMCs. There is also evidence that 5-HT can influence vasoconstriction and remodelling via $K_V1.5$ channel inhibition.[44] Furthermore, 5-HT can also inhibit BMP signalling via stimulation of angiopoeitin 1 and Tie2 (a receptor tyrosine kinase expressed principally on vascular endothelium), thus releasing inhibition of TGF-β-mediated ALK-5 phosphorylation of SMAD2/3, thereby providing a proproliferative, pro-inflammatory and pro-fibrotic environment within the pulmonary vasculature. The central role for 5-HT and how TPH1 inhibition may influence the many pathways and pathologies of PAH are shown in Fig. 31.3.

However, potential challenges remain for TPH1 as a target. Gaining selectivity for TPH1 over the closely conserved TPH2 – the dominant isoform responsible for 5-HT production in the central nervous system – may be required for avoiding behavioural side effects. Indeed, selectivity over the other members of the aromatic

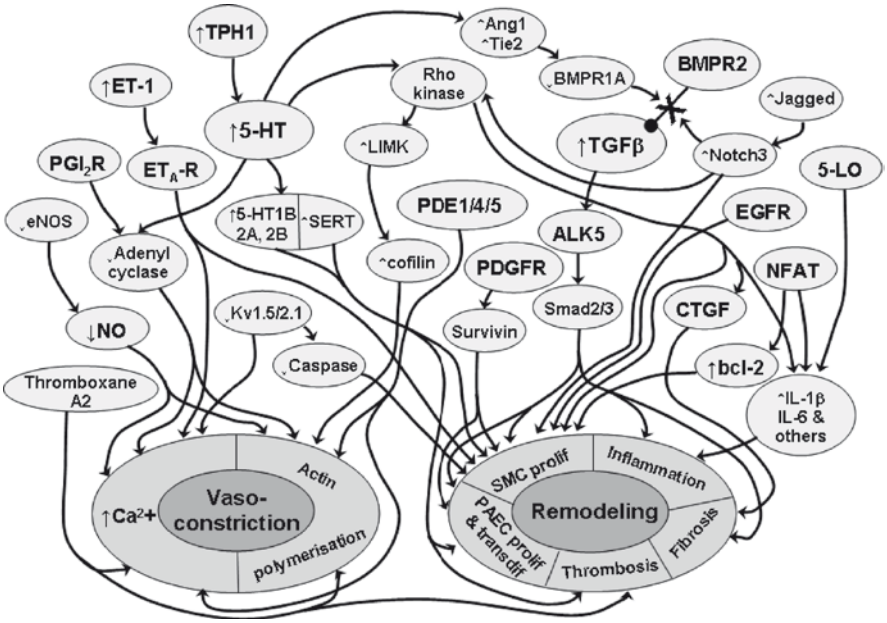

Fig. 31.3 The pathophysiology of PAH can be viewed as a combination of both vasoconstriction and vascular remodelling. Vasoconstriction is dependent on increases in intracellular Ca^{2+} and actin polymerisation, whereas remodelling processes include smooth muscle cell proliferation, endothelial cell proliferation and transdifferentiation and inflammatory, fibrotic and thrombotic mechanisms. A simplified diagram of where signalling mechanisms influence the pathophysiology of PAH is shown. The bias of current therapies toward vasoconstrictive elements of the disease can be seen. Furthermore, interconnections between pathways highlight potential nodes within the disease and therefore targets that may represent opportunities to have an impact on both vasoconstrictive and remodelling aspects of PAH (e.g. 5-HT can act directly on the remodelling of smooth muscle and endothelial cells as well as thrombotic mechanisms yet may also influence vascular tone via adenyl cyclase and RhoA/ROK pathways, as well as inflammation and fibrosis via interaction with the BMP/TGF-β axis)

amino acid hydroxylase family, tyrosine and phenylalanine hydroxylase would be a must for any therapeutic compound. An alternative, or additive, strategy may be to design a compound with restricted blood–brain barrier penetrance, thus not only avoiding the site of action of related hydroxylases but also negating any potential role for TPH1 in brain 5-HT production.

4 Conclusions

Where the current therapeutic options are featured within a scheme of pathological mechanisms, compared to some of the proposed targets discussed, is shown in Fig. 31.3. It can be seen that the proposed mechanism of action for antagonists of prostacyclin, endothelin or PDE-5 predominantly influence vasoconstrictive elements

of PAH pathology. Inhibition of PDGF, on the other hand, is largely restricted to affecting PASMC proliferation and PAEC proliferation and transdifferentiation. It is apparent that RhoA/ROK inhibition has the potential to provide profound improvements in haemodynamic parameters in preclinical models and patients with PAH. Indeed, it can be argued that the apparent modest efficacy observed with current vasodilators simply reflects inefficiencies in a relevant mode of action – could achieving greater vasodilation (via RhoA/ROK inhibition) dramatically improve efficacy? However, the dramatic effects of chronic RhoA/ROK inhibition may also be related to an impact on PASMC proliferation, inflammation or CTGF-mediated fibrosis. Inhibition of ALK-5 signalling also represents a largely anti-remodelling therapy, although there is evidence that sites of action might also include inflammatory and fibrotic elements of the disease. The role of inflammatory targets, although undoubtedly contributing to disease pathologies, is controversial with regard to therapeutic value.

Further investigation in preclinical models and patient tissue/biomarker analysis, potentially utilizing the more selective generation of anti-inflammatory therapeutics becoming available, may determine which of the multitude of inflammatory elements is most important in PAH and whether inhibition might lead to clinical improvement. Potassium channel agonism may have an impact on both vasoconstrictor and remodelling aspects of PAH. However, rescue of the K_V channels whose decrease in expression and function is associated with the disease is a challenge for classical pharmacologic methods. An alternative strategy may be to increase the function of surrogate K channels in the hope of compensating for the loss of others during disease development. The feasibility of this hypothesis is yet to be thoroughly investigated.

The inhibition of Notch3 represents another potential functional node within PAH. Direct effects on PASMC proliferation may be further compounded by interaction with both BMP and RhoA/ROK signalling pathways, thereby indirectly influencing PAEC proliferation, inflammation, fibrosis and actin polymerisation. Although broad-spectrum Notch signalling inhibition can be achieved by some γ-secretase antagonists, therapeutic use may be limited by the range of side effects observed.[62] Strategies to selectively inhibit Notch3 and thus benefit from targeting a restricted expression profile would almost certainly improve safety concerns.

Inhibition of the 5-HT pathway has been proposed as a therapeutic strategy for PAH for a number of years, perhaps unsurprisingly when interactions between this mediator and every pathological mechanism can be demonstrated. It can be argued that the relatively slow progress of therapeutics targeting 5-HT is due to the complex and compensatory nature of the receptors and transporter through which 5-HT functions. It is possible that inhibiting peripheral 5-HT synthesis via TPH1 antagonism may provide a more successful therapeutic strategy.

The aforementioned targets of remodelling within PAH are far from exhaustive but perhaps represent many of the areas under current investigation and development. It is hoped and anticipated that therapies that modify remodelling aspects of the disease will have a more profound impact on morbidity than current vasodilator strategies. The aggressive nature of PAH has broadened the acceptable adverse

effect profile of more novel targets. However, as novel, more effective therapies are developed, so will the life expectancy of PAH patients increase, which will in turn increase the need for compounds with improved safety characteristics A 5-year exposure safety risk is of little consequence to patients hoping to extend life by 2 years, unless therapies become effective enough to extend life beyond 5 years, thus making such a long term safety risk clinically relevant. It is clear, therefore, that despite recent advances, there is a long way to go before a range of therapies can provide long-term clinical benefit to a patient with PAH.

References

1. Colson P, Saussine M, Séguin JR, Cuchet D, Chaptal PA, Roquefeuil B (1992) Hemodynamic effects of anesthesia in patients chronically treated with angiotensin-converting enzyme inhibitors. Anesth Analg 74:805–808
2. Narine L, Hague LK, Walker JH et al (2005) Cost-minimization analysis of treprostinil vs. epoprostenol as an alternate to oral therapy non-responders for the treatment of pulmonary arterial hypertension. Curr Med Res Opin 21:2007–2016
3. Kenyon KW, Nappi JM (2003) Bosentan for the treatment of pulmonary arterial hypertension. Ann Pharmacother 37:1055–1062
4. Gilbert C, Brown MCJ, Cappelleri JC, Carlsson M, McKenna SP (2009) Estimating a minimally important difference in pulmonary arterial hypertension following treatment with sildenafil. Chest 135:137–142
5. Teichert-Kuliszewska K, Kutryk MJB, Kuliszewski MA et al (2006) Bone morphogenetic protein receptor-2 signaling promotes pulmonary arterial endothelial cell survival: implications for loss-of-function mutations in the pathogenesis of pulmonary hypertension. Circ Res 98:209–217
6. Yang J, Davies RJ, Southwood M et al (2008) Mutations in bone morphogenetic protein type II receptor cause dysregulation of Id gene expression in pulmonary artery smooth muscle cells: implications for familial pulmonary arterial hypertension. Circ Res 102:1212–1221
7. Bonnet S, Rochefort G, Sutendra G et al (2007) The nuclear factor of activated T cells in pulmonary arterial hypertension can be therapeutically targeted. Proc Natl Acad Sci U S A 104:11418–11423
8. Merklinger SL, Jones PL, Martinez EC, Rabinovitch M (2005) Epidermal growth factor receptor blockade mediates smooth muscle cell apoptosis and improves survival in rats with pulmonary hypertension. Circulation 112:423–431
9. Sakao S, Taraseviciene-Stewart L, Lee JD, Wood K, Cool CD, Voelkel NF (2005) Initial apoptosis is followed by increased proliferation of apoptosis-resistant endothelial cells. FASEB J 19:1178–1180
10. Arciniegas E, Frid MG, Douglas IS, Stenmark KR (2007) Perspectives on endothelial-to-mesenchymal transition: potential contribution to vascular remodeling in chronic pulmonary hypertension. Am J Physiol Lung Cell Mol Physiol 293:L1–L8
11. Heldin CH, Westermark B (1999) Mechanism of action and in vivo role of platelet-derived growth factor. Physiol Rev 79:1283–1316
12. Balasubramaniam V, Le Cras TD, Ivy DD, Grover TR, Kinsella JP, Abman SH (2003) Role of platelet-derived growth factor in vascular remodeling during pulmonary hypertension in the ovine fetus. Am J Physiol Lung Cell Mol Physiol 284:L826–L833
13. Perros F, Montani D, Dorfmuller P et al (2008) Platelet-derived growth factor expression and function in idiopathic pulmonary arterial hypertension. Am J Respir Crit Care Med 178:81–88
14. Schermuly RT, Dony E, Ghofrani HA et al (2005) Reversal of experimental pulmonary hypertension by PDGF inhibition. J Clin Invest 115:2811–2821
15. Barst RJ (2005) PDGF signaling in pulmonary arterial hypertension. J Clin Invest 115:2691–2694

16. Wang S, Wilkes MC, Leof EB, Hirschberg R (2005) Imatinib mesylate blocks a non-Smad TGF-β pathway and reduces renal fibrogenesis in vivo. FASEB J 19:1–11
17. Nagaoka T, Fagan KA, Gebb SA et al (2005) Inhaled Rho kinase inhibitors are potent and selective vasodilators in rat pulmonary hypertension. Am J Respir Crit Care Med 171:494–499
18. Kataoka C, Egashira K, Ishibashi M et al (2004) Novel anti-inflammatory actions of amlodipine in a rat model of arteriosclerosis induced by long-term inhibition of nitric oxide synthesis. Am J Physiol Heart Circ Physiol 286:H768–H774
19. Watts KL, Spiteri MA (2004) Connective tissue growth factor expression and induction by transforming growth factor-β is abrogated by simvastatin via a Rho signaling mechanism. Am J Physiol Lung Cell Mol Physiol 287:L1323–L1332
20. Jiang BHM, Tawara SM, Abe KM, Takaki AP, Fukumoto YM, Shimokawa HM (2007) Acute vasodilator effect of fasudil, a Rho-kinase inhibitor, in monocrotaline-induced pulmonary hypertension in rats. J Cardiovasc Pharmacol 49:85–89
21. Oka M, Fagan KA, Jones PL, McMurtry IF (2008) Therapeutic potential of RhoA/Rho kinase inhibitors in pulmonary hypertension. Br J Pharmacol 155:444–454
22. Chapados R, Abe K, Ihida-Stansbury K et al (2006) ROK controls matrix synthesis in vascular smooth muscle cells: coupling vasoconstriction to vascular remodeling. Circ Res 99:837–844
23. Tawara SM, Fukumoto YM, Shimokawa HM (2007) Effects of combined therapy with a Rho-kinase inhibitor and prostacyclin on monocrotaline-induced pulmonary hypertension in rats. J Cardiovasc Pharmacol 50:195–200
24. Kao PN (2005) Simvastatin treatment of pulmonary hypertension: an observational case series. Chest 127:1446–1452
25. Frid MG, Brunetti JA, Burke DL et al (2006) Hypoxia-induced pulmonary vascular remodeling requires recruitment of circulating mesenchymal precursors of a monocyte/macrophage lineage. Am J Pathol 168:659–669
26. Gillespie MN, Goldblum SE, Cohen DA, McClain CJ (1988) Interleukin 1 bioactivity in the lungs of rats with monocrotaline-induced pulmonary hypertension. Proc Soc Exp Biol Med 187:26–32
27. Perkett EA, Lyons RM, Moses HL, Brigham KL, Meyrick B (1990) Transforming growth factor-β activity in sheep lung lymph during the development of pulmonary hypertension. J Clin Invest 86:1459–1464
28. Tuder RM, Groves B, Badesch DB, Voelkel NF (1994) Exuberant endothelial cell growth and elements of inflammation are present in plexiform lesions of pulmonary hypertension. Am J Pathol 144:275–285
29. Dorfmuller P, Perros F, Balabanian K, Humbert M (2003) Inflammation in pulmonary arterial hypertension. Eur Respir J 22:358–363
30. Balabanian K, Foussat A, Dorfmuller P et al (2002) CX3C Chemokine fractalkine in pulmonary arterial hypertension. Am J Respir Crit Care Med 165:1419–1425
31. Daley E, Emson C, Guignabert C et al (2008) Pulmonary arterial remodeling induced by a Th2 immune response. J Exp Med 205:361–372
32. Ulrich S, Nicolls MR, Taraseviciene L, Speich R, Voelkel N (2008) Increased regulatory and decreased $CD8^+$ cytotoxic T cells in the blood of patients with idiopathic pulmonary arterial hypertension. Respiration 75:272–280
33. Taraseviciene-Stewart L, Nicolls MR, Kraskauskas D et al (2007) Absence of T cells confers increased pulmonary arterial hypertension and vascular remodeling. Am J Respir Crit Care Med 175:1280–1289
34. Zhang TT, Okkenhaug K, Nashed BF et al (2008) Genetic or pharmaceutical blockade of p110δ phosphoinositide 3-kinase enhances IgE production. J Allergy Clin Immunol 122:811–819
35. Hagen M, Fagan K, Steudel W, Carr M, Lane K, Rodman DM, West J (2007) Interaction of interleukin-6 and the BMP pathway in pulmonary smooth muscle. Am J Physiol Lung Cell Mol Physiol 292:L1473–L1479
36. Song Y, Coleman L, Shi J et al (2008) Inflammation, endothelial injury, and persistent pulmonary hypertension in heterozygous BMPR2-mutant mice. Am J Physiol Heart Circ Physiol 295:H677–H90

37. Yuan XJ (1995) Voltage-gated K^+ currents regulate resting membrane potential and $[Ca^{2+}]_i$ in pulmonary arterial myocytes. Circ Res 77:370–378
38. Archer SL, Souil E, Dinh-Xuan AT et al (1998) Molecular identification of the role of voltage-gated K^+ channels, $K_{V1\ 5\ and\ KV}2.1$, in hypoxic pulmonary vasoconstriction and control of resting membrane potential in rat pulmonary artery myocytes. J Clin Invest 101:2319–2330
39. Cogolludo A, Moreno L, Bosca L, Tamargo J, Perez-Vizcaino F (2003) Thromboxane A_2-induced inhibition of voltage-gated K^+ channels and pulmonary vasoconstriction: role of protein kinase Cς. Circ Res 93:656–663
40. Shimoda LA, Sylvester JT, Sham JSK (1998) Inhibition of voltage-gated K^+ current in rat intrapulmonary arterial myocytes by endothelin-1. Am J Physiol Lung Cell Mol Physiol 274:L842–L853
41. Weir EK, Reeve HL, Huang JMC, Michelakis E, Nelson DP, Hampl V, Archer SL (1996) Anorexic agents aminorex, fenfluramine, and dexfenfluramine inhibit potassium current in rat pulmonary vascular smooth muscle and cause pulmonary vasoconstriction. Circulation 94:2216–2220
42. Remillard CV, Tigno DD, Platoshyn O et al (2007) Function of $K_V1.5$ channels and genetic variations of KCNA5 in patients with idiopathic pulmonary arterial hypertension. Am J Physiol Cell Physiol 292:C1837–C1853
43. Pozeg ZI, Michelakis ED, McMurtry MS et al (2003) In vivo gene transfer of the O_2-sensitive potassium channel $K_V1.5$ reduces pulmonary hypertension and restores hypoxic pulmonary vasoconstriction in chronically hypoxic rats. Circulation 107:2037–2044
44. Cogolludo A, Moreno L, Lodi F, Frazziano G, Cobeno L, Tamargo J, Perez-Vizcaino F (2006) Serotonin inhibits voltage-gated K^+ currents in pulmonary artery smooth muscle cells: role of $5\text{-}HT_{2A}$ receptors, caveolin-1, and $K_V1.5$ channel internalization. Circ Res 98:931–938
45. Ekhterae D, Platoshyn O, Zhang S, Remillard CV, Yuan JX-J (2003) Apoptosis repressor with caspase domain inhibits cardiomyocyte apoptosis by reducing K^+ currents. Am J Physiol Cell Physiol 284:C1405–C1410
46. McMurtry MS, Bonnet S, Wu X, Dyck JRB, Haromy A, Hashimoto K, Michelakis ED (2004) Dichloroacetate prevents and reverses pulmonary hypertension by inducing pulmonary artery smooth muscle cell apoptosis. Circ Res 95:830–840
47. Kaufmann P, Engelstad K, Wei Y et al (2006) Dichloroacetate causes toxic neuropathy in MELAS: a randomized, controlled clinical trial. Neurology 66:324–330
48. Harper JA, Yuan JS, Tan JB, Visan I, Guidos CJ (2003) Notch signaling in development and disease. Clin Genet 64:461–472
49. Domenga V, Fardoux P, Lacombe P et al (2004) Notch3 is required for arterial identity and maturation of vascular smooth muscle cells. Genes Dev 18:2730–2735
50. Campos AH, Wang W, Pollman MJ, Gibbons GH (2002) Determinants of Notch-3 receptor expression and signaling in vascular smooth muscle cells: implications in cell-cycle regulation. Circ Res 91:999–1006
51. Lilly B, Kennard S (2009) Differential gene expression in a coculture model of angiogenesis reveals modulation of select pathways and a role for Notch signaling. Physiol Genomics 36:69–78
52. Belin de Chantemele EJ, Retailleau K, Pinaud F et al (2008) Notch3 is a major regulator of vascular tone in cerebral and tail resistance arteries. Arterioscler Thromb Vasc Biol 28:2216–2224
53. Bellavia D, Checquolo S, Campese AF, Felli MP, Gulino A, Screpanti I (2008) Notch3: from subtle structural differences to functional diversity. Oncogene 27:5092–5098
54. Hervé P, Launay JM, Scrobohaci ML et al (1995) Increased plasma serotonin in primary pulmonary hypertension. Am J Med 99:249–254
55. MacLean MR, Hervé P, Eddahibi S, Adnot S (2000). 5-Hydroxytryptamine and the pulmonary circulation: receptors, transporters and relevance to pulmonary arterial hypertension. Br J Pharmacol 131:161–168
56. Willers ED, Newman JH, Loyd JE et al (2006) Serotonin transporter polymorphisms in familial and idiopathic pulmonary arterial hypertension. Am J Respir Crit Care Med 173:798–802
57. Guignabert C, Izikki M, Tu LI et al (2006) Transgenic mice overexpressing the 5-hydroxytryptamine transporter gene in smooth muscle develop pulmonary hypertension. Circ Res 98:1323–1330

58. Hoyer D, Hannon JP, Martin GR (2002) Molecular, pharmacological and functional diversity of 5-HT receptors. Pharmacol Biochem Behav 71:533–554
59. Eddahibi S, Guignabert C, Barlier-Mur AM et al (2006) Cross talk between endothelial and smooth muscle cells in pulmonary hypertension: critical role for serotonin-induced smooth muscle hyperplasia. Circulation 113:1857–1864
60. Morecroft I, Dempsie Y, Bader M et al (2007) Effect of tryptophan hydroxylase 1 deficiency on the development of hypoxia-induced pulmonary hypertension. Hypertension 49:232–236
61. Kay JM, Keane PM, Suyama KL (1985) Pulmonary hypertension induced in rats by monocrotaline and chronic hypoxia is reduced by p-chlorophenylalanine. Respiration 47:48–56
62. Pissarnitski D (2007) Advances in gamma-secretase modulation. Curr Opin Drug Discov Dev 10:392–402

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