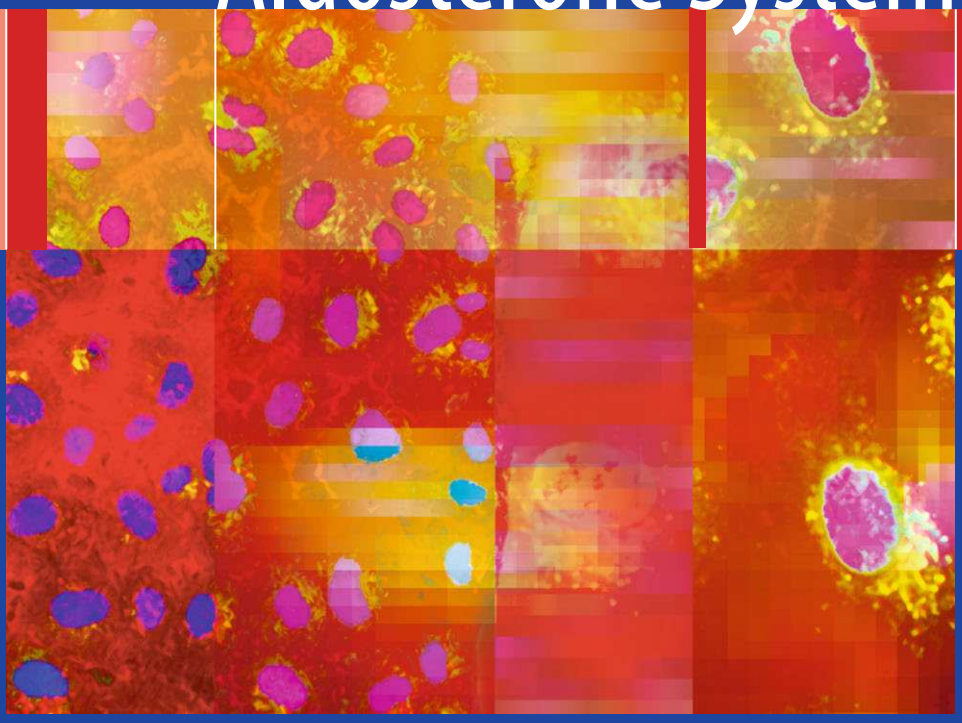
Edward D. Frohlich
Richard N. Re *Editors*

The Local Cardiac Renin–Angiotensin Aldosterone System



Second Edition

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Edward D. Frohlich · Richard N. Re
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Preface

With great pleasure we present our Third International Workshop on The Local Cardiac Renin–Angiotensin–Aldosterone System (RAAS), held at the Ochsner Clinic Foundation in November 2008. When this meeting was first organized in 2002, we were pleased that all who were invited to participate in our first workshop attended our program; and what is most satisfying, all of these participants returned for the second and this, the third workshop. In addition, we are delighted that a number of additional workers in this growing field of investigation joined us for this third program.

Over the years since our first workshop, there is little doubt as to the existence of a local RAAS in the heart. Indeed, there is substantial evidence as to the existence of other local systems in brain, vessels, adrenal, and, as presented in the current workshop, the kidneys. What is even more stimulating intellectually, through duplication of local RAAS systems a remarkable yin-yang biological balance is provided by nature. Thus, in the current third workshop considerable evidence has accumulated over the years for complicated local systems that serve to provide exquisite and unique local control of local organ functions.

This notion that local renin–angiotensin systems (RAS) operate in a variety of tissues has gained considerable importance over recent years. Although not all components of the RAAS need be synthesized in a particular tissue, local regulation of the production of angiotensin II and other angiotensin-related peptides can, nonetheless, reside at the tissue level by virtue of differential uptake of some system components and regulated synthesis of others. Moreover, the recognition that renin receptors exist in target tissues expands the possible physiological implication of local RASs by defining a new mechanism through which (pro)renin can alter tissue biology. In addition, the angiotensin (1-7)/ACE2 arm of the RAS is proving to offer important new insights into the workings of these systems in health and disease. Similarly, the local actions of aldosterone—and the possibility of the extra-adrenal synthesis of this hormone—further expand the role of the local RASs. The AT-2 angiotensin receptor is also proving to be important in previously unappreciated ways in mediating the tissue effects of the RASs. So too, new insights into the workings of the AT-1 receptor offer the prospect of better understanding the local regulation of angiotensin action. Finally, a large and growing body of evidence has recently been developed to indicate the intracellular or intracrine action of angiotensin II

and other RAS components, thereby extending the notion of the local RASs to the intracellular milieu.

This monograph is dedicated to disseminating new findings on all these levels with a focus on the local RAASs of the cardiovascular system and kidney. It is derived from the papers presented and discussed at the Third International Workshop on The Local Cardiac Renin Angiotensin Aldosterone System held November 12–14, 2008, on the campus of the Ochsner Clinic Foundation in New Orleans, Louisiana, and builds on the reports of the Ochsner Workshop of 2002 and 2004. The editors and organizers once again are extremely pleased with the willingness of outstanding investigators in the area of RAAS biology to participate both in the symposium and in the production of this monograph. We thank them for their willingness to share their latest research findings. As a result of their efforts, the editors are confident the resulting monograph will prove to be of considerable value to anyone interested in this emerging and important field of inquiry.

Finally, we wish to extend appreciation to the AstraZeneca and Forest Pharmaceutical firms, which provided support to this workshop. There were no statements of commercial interests in any of the participants' contributions before, during, or after the workshop. And, finally we wish to express the personal appreciation of the participants for the administrative support of the workshop by our staff, Mrs. Lillian Buffa and Ms. Caramia Fairchild.

New Orleans, Louisiana
20 March 2009

Edward D. Frohlich
Richard N. Re

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Chapter 1
Are Local Renin–Angiotensin Systems the Focal Points for Understanding Salt Sensitivity in Hypertension?

Edward D. Frohlich

Abstract Salt has had a prominent role in the history of man. Initially involving social, economic, and political aspects of human endeavor, in more recent decades salt has become extremely important in its role in the pathogenesis of cardiovascular and renal diseases. The magnitude of this relationship is of tremendous significance, affecting the health of billions of people throughout the world. Our laboratory studies in the adult spontaneously hypertensive rat and in its normotensive control Wistar Kyoto rat over the past 30 to 40 years have clearly demonstrated that in addition to elevating arterial pressure slightly, but significantly, long term salt loading produced severe structural and functional derangements of the vital organs. These salt induced changes have resulted in severe fibrosis (with deposition of hydroxyproline, type 1collagen), ischemia of both ventricles (the hypertrophied left as well as the non-hypertrophied right), and impaired diastolic ventricular function in the presence of preserved systolic function. The aorta demonstrated severe fibrosis and impaired distensibility and pulse wave velocity. Furthermore, the kidneys demonstrated severe changes of nephrosclerosis manifested by marked ischemia, fibrosis, small cell infiltration, glomerular sclerosis, increased total arteriolar resistance associated with afferent and efferent glomerular resistances with increased glomerular hydrostatic pressure, and marked proteinuria. The changes are typical of diastolic functional impairment of the heart and end-stage renal disease in patients with end-stage renal disease that were dramatically prevented and/or reversed by either of two angiotensin II (type 1) receptor blocking agents. These salt induced cardiac, vascular and renal structural and functional findings are strikingly similar to the target organ involvements in patients with essential hypertension associated with suppression of the endocrine rennin–angiotensin system mediated through the juxtaglomerular apparatus, We therefore suggest that these disastrous effects of salt loading are mediated through local cardiac, vascular, and renal angiotensin systems in these organs. They are dramatically supported by a large recent multicenter

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clinical trial involving prehypertensive patients who were maintained on their usual salt loaded diets and were compared with similar patients who received a salt restricted diet. Further studies are in progress to elaborate this attractive and novel mechanism of action.

Salt has had a prominent role in the history of man. Initially involving social, economic, and political aspects of human endeavor, in more recent decades, salt has become extremely important in its role in the pathogenesis of several diseases. This relatively more recent health concern relates to the role of salt in a multiplicity of cardiovascular, renal, endocrine, and neurological diseases. The magnitude of this relationship is of tremendous significance and affects the well-being of billions of people throughout the world although the underlying disease mechanisms are inadequately understood[1].

The association of salt and disease was initially based upon astute clinical observations of patients with hypertension and cardiovascular disease[2]. This relationship was subsequently supported by many epidemiological studies of large population groups which presented substantial evidence demonstrating that the greater the dietary salt intake in less developed as well as more acculturated or industrialized societies, the greater the prevalence of hypertension[3–5]. However, this relationship has been more difficult to show in the everyday clinical practice of medicine in which only a minority of patients (perhaps a third or less) with essential hypertension demonstrate this close relationship. Thus, a disturbing conundrum exists which heretofore has been unexplained[6]. In part, this may be related to two underlying factors: first, hypertensive disease has been defined epidemiologically by blood pressure measurements (albeit carefully obtained) and, second, by the general definition of "hypertension" that is based on the term "salt sensitivity" of blood pressure[7]. This latter term relies exclusively upon the response of arterial pressure to salt loading, a situation that depends upon rather rigidly defined short-term clinical procedures[7]. On the other hand, an alternative means for defining salt sensitivity could also be demonstrated by a significant reduction of arterial pressure and its consequences with sodium or salt withdrawal; but this means also has not ideally been tested systematically[8].

Thus, this situation continues to exist; but it can be approached more appropriately by re-evaluation of the definition of "hypertension" that considers hypertension as not simply the significant elevation of arterial pressure. Hypertension also depends upon the precise demonstration of the structural and functional alterations of the target organ of disease (i.e., heart, aorta, vessels, kidney, and brain) as well with the elevated arterial pressure (of course, associated with evidence of chronic salt overload)[6,9]. Indeed, appreciation of this obvious necessity has been the focus of our experimental investigative activities dealing with salt excess in hypertension over the past 40 years[10–16]. To this end, we have restricted our experimental efforts to studies of the spontaneously hypertensive rat (SHR), a strain which demonstrates naturally developing hypertension without the necessity of other experimental interventions such as ablation of renal mass, other dietary manipulations, or exogenously administered drugs such as steroids[17].

Experimental Investigations

The results of our studies have clearly demonstrated that salt excess, no matter whether by 4, 6, or 8% salt loading, consistently and significantly elevates arterial pressure (if not excessively) in the SHR. Furthermore, these responses have consistently and impressively been associated with cardiac, vascular, and renal pathophysiological changes that are identical to those changes which occur in the patient with essential hypertension (e.g., impaired arterial distensibility, left ventricular hypertrophy with primarily diastolic ventricular dysfunction). In the younger adult SHR, 25% developed systolic dysfunction with cardiac failure, but in all of the older adult SHRs and the remaining 75% of the younger adults developed diastolic dysfunction with preserved systolic function was demonstrated and was also associated with impaired aortic distensibility and nephrosclerosis with end-stage renal disease[15,16]. In addition to the latter changes in the kidney, our renal micropuncture studies (after prolonged salt loading) demonstrated renal arterial resistance increase, ischemia, afferent and efferent glomerular arteriolar constriction, increased glomerular hydrostatic pressure, severe proteinuria, increased serum creatinine and uric acid concentrations, and end-stage renal disease[16].

Clinical Investigations

In addition to the many epidemiological studies demonstrating a highly significant association between dietary intake of salt (i.e., sodium) and those clinical reports demonstrating evidence of salt sensitivity in patients, there have been a number of clinical reports that have demonstrated adverse clinical effects of salt loading. These studies included the effects of salt on arterial pulse pressure, distensibility, and pulse wave velocity[18,19]; ventricular relaxation[20]; renal function and proteinuria[21,22]; and other target organ damage in normotensive and prehypertensive individuals[23] as well as in patients with essential hypertension.

Most pertinent to this discussion was the first multicenter clinical epidemiological study, which only recently demonstrated that dietary restriction of sodium resulted in a significant reduction in cardiovascular morbidity and mortality. This study, conducted in two parts, the Trial of Hypertension Prevention (TOPH) I and II, involved 2,382 participants with "prehypertension" receiving either a sodium-restricted diet or a control normal diet which was not restricted in its sodium content[23]. The clinical outcomes of this study included stroke, myocardial infarction, coronary artery bypass grafting, percutaneous transluminal angioplasty, and cardiovascular death. Patients who had bypass grafts and angioplasty prior to the initiation of the study were excluded[23]. The findings convincingly demonstrated that the risk of cardiovascular events was about 35% lower in those prehypertensive individuals who received the sodium-restricted diet. Earlier prospective evidence supported the earlier epidemiological reports in which only a single determination of urinary sodium excretion was used to demonstrate diminished risk of stroke and coronary heart disease[24,25].

Hypothesis

A reasonable question therefore follows as to an explanation for our findings which demonstrated that salt loading produced an elevation of arterial pressure associated with pathophysiological changes that were identical to those seen in patients with essential hypertension or those "prehypertensive" individuals who received their unrestricted salt-excess diets[14–16,23]. These controlled experimental studies demonstrated changes including impaired large arterial distensibility, diastolic ventricular dysfunction with preserved systolic function, and nephrosclerosis with end-stage renal disease, which are the most common end-points of long-standing essential hypertensive disease[26,27]. Moreover, each of these reproduced experimental observations has been prevented by the co-administration of angiotensin II type I receptor blocking agents even though arterial pressure was not significantly reduced by this treatment[15,16,22]. The most plausible explanation for these findings, to our way of thinking, is that the therapeutic agents used were able to suppress the action of angiotensin II on the target organs of hypertensive cardiovascular and renal disease and, consequently, the elaboration of the structural and functional derangements of hypertension[6,15–17,26,27].

However, classic salt loading is well known to suppress the release of renin from the juxtaglomerular apparatus of the kidney and the subsequent decrease in the synthesis of angiotensin II. Our experimental findings showed that salt loading only slightly, but significantly, raised arterial pressure even though it also produced severely deranged aortic, cardiovascular, and renal diseases, which were markedly prevented individually by two different angiotensin II receptor blocking drugs in separate studies[15,16,22]. We suggest that these therapeutic agents did, in fact, act on angiotensin II type I receptors in these target organs but not by interfering with the classic renal endocrine action of renin. Thus, we hypothesize that the prolonged salt loading stimulated local renin–angiotensin–aldosterone systems in the aorta and smaller arteries; heart, and kidneys. There are much compelling recent data that support the notion that there are operable local renin-angiotensin II type 1 systems, which have been demonstrated in the target organs of hypertension studied in our studies[28–32]. Much work is necessary to demonstrate that these local systems were stimulated experimentally by salt loading, that these systems can be inhibited by angiotensin II receptor blocking drugs, and that hypertensive cardiovascular and renal diseases can similarly be prevented, inhibited, or reversed by these drugs in patients with prehypertension or with established essential hypertension.

There are several biological mechanisms which have been postulated that may explain the above findings. Our hypothesis suggests that local renin-angiotensin systems existing in the heart, arteries, kidney, and other organs are stimulated to initiate mitogenesis, collagen synthesis and fibrosis, apoptosis, and other possible pathological events[6,9]. Among the other biological actions that have been reported are salt-related mitogenesis of the cardiomyocytes; exaggerated accumulation of fibrillar collagen within the extracellular ventricular matrix and surrounding arterioles within the ventricle that are, in part, independent of the hemodynamic load; modulation of the hemodynamic response to norepinephrine, implying overactive

adrenergic function in response to sodium excess; and sodium ion facilitation of a possible role of certain growth-promoting hormones and factors[33]. However, none of these mechanisms have been prevented by an angiotensin II type 1 receptor antagonist.

Hopefully, our data and those of others will stimulate further studies that will provide additional evidence demonstrating that the mechanisms underlying the actions and events which involve the local renin-angiotensin systems in promoting the disastrous adverse clinical outcomes result from dietary sodium excess.

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Chapter 2
Newer Insights into the Biochemical Physiology of the Renin–Angiotensin System: Role of Angiotensin-(1-7), Angiotensin Converting Enzyme 2, and Angiotensin-(1-12)

Carlos M. Ferrario, Jewell A. Jessup, and Jasmina Varagic

Abstract Knowledge of the mechanisms by which the rennin–angiotensin system contributes to cardiovascular pathology continues to advance at a rapid pace as newer methods and therapies uncover the nature of this complex system and its fundamental role in the regulation of blood pressure and tissue function. The characterization of the biochemical pathways and functions mediated by angiotensin-(1-7) [Ang-(1-7)], angiotensin converting enzyme 2 (ACE2), and the mas receptor has revealed a vasodepressor and antiproliferative axis that within the rennin–angiotensin system opposes the biological actions of angiotensin II (Ang II). In addition, new research expands on this knowledge by demonstrating additional mechanisms for the formation of Ang II and Ang-(1-7) through the existence of an alternate form of the angiotensinogen substrate [angiotensin-(1-12)] which generates Ang II and even Ang-(1-7) through a non-renin dependent action. Altogether, this research paves the way for a better understanding of the intracellular mechanisms involved in the synthesis of angiotensin peptides and its consequences in terms of cell function in both physiology and pathology.

Introduction

Knowledge of the mechanisms contributing to the pathogenesis of cardiovascular disease is today at a crossroad, possibly one of its most important stages, due to rapid advances in genetics, cellular signaling mechanisms, and the addition of new therapies. Concepts, often heavily weighted by a reductionist approach to accepting the multi-faceted nature of the mechanisms contributing to organ changes in the evolution of chronic disease processes, have been confronted by new discoveries

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that do not match previous tenets[1,2]. Advances in molecular biology are bringing to the physician new and sometimes bewildering views, which he has to learn and judge in relation to clinical facts and to pressures derived from the new problems generated by advancing technology, earlier diagnosis, and longer survival. The evolution of knowledge on the contribution that the renin–angiotensin system has in the regulation of tissue perfusion in both health and disease is a good example. The past decade brought to the forefront the complexity of the renin-angiotensin system, which is more elaborate than originally accepted. The basic research knowledge of the role of angiotensin II (Ang II) in hypertension, vascular disease, and lipid and carbohydrate metabolism does not necessarily match with the outcomes of clinical trials testing the system's contribution by way of using angiotensin converting enzyme (ACE) inhibitors[1,2], Ang II receptor blockers (ARBs)[3], and now the new class of direct renin inhibitors[4–8]. It will be inappropriate to assume that this relative gap between the lessons that are learnt from testing the effects of these agents in the clinical setting and the information gained from meticulous studies of the renin-angiotensin system in animal models and cell systems suggests that one or the other has gone astray. What we need to remember is that homeostasis, in both health and disease processes, depends on the interplay of multiple regulatory mechanisms, which in the normal state act in coordination while they may become discordant in disease states.

In this chapter, we will address these issues from a viewpoint that for one of us (CMF) originates from perspectives gained from his association with Dr. Irvine H Page and from the research we conducted since the first demonstration of the biological actions of angiotensin-(1-7). Throughout this time, the slow process of unraveling pieces of this puzzle provides a more cogent understanding of the harmonious and dis-harmonious ways by which the renin-angiotensin system works to regulate normal blood pressure and its contribution to the expression of the disease, we call, essential hypertension.

A Revolving Story

Although a discussion of the biochemical pathways accounting for the formation of angiotensin peptides should begin with a description of the role of renin in the formation of angiotensin I (Ang I), for our objective we will begin with the discovery and analysis of the functions of the heptapeptide angiotensin-(1-7) [Ang-(1-7)], since its characterization became the stepping stone for a new understanding of the renin-angiotensin system. At the time of the first report of a biological effect of Ang-(1-7)[9], investigators were adamantly focused on finding a receptor for the actions of Ang II. Work on Ang II analogs and Ang II peptide antagonists suggested that the Pro^7–Phe^8 bond of the Ang II molecule was an essential requisite for binding to the as yet to be identified receptor[10–12]. Therefore, our first report that Ang-(1-7), having a truncated C-terminus, showed biological activity did not meet with any enthusiasm. Over the ensuing years, and as reviewed elsewhere, our laboratory continued to unravel the participation of Ang-(1-7) in the regulation of

blood pressure[13–33], characterize the effects of Ang-(1-7) in blocking the proliferative actions of Ang II[34–36], and decipher the biochemical pathways of Ang-(1-7) processing and metabolism[18,37–40]. Our studies and those of others further demonstrated a role for Ang-(1-7) in mediating a part of the antihypertensive actions of ACE inhibitors and ARBs[21–23,31,41,42]. As work progressed, the concept advanced that Ang-(1-7) may act in tissues as a paracrine hormone opposing the actions of Ang II[43,44]. A paracrine role for this component of the renin-angiotensin system explains the relative higher concentrations required for Ang-(1-7) effects, as it is known that at the vicinity of a tissue receptor, the concentration of the ligand may be in the nanomolar range. The characterization of the *mas*-receptor as the binding site expressing the cellular actions of Ang-(1-7) completed a critical step in defining the role of Ang-(1-7) in cardiovascular homeostasis and opened new avenues for exploring alternate approaches in competing against the pathological actions of Ang II[45,46].

A second critical step in gaining acceptance for the functional activity of Ang-(1-7) came about from the identification of angiotensin converting enzyme 2

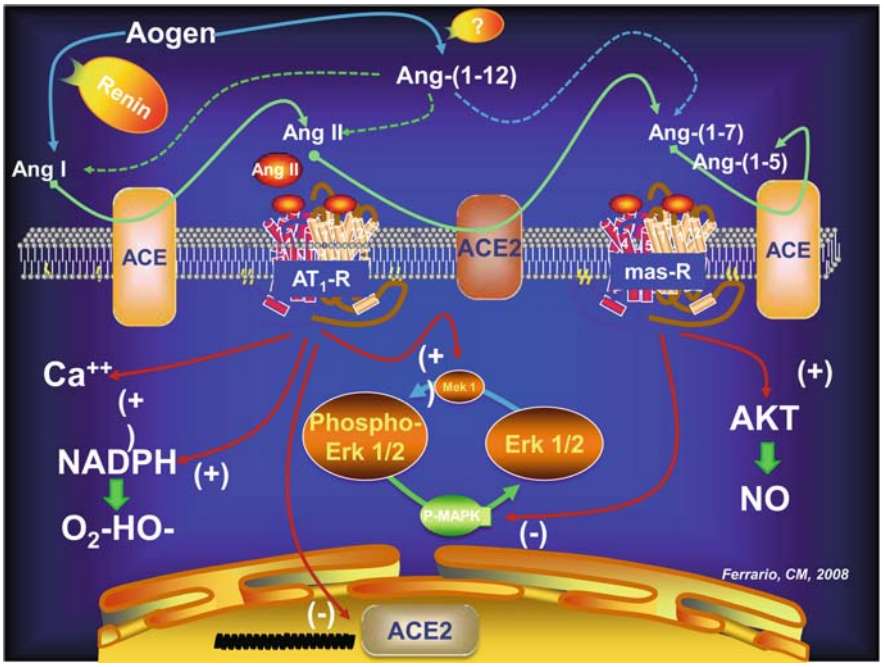

Fig. 2.1 Schematic diagram illustrating the pathways for the formation of angiotensin peptides and their actions on cellular signaling mechanisms. Abbreviations other than those described in the text are NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; O_2—HO—, a form of active oxygen species or free radical; AKT, protein kinase B; MEK 1, dual threonine and tyrosine recognition kinase that phosphorylates and activates mitogen-activated protein kinase (MAPK); ERK 1 and ERK 2, mitogen-activated protein or extracellular signal-regulated kinase p^{44ERK1} and p^{42ERK2}

(ACE2) by two separate laboratories[47,48]. These studies led to the demonstration of a high efficiency of ACE2 in converting Ang II into Ang-(1-7)[49–51], the demonstration that Ang II negatively regulated ACE2 gene expression[52–55] and that genetic or molecular approaches to suppress ACE2 function were associated with cardiac abnormalities, vascular proliferative responses, and worsening of type 2 diabetes[56–62]. Several review articles detail the knowledge that was gained from exploring the role of ACE2 in cardiovascular pathology, its interaction with Ang-(1-7), and its role as the cellular entry point for the severe acute respiratory syndrome (SARS) virus[63–65]. Figure 2.1 illustrates the biochemical pathways leading to the formation of the biologically active angiotensin peptides.

New Precursor of Angiotensin Peptides

Our original view of the RAS as a complex system entailing several levels of regulation and processing expanded with the identification of proangiotensin-12 [angiotensin-(1-12), Ang-(1-12)] as an upstream propeptide to Ang I. Nagata et al.[66] first isolated this novel angiotensinogen-derived peptide from the rat small intestine. Consisting of 12 amino acids, this peptide was termed proangiotensin-12 [Ang-(1-12)], based on its possible role as an Ang II precursor. Ang-(1-12) constricted aortic strips and, when infused intravenously, raised blood pressure in rats[66]. The vasoconstrictor response to Ang-(1-12) was abolished by either captopril or the Ang II type I receptor blocker, CV-11974. Over the years, questions arose regarding the capability of tissues other than the kidneys to synthesize Ang II, in part because gene expression for some of the RAS components occurs at low levels (i.e., renin and Aogen). The heart is a critical example. Although a large body of evidence suggests a participation of local tissue RAS in the regulation of cardiac function and remodeling[67–76], most studies showed low levels of gene expression for both cardiac renin and Aogen. Neither the identification of renin in cardiac mast cells[77] nor the finding of renin activation by prorenin binding to the prorenin/renin receptor[78–80] can be construed as evidence for local production of cellular renin, as an uptake mechanism from the blood compartment cannot be excluded.

Many cell types in myocardial tissue, including cardiomyocytes, contain receptors for Ang II, but as indicated above the activation of these receptors requires angiotensin concentrations in the micromolar range, which do not occur in plasma in vivo. However, angiotensins formed locally in the heart can activate these receptors in a paracrine and autocrine mode[81]. Indeed, recent studies have provided compelling evidence for the existence of an intracellular RAS that functions as an *autocrine* system[67,68,74–76,81]. It has been suggested that intracellular generation of Ang II may occur via a non-renin pathway with rerouting of Aogen to other subcellular structures[67,68,71,74–76,81–89]. With this in mind, we began to explore the potential role of Ang-(1-12) in the formation of angiotensin peptides as well as to inquire into the mechanisms that may regulate the processing of Aogen into

Ang-(1-12). Work in progress documents the expression of Ang-(1-12) in cardiac myocytes of Wistar Kyoto (WKY) rats, increased expression of Ang-(1-12) in the spontaneously hypertensive rat (SHR)[90], and evidence that Ang-(1-12) is a functional substrate for the formation of Ang I, Ang II, and even Ang-(1-7) in the isolated heart from several rat strains[91]. In rodents, the sequence of Ang-(1-12) is Asp^{1}-Arg^{2}-Val^{3}-Tyr^{4}-Ile^{5}-His^{6}-Pro^{7}-Phe^{8}-His^{9}-Leu^{10}-Leu^{11}-Tyr^{12}. Since renin specifically cleaves the Leu^{10}–Leu^{11} bond of rat Aogen to form Ang I, the cleavage between the two aromatic residues Tyr^{12}–Tyr^{13} for the liberation of Ang-(1-12) may not be accounted for by the action of renin. Assessment of the processing of Ang-(1-12) into angiotensin peptides in the isolated heart confirmed that renin is not involved in processing Ang-(1-12) into Ang I. In addition, we have recently extended these observations in rats in which bilateral nephrectomy was employed as a tool for elimination of renal renin[92]. The anephric state resulted in divergent effects on circulating and cardiac content of Ang-(1-12), Ang I, and Ang II since these peptides fell in the plasma, but increased markedly in the left ventricle of 48 h bilateral nephrectomized WKY rats compared to sham-operated controls. A 34% decrease in plasma Ang-(1-12) levels 48 h post-nephrectomy was associated with a 78 and 66% decrease in plasma Ang I and Ang II, respectively ($p < 0.05$ versus sham-animals). In contrast, cardiac content of Ang-(1-12) in anephric rats averaged 276 ± 24 fmol/mg compared to 144 ± 20 fmol/mg in sham-operated controls ($p < 0.005$). A representative example of Ang-(1-12) in cardiac myocytes is illustrated in Fig 2.2.

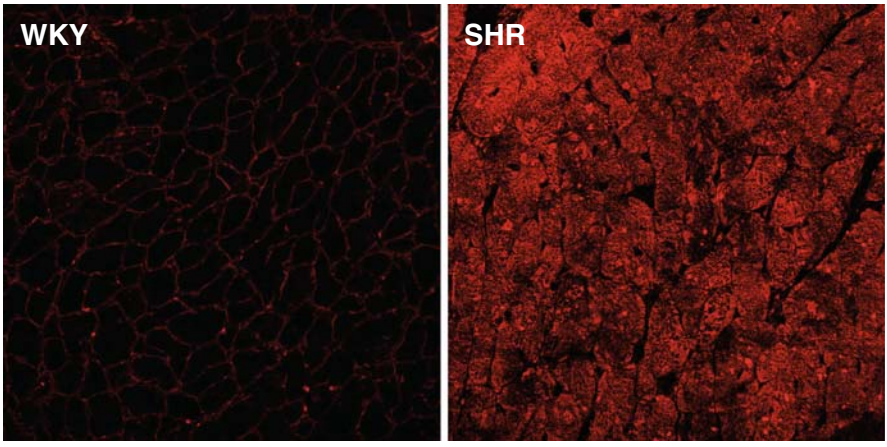

Fig. 2.2 Representative Ang-(1-12) immunofluorescence in the left ventricle of WKY and SHR rats. Data from our reference[90]

Further research on Ang-(1-12) may resolve the nature of the precursor protein accounting for the local synthesis of angiotensins (Ang II and Ang-(1-7)) in cardiovascular tissues, particularly the heart. The lower molecular weight Ang-(1-12) peptide (compared to Aogen) may represent a stored form of a precursor

to angiotensins formation since its amino acid sequence is composed of 12 rather than the 458 amino acids of the parent Aogen compound. These findings are also germane to the demonstration by others of the existence of an intrinsic mechanism for the intracellular generation of Ang II in cardiac myocytes that is independent from uptake from the circulation or the interstitial environment[68,82].

Conclusions

Our past work on Ang-(1-7) and ACE2 has been seminal in bringing about a better understanding of the biochemical and functional role of the renin-angiotensin system in cardiovascular regulation. Furthermore, the discovery of the biological actions of Ang-(1-7) provided the underpinning for the latter characterization of ACE2 and the establishment of the *mas*-receptor as a component of the system. Altogether, these studies contributed much to the affirmation of a role of this system in tissues and established a newer understanding of how the counter regulatory actions of Ang-(1-7) oppose the effects of Ang II on arterial pressure and cardiovascular remodeling. A new phase in unraveling the biochemical pathways that determine the generation of angiotensin peptides arises from studies now identifying Ang-(1-12) as an alternate Ang I forming substrate for the cellular processing of Ang II and even Ang-(1-7). The discovery of Ang-(1-12) as an endogenous substrate for the formation of angiotensin peptides may be critical to the understanding of intracellular mechanisms associated with the actions of the renin-angiotensin system in health and disease. It will be incorrect to argue that characterization of this potentially alternate substrate may have no major consequences in terms of the functions of the biologically active angiotensins. First, characterization of Ang-(1-12) as a cellular substrate may explain the observations (both clinical and experimental) of incomplete blockade of Ang II formation with the currently approved orally active renin inhibitor [aliskiren (Tekturna®)]. We have reviewed in detail the data[93] that demonstrates that the antihypertensive effects of aliskiren are not greater than those obtained with amlodipine[94] and that the addition of an angiotensin receptor blocker (ARB) markedly potentiates the response to renin inhibition[4,7]. These data suggest that aliskiren does not eliminate formation of Ang II. Further evidence for an incomplete blockade of Ang II generation was obtained in normal volunteers in whom the reduction of plasma Ang II following administration of Ang II was not complete as plasma Ang II levels returned toward control values within 12 h after oral administration[95]. To date no studies of the fate of Ang II following direct renin inhibition have been performed in essential hypertensive subjects. Thus, our recent studies on Ang-(1-12) have much significance in determining the efficacy of direct renin inhibition as the current data suggest that Ang-(1-12) generation is not dependent on renin.

If we are correct in assuming that the primary factor involved in the expression of hypertension and the remodeling of the heart and blood vessels originates from the rupture of the equilibrium between on one hand the ACE/Ang II/AT_1 receptor axis and on the other the state of the counterbalance actions of ACE2/Ang-(1-7)

/*mas*-receptor axis, a further inquiry into the mechanisms that result in reduced expression of the later regulatory arm of the renin-angiotensin system should lead to new therapies and a better understanding of the cellular processes at which these two systems act to maintain homeostasis.

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Chapter 3
Renin, Prorenin, and the (Pro)renin Receptor

Genevieve Nguyen and Aurelie Contrepas

Abstract The discovery of a receptor for renin and for its inactive precursor prorenin, and the introduction of renin inhibitors in therapeutic, has renewed the interest for the physiology of the renin-angiotensin system (RAS) and has brought prorenin back in the spotlight. The receptor known as (P)RR for (pro)renin receptor binds both renin and prorenin, and binding triggers intracellular signaling involving the MAP kinases ERK1/2 and p38. The MAP kinases activation in turn upregulates the expression of profibrotic genes, potentially leading to fibrosis, growth, and remodeling. Simultaneously, binding of renin to (P)RR increases its angiotensin I generating activity, whereas binding of prorenin induces the inactive prorenin to become enzymatically active. These biochemical characteristics of (pro)renin binding to (P)RR allow to distinguish two aspects for the new (pro)renin/(P)RR system, an angiotensin-independent function related to the intracellular signaling and its downstream effects, and an angiotensin-dependent aspect related to the increased generation of angiotensin I on the cell surface. Ongoing experimental studies should now determine which of the two aspects is the most important in pathological situations.

Abbreviations

(pro)renin:	designate renin and prorenin
AOG:	angiotensinogen
Ang I and Ang II:	angiotensin I and angiotensin II
ACE:	angiotensin converting enzyme
HRP:	handle region peptide
(P)RRB:	(pro)renin receptor blocker

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Introduction

The discovery of a specific receptor for renin and for its precursor, prorenin, has modified our concept of renin being just an enzyme responsible for the cleavage of angiotensinogen and of prorenin being just an "inactive" proenzyme. The receptor named (P)RR binds with similar affinity renin and prorenin. Binding to the receptor allows these enzymes to display increased enzymatic activity on the cell surface and to trigger intracellular signaling that in turn modifies gene expression. This implies that renin may also be considered as a hormone and that a function was finally found for prorenin. Information on the role of the (P)RR in organ damage was obtained only recently and experimental models suggest that (P)RR may play a role in the development of high blood pressure and of glomerulosclerosis, in cardiac fibrosis, and in diabetic nephropathy and retinopathy by "non-proteolytically" activating prorenin. Importantly, blocking prorenin/(P)RR interaction with a putative (P)RR blocker called "handle region peptide" (HRP) was claimed not only to prevent diabetic nephropathy but also to reverse the glomerulosclerosis of diabetic nephropathy. If this is true, then it would make (P)RR a major therapeutic goal.

Renin and Prorenin

The term "renin" is used to cover two entities:

- renin, the mature enzyme which is catalytically active in solution, and
- prorenin, the proenzyme form of renin, which is virtually inactive in solution.

Prorenin is synthesized in many organs, the kidney of course, but also the eye, the brain, the adrenal gland, the submandibular gland, the glands of the reproductive system, and the adipose tissue. All these tissues are able to secrete inactive prorenin in the surrounding milieu and in plasma, but the only tissue able to mature and secrete active renin is the kidney. Indeed, prorenin but not renin is still detectable in blood after bilateral nephrectomy, although prorenin levels are lower than in normal subjects indicating that, although kidney is the main if not the only source of renin in the body, other tissues are able to release prorenin in the circulation[7,19].

Renin

Renin is an aspartyl protease with a typical structure made of two lobes. The cleft in between the lobes contains the active site characterized by two catalytic aspartic residues. Renin is a highly specific enzyme and has only one known substrate, angiotensinogen (AOG). Renin cleaves AOG to generate angiotensin (Ang) I that is converted into Ang II by the angiotensin converting enzyme. In addition to its substrate specificity, renin catalytic activity is species-specific and renin can only

cleave AOG of the same species. Renin is synthesized by the renin-producing cells of the juxtaglomerular apparatus (JGA) and is stored as active enzyme in secretory granules from which it is released upon acute stimulation of the JGA. Renin has also been called "active" renin in opposition to the enzymatically "inactive" form of renin, prorenin[6].

Prorenin

Being the precursor of renin, prorenin was assumed to have no function of its own and yet, it represents 70–90% of total renin in human plasma. The absence of enzymatic activity of prorenin is due to the fact that a 43-amino acid N-terminal prosegment covers the cleft of the active site. Unlike the proenzymes of trypsin and of cathepsin D, prorenin does not undergo auto-activation in the plasma and its activation takes place under two circumstances: a proteolytic activation by a proconvertase whose identity is still not established and that removes the prosegment, an irreversible process that occurs in physiology in the renin-producing cells of the juxtaglomerular apparatus exclusively; and non-proteolytic activation in a test tube by exposure to low pH (pH < 3.0) or cold (4°C) and which can be imagined as a reversible unfolding of the prosegment. In plasma and in physiological conditions, however, approximately 2% of prorenin is in the open, active form and can display enzymatic activity, whereas 98% is in closed and inactive form[6].

In contrast to renin, prorenin is released constitutively and renin and prorenin levels are usually well correlated. However, under some physiopathological circumstances such as pregnancy and diabetes, prorenin levels exceed by far those of renin. In diabetes mellitus complicated by retinopathy and nephropathy prorenin is increased out of proportion to renin and this increase starts before the occurrence of microalbuminuria, so that prorenin level was suggested to be a marker of microvascular complications in diabetic patients[23,49]. Pregnant women also have high plasma prorenin levels, likely derived from the ovaries. The reason for the elevated prorenin levels in diabetes and pregnancy is unknown.

The (Pro)renin Receptor

Interestingly, the renal vasodilator response to captopril in diabetic subjects correlated better with plasma prorenin than plasma renin[42]. Possibly therefore, prorenin rather than renin is responsible for tissue angiotensin generation despite the absence of prorenin–renin conversion that cannot occur elsewhere than in the JGA cells[20]. In support of this concept, transgenic rodents with inducible prorenin expression in the liver display increased cardiac Ang I levels, cardiac hypertrophy, hypertension, and/or vascular damage without evidence for increased circulating renin or angiotensin[31,32,48]. More surprisingly, increased tissue Ang I formation occurred even when expressing a non-activatable prorenin variant mutated in the site of cleavage of the prosegment[25]. Therefore it seems logical to assume that prorenin

accumulates in tissues, e.g., via a receptor-dependent mechanism, where it can be activated in a non-proteolytic manner.

Several proteins able to bind renin and prorenin have been described, an intracellular renin-binding protein (RnBP)[24] and the mannose 6-phosphate/insulin-like growth factor II receptor (M6P/IGF2R)[34,46,47]. The intracellular RnBP was found to be an inhibitor of renin activity and its deletion affected neither blood pressure nor plasma renin[40] and it is now believed that the M6P/IGF2R is a clearance receptor for renin/prorenin[35]. This leaves the (pro)renin receptor [(P)RR] as the most promising candidate for the tissue uptake of circulating renin/prorenin.

Biochemistry of the (P)RR

The (pro)renin receptor is a 350 amino acid receptor with a single transmembrane domain, like receptors for growth factors[29]. There is no homology with any known protein based on the nucleotide and the amino acid sequence of (P)RR. Homologies in the tertiary structure have not yet been determined due to the lack of knowledge on the crystal structure of (P)RR. The receptor binds both renin and prorenin, with affinities in the nanomolar range, and the encoding gene, called *ATP6AP2* (see below), is located on the X chromosome in locus p11.4.

The initial characteristics of the (P)RR were as follows:

1. Renin and prorenin bound to the receptor are not internalized or degraded but remain on the cell surface.
2. Renin bound to the receptor displays increased catalytic activity as compared to renin in solution.
3. Receptor-bound prorenin displays Ang I generating activity in the absence of cleavage of the prosegment, most likely due to a conformational change induced by binding and non-proteolytic activation of prorenin.
4. (Pro)renin binding triggers intracellular signalization involving the mitogen-activated protein (MAP) kinase ERK1/2 and p38.

Further studies confirmed ERK1/2 phosphorylation and showed that it was due to MEK phosphorylation and provoked Elk phosphorylation[8,11,12,33]. Moreover, ERK1/2 activation resulted in the upregulation of transforming growth factor β_1 gene expression, the subsequent upregulation of genes coding for profibrotic molecules such as plasminogen-activator inhibitor-1, fibronectin, and collagens, and the induction of mesangial cell proliferation[10–12]. The ERK1/2 pathway is not the only signaling pathway linked to the (P)RR as the receptor also appears to activate the MAP kinase p38-heat shock protein 27 cascade[36] and the PI3K-p85 pathway[38]. Importantly, the latter results in the nuclear translocation of the promyelocytic zinc finger transcription factor, which downregulates the expression of the (P)RR itself[38,39]. In other words: high (pro)renin levels will suppress (P)RR expression, thereby preventing excessive receptor activation.

Prorenin binding and its subsequent non-proteolytic activation was confirmed in both primary cells[2] and in cells with transient overexpression of (P)RR.[27] Data in rat aortic vascular smooth muscle cells overexpressing the human (P)RR suggested that prorenin binds with higher affinity to the receptor than renin, so that in vivo, prorenin might be the endogenous agonist of the receptor[2]. The fact that both prorenin and renin are capable of binding to the (P)RR implies that the domains involved in the interaction between (P)RR and the (pro)renin molecule are different from the active site and are not restricted to the prosegment of prorenin. Unfortunately, due to the difficulties in generating purified recombinant (P)RR, no structure–function studies are currently available that would allow the identification of the domains of the (P)RR and (pro)renin involved in binding. In the absence of such structure–function studies or of a X-ray crystallographic structure of the (P)RR, it is difficult to design antagonists for the (P)RR.

Nevertheless, Suzuki et al.[43] made the interesting observation that, when bound to prorenin, an antibody against the sequence $I^{11P}FLKR^{15P}$ of the prosegment was able to open the pro-fragment yielding a "non-proteolytically" activated prorenin in a manner similar to the putative mechanism of (P)RR binding-induced prorenin activation. They named this region of the prosegment the "handle" region. Based on this observation, Ichihara et al.[13] tested a 10 amino acid peptide encompassing the handle region and called HRP for handle region peptide, as a blocker of prorenin–(P)RR binding. In diabetic rodents, they reasoned that diabetes would increase prorenin synthesis, thus creating optimal conditions to test the efficacy of HRP in vivo. Indeed, HRP could totally prevent or even reverse diabetic nephropathy[13,15,44] and blocked ischemia-induced retinal neovascularization and ocular inflammation in endotoxin-induced uveitis[37]. Moreover, it diminished cardiac fibrosis in stroke-prone spontaneously hypertensive rats[14]. Taken together, these data strongly suggest that the prorenin–(P)RR axis plays an essential role in end-organ damage in diabetic and inflammatory pathologies. HRP was subsequently renamed a (P)RR "blocker."

However, in vitro and in vivo studies by others did not reproduce the protective effect of HRP on organ damages well, as they did not support the inhibition of prorenin binding to its receptor by HRP[2,8,26]. Even more surprising, a FITC-labeled HRP also bound to cells devoid of the (P)RR on the plasma membrane[8]. If there is no demonstration that HRP can really block (pro)renin binding to the (P)RR, then one may wonder why it is so successful if not blocking renin–(P)RR interaction. At this moment, it cannot be ruled out that HRP also exerts other non-(P)RR-related effects, particularly in diabetic animals. Clearly, more work is needed to unravel its mechanism of action, before HRP can truly be called a (P)RR blocker.

(P)RR in Experimental Models of Cardiovascular and Renal Diseases

The high blood pressure occurring in a transgenic rat model targeting human (P)RR expression to vascular smooth muscle cells suggests a pathological role of the (P)RR in raising blood pressure[4]. Ubiquitous over-expression of the human (P)RR resulted

in proteinuria and glomerulosclerosis[16] and in cyclooxygenase-2 upregulation[17]. Both targeted and ubiquitous (P)RR expression left the plasma levels of renin and angiotensin unaltered, but did cause a rise in plasma aldosterone. Finally, in a Goldblatt model of hypertension, a parallel increase in (P)RR and renin was suggested to be profibrotic in the clipped kidney[18] and an increase of (P)RR expression was described in diabetic rats[41].

Although the claimed beneficial effects of HRP in diabetic rodents and stroke-prone spontaneously hypertensive rats are suggestive of a role of the (P)RR in fibrosis and glomerulosclerosis, no increased (P)RR expression was described in these models[13–15]. In addition, it should be noted that glomerulosclerosis did not occur in transgenic *ren-2* rats with inducible prorenin expression[29] despite the fact that such rats following induction displayed 200-fold higher prorenin levels, with no change in renin. This argues against the concept that prorenin, through a direct interaction with its receptor, induces glomerulosclerosis.

Of the two means classically used to establish the role of a receptor in pathology, the antagonist, HRP, is still speculative and the total knockout of the (P)RR is, surprisingly for a component of the renin–angiotensin system (RAS), not possible[3]. Therefore, the generation of (P)RR conditional knockout mice is becoming mandatory and such animals will allow to further establish the role of (P)RR in disease.

Unexpected Properties and Ontogeny of the (P)RR

There is only one gene called *ATP6ap2* coding for the full-length protein known as (P)RR. All other truncated forms of (P)RR derive from intracellular processing of the full-length form. The reason why the (P)RR gene is called *ATP6ap2* was because a truncated form of the (P)RR, composed of the transmembrane and cytoplasmic domains of (P)RR, had been co-purified with a vacuolar H^+-ATPase (V-ATPase)[22]. This V-ATPase is a complex, 13-subunit-protein, essential to maintain an acidic pH in intracellular vesicles and to regulate cellular pH homeostasis[30], but (P)RR is not a subunit of this V-ATPase.

The necessity of an intact *(P)RR/ATP6ap2* gene in early development is stressed by the observations that in zebra fish, the mutation of *(P)RR/ATP6ap2* gene provoked the death of the fish before the end of the embryogenesis[1] and that in rodents *(P)RR/ATP6ap2* gene expression is ubiquitous and early in development[5]. Whereas renin expression can be detected in large intrarenal arteries only at 15.5 days of gestation, (P)RR mRNA is already present on day 12 in the ureteric bud and at later stages in vesicles and S-shaped bodies (Fig. 3.2). In newborn mice (P)RR expression is high in epithelial cells of distal, proximal, and collecting tubules and low in glomeruli and arteries[5]. These observations in zebra fish and in the developing kidney suggest that the (P)RR has functions essential for cell survival and proliferation that are unrelated to the RAS.

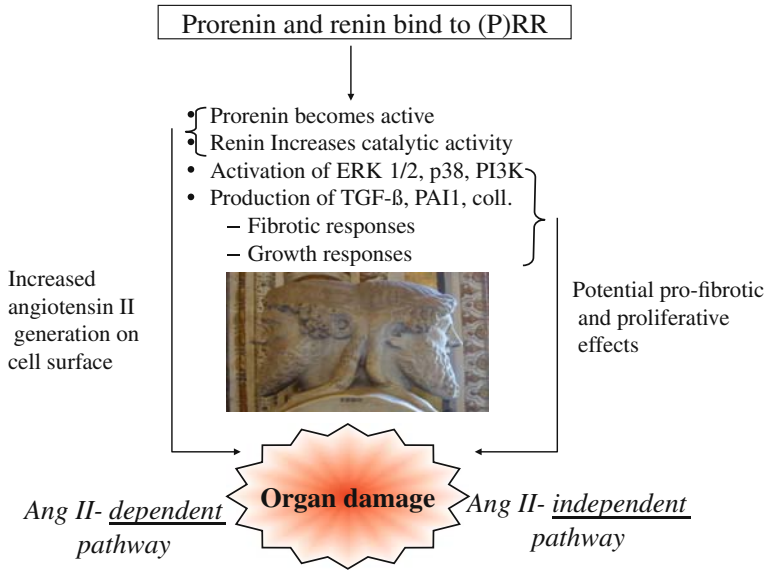

Fig. 3.1 Schematic representation of the angiotensin II-dependent and -independent consequences of (pro)renin binding to (P)RR and of (P)RR activation. Adapted from Nguyen and Danser[28]

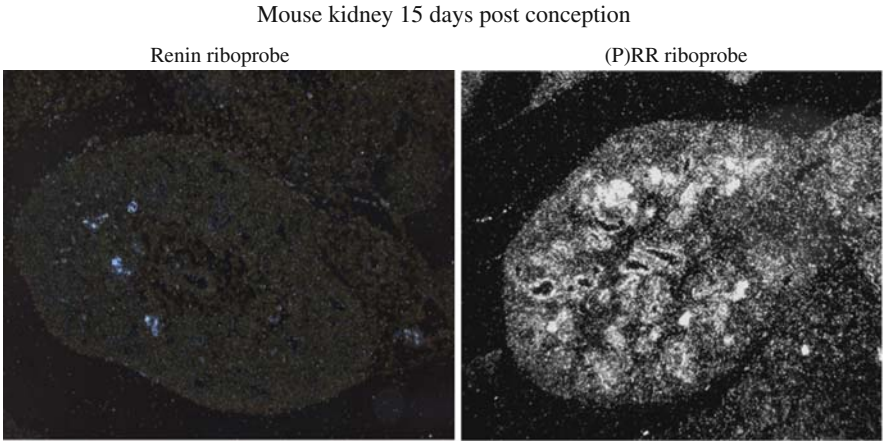

Fig. 3.2 In situ hybridization with ^{35}S-labeled mouse (P)RR (*left*) and mouse renin (*right*) riboprobes

Analysis of the sequence of (P)RR coding cDNA shows that sequence coding for the transmembrane and the intracellular domain putatively associated with the V-ATPase is remarkably conserved between invertebrates and vertebrates, whereas the cDNA sequence coding for the extracellular domain responsible for renin and prorenin binding is conserved in vertebrates only[21]. This leads to the postulate that

the *(P)RR/ATP6ap2* gene may result from the fusion of two genes, an ancient gene (corresponding with the C-terminus) coding for a protein essential for cell survival and a more recent gene in vertebrates (corresponding with the N-terminus) which binds renin and prorenin[3]. However, to date, we have no arguments to confirm or to infirm that the (P)RR role in cell survival is related to V-ATPase activity.

Conclusion

The discovery of the (P)RR has confirmed the hypothesis of Tigerstedt and Bergman more than a century ago that renin is a hormone[45]. Now, the (P)RR also endows prorenin with a function that was suspected over 25 years ago by Luetscher and Wilson in diabetic patients (1985). Experimental studies suggest that the (P)RR might be a major target in cardiovascular disease and in diabetes-induced organ damage, and tissue-specific knockout of (P)RR should soon establish whether the (P)RR plays a role in cardiovascular pathologies and in diabetes and to what degree HRP exerts (P)RR-dependent effects.

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Chapter 4
Intracellular Accumulation and Nuclear Trafficking of Angiotensin II and the Angiotensin II Type 1 Receptor

Julia L. Cook and Richard N. Re

Abstract Despite the fact that the concept of an intracellular or intracrine renin-angiotensin system has gained general acceptance over the last decade, several questions continue to trouble this research area. Empirical evidence supports the existence of intracellular angiotensin II peptide and nuclear angiotensin II type 1 receptor, translocation of the plasma membrane receptor to the nucleus, and functionality of the intracellular RAS components. However, the mechanism by which intracellular angiotensin II is generated or alternatively internalized and reutilized, and the means by which the AT_1 receptor is transported to the nuclear membrane remain unclear. This paper reviews some of the relevant literature and ideas related to intracellular trafficking of angiotensin II and its cognate receptors and presents new supporting data for alternative mechanisms by which the receptors and angiotensin II might accumulate in the nuclear compartment.

In addition to the well-characterized circulating and tissue renin–angiotensin systems (RAS), increasingly, particularly over the last decade, evidence has accumulated that supports the existence of an intracellular or intracrine RAS. The first data championing an intracellular function for angiotensin II (Ang II) was published in 1971, when Robertson and Khairallah showed that labeled Ang II, injected into rat heart, associated with nuclei of smooth muscle cells, endothelial and cardiac muscle cells[1]. Richard Re and colleagues at Ochsner Foundation corroborated those study results by showing that labeled Ang II binds to isolated rat liver and spleen nuclei in a manner that is competed by cold Ang II, competed less effectively by Ang I, and insensitive to neurotensin[2]. Those studies were consistent, therefore, with the existence of a nuclear receptor specific for Ang II. Over the next decade, other laboratories verified that Ang II binds to isolated rat liver nuclei through a specific receptor, that the binding is sensitive to the AT_1 receptor (AT_1R) blocker, losartan,

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but independent of the AT_2R blocker, PD123177, and that the binding is of functional consequence[3–6].

Collectively, the studies on isolated nuclei and intact liver support the existence of a functionally significant intracellular Ang II receptor that is AT_1-like and similar enough to the AT_1 receptor that it likely represents a post-translational modification product of the prototypical plasma membrane receptor.

Nuclear Membrane-Associated AT_1 Receptor

The studies addressed above support the existence of a nuclear AT_1-like receptor that binds Ang II and stimulates cellular events. Because several lines of evidence suggest that at least one variety of nuclear Ang II:AT_1R binding is sensitive to AT_1R blockers (ARBs)[3,6] it implies that this particular Ang II:AT_1R interaction occurs through the conventional ligand-binding pocket in the receptor "extracellular domain" and, as such, that the AT_1R retains a classical native conformation in the nuclear membrane (i.e., similar to its conformation in the plasma membrane). The diffusion-retention model for nuclear trafficking predicts that transmembrane or integral membrane proteins in the endoplasmic reticulum (ER) can diffuse laterally in a retrograde direction from the ER to the outer nuclear membrane and then through the phospholipid bilayer flanking the nuclear pores and into the inner nuclear membrane (Fig. 4.1a). This model further predicts that proteins will only be retained in the inner nuclear membrane at significant levels if the proteins bind to nucleosolic proteins, chromatin, nuclear matrix, or other intranuclear structures. Note that the AT_1R *does* accumulate, in intact cells, to high levels in nuclear membranes (Fig. 4.2). AT_1R in theory, therefore, could accumulate in the inner nuclear membrane by retrograde trafficking from the ER.

In studies utilizing isolated nuclei, Ang II introduced in the incubation buffer has access to the intranuclear membrane space through ruptures remaining in the ER:outer nuclear membrane junctions following removal of the ER (see Fig. 4.1b). How might Ang II access nuclear membrane-associated AT_1 receptor in *intact* cells?

The simplest model for the existence of intracellular Ang II, and that probably best accepted by scientists working in this area, relies on the knowledge that Ang II, within ligand:receptor complexes, is internalized via clathrin-coated pit-mediated endocytosis. Since it is well known that contents of endosomes can leak into the cytosol, Ang II in the cytosol could access binding sites within the nucleosol via nuclear pores. To our collective knowledge, however, no model has been proposed

Fig. 4.1 Diagram of the nuclear envelope. The nuclear envelope is illustrated in cross-section. (**a**) Receptors from the ER can traffic through the outer nuclear membrane, pore membrane domain, and into the inner nuclear membrane. Receptors can be maintained in the inner membrane by attachments to the lamina or chromatin. (**b**) In isolated nuclei preparations, lacerations within the ER allow entry, to the inner nuclear membrane space, of salts and peptides such as Ang II that are present in the buffer

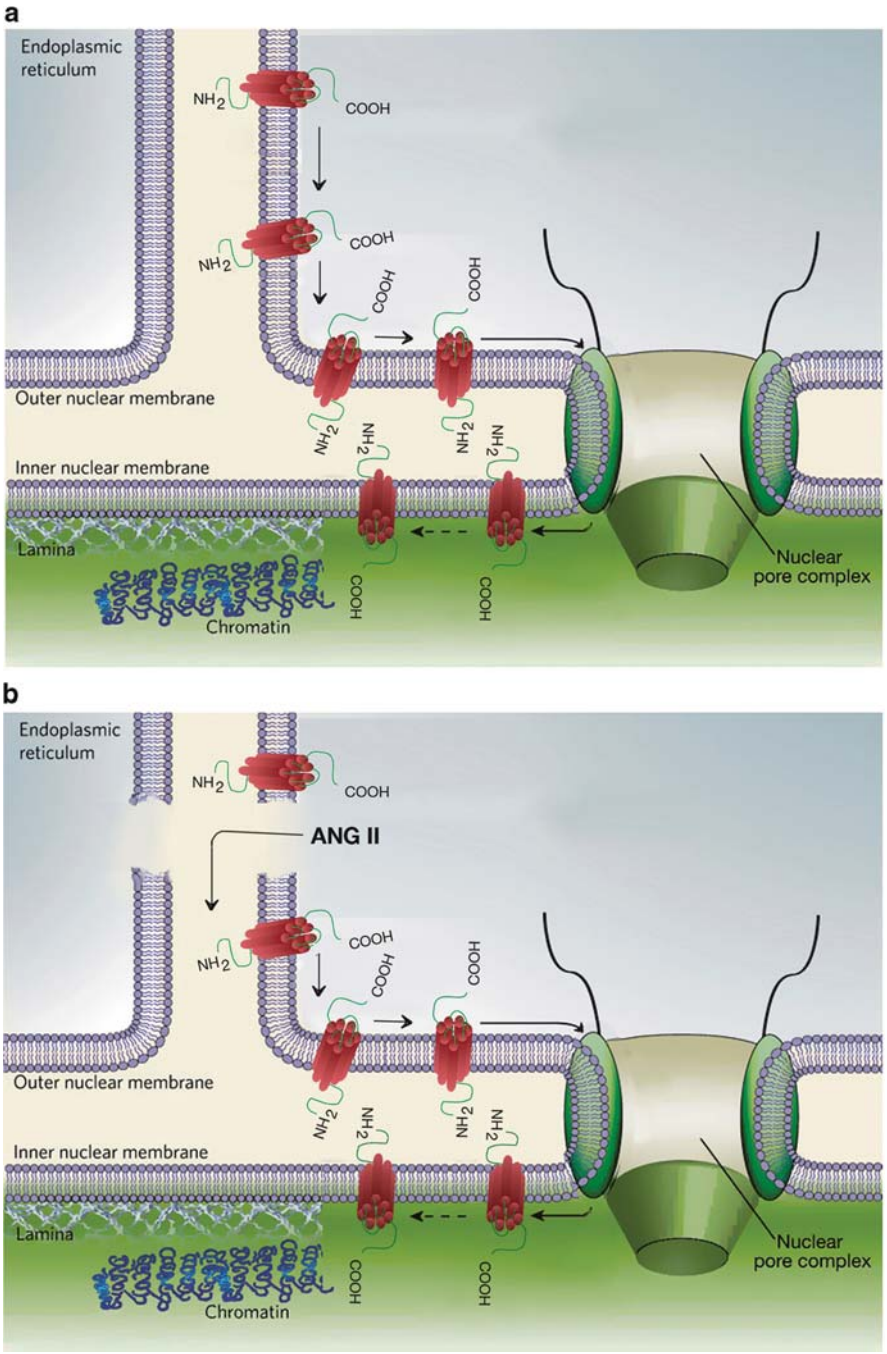

Fig. 4.1 (Continued)

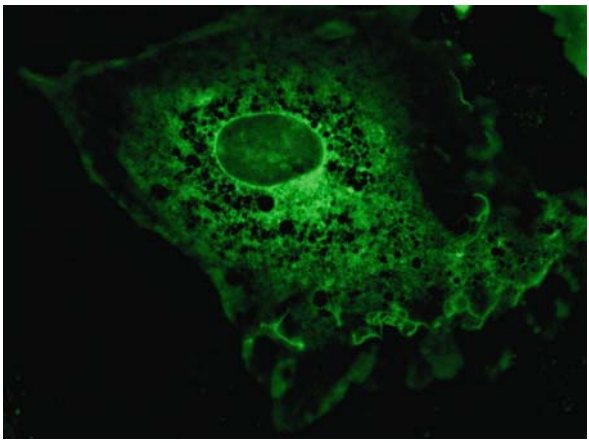

Fig. 4.2 Transfected AT_1R accumulates in the nuclear membrane. Vascular smooth muscle cell line A10 was transfected with *pAT1 R/EGFP* and imaged at 48 h post-transfection

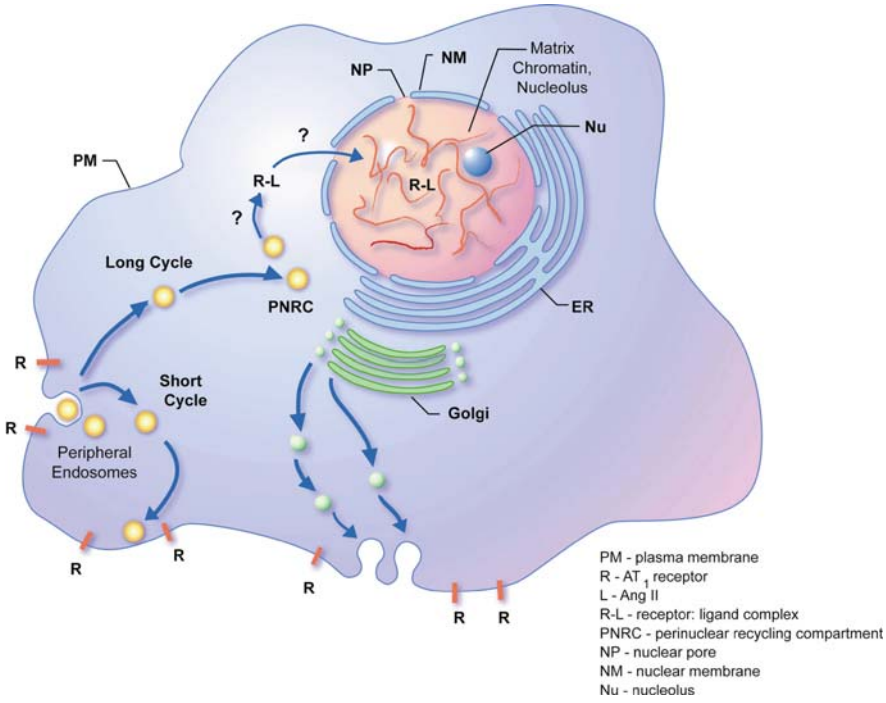

Fig. 4.3 Internalized AT_1R recycles through both long and short recycling pathways

to address Ang II transport to the intranuclear membrane space. We propose that Ang II may reach the intranuclear membrane space by way of recycling endosomes.

AT_1R/EYFP (AT_1R fused upstream of enhanced yellow fluorescent protein) is reported to be internalized via clathrin-coated pits following ligand binding and then

to traffic through two different endocytotic pathways, in HEK 293 cells[7]. Some of the receptors traffic through endosomes of the early (short) recycling pathway, others through the late (long) recycling pathway to the perinuclear recycling compartment (PNRC) (Fig. 4.3). The purpose of the long recycling pathway is not clear but it appears to slow the rate of return of receptor to the plasma membrane and it appears to be functionally distinct from the short pathway. By way of example, the endothelin receptors, ET(A) and ET(B), in CHO-K1 and COS cells, follow different routes after ET stimulation[8, 9]. While ET(A) follows the long recycling pathway and colocalizes with transferrin in the PNRC, ET(B) is targeted to the lysosomes after ET-induced internalization. ET(A) receptors are also present on the nuclear envelope membrane and induce nuclear free calcium upon application of cytosolic ET-1 suggesting that the peptide may exert part of its biological function through intracellular nuclear membranes[8]. In studies of cardiac ventricular myocytes, ET(A) has been detected on plasma membrane and to a lesser extent on the nucleus whereas ET(B) is localized to nucleus. In isolated cardiac myocyte nuclei, ET-1 is shown to increase nuclear calcium[10]. Bkaily and coworkers have also shown the existence of ET-1 receptors on nuclear membranes of VSMCs, stimulation of which regulates free nucleoplasmic calcium levels, further suggesting the existence of functionally important second messenger pathways at the level of the nuclear membrane[11].

The AT_1, vasopressin, and endothelin G protein-coupled receptors (GPCRs) as well as the TNF-α, EGF, and insulin receptors have been found associated with endosomes of the PNRC. The large endosomes of the PNRC are not only localized proximal to the nucleus, but at least some of the reported contents of the PNRC endosomes, including the EGF receptor[12–14], insulin receptor[15–17], ET receptor[11], and AT_1 receptor, can reside in the nucleus and may have active nuclear functions. Collectively, the above data suggest to us that the PNRC endosomes may act as vehicles for delivery of receptors, growth factors, and chemokines destined for the nucleus.

To investigate this possibility, we first generated a fluorescent marker of the PNRC. The Rab11 small GTP-binding protein is a specific marker of the PNRC[7]. We generated an expression plasmid encoding a fluorescent fusion protein of Rab11 with HcRed (*pHcRed/Rab11*) and transfected it into COS-7 cells to verify that the corresponding protein localizes specifically to perinuclear endosomes (Fig. 4.4). We found Rab11 to accumulate almost exclusively in large juxtanuclear endosomes consistent with the PNRC.

We next cotransfected *pHcRed/Rab11* with *pAT$_1$R/EYFP* into COS-7 cells (Fig. 4.5). Co-localization of the green AT_1R with red Rab11 is indicated by bright yellow fluorescence in the merged red and green images. AT_1R accumulates, therefore, in the PNRC. The subcellular location of the PNRC, observed in the two studies immediately above, suggested to us that it might overlap with the Golgi apparatus. We proceeded, therefore, to transfect COS cells with *pHcRed/Rab11* and *pECFP-Golgi* (Clontech, enhanced cyan fluorescent marker of the Golgi) (Fig. 4.6). Interestingly, we found that many of the membranes of the Golgi (blue) and the PNRC (red) overlap (magenta color) suggesting at least a partial fusion of the compartments, which would permit an exchange of membrane-imbedded and

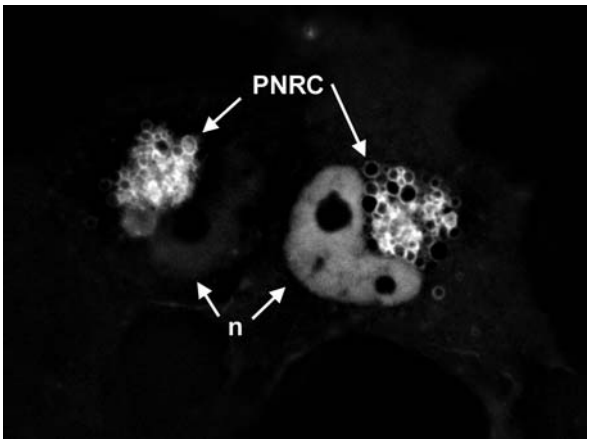

Fig. 4.4 Image of the perinuclear recycling compartment. COS-7 cells, 24 h post-transfection with *pHcRed/Rab11*. *Arrows* indicate the endosomes of the perinuclear recycling compartment (PNRC) and nucleus (n)

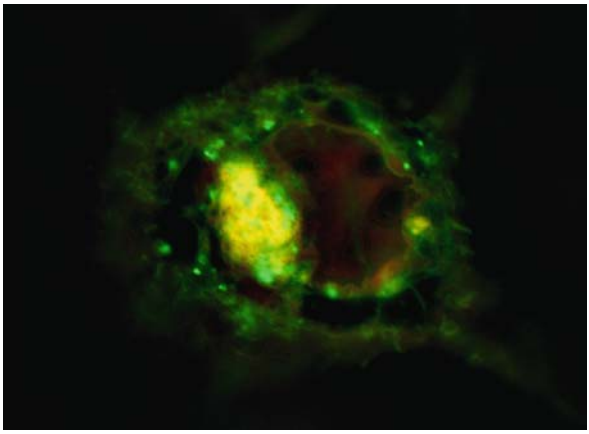

Fig. 4.5 AT_1R accumulates in the PNRC. COS-7 cells, 48 h post-transfection with *pAT1R/EYFP* (*green*) and *pHcRed/Rab11* (*red*). *Yellow color* indicates colocalized *red* and *green* proteins

lumenal materials. AT_1R and Ang II, derived from the PNRC endosomes, could be transported to the Golgi and, in a retrograde manner, back to the ER via COP1 vesicles[18]. The ER, as discussed above, is contiguous with the nuclear membrane, allowing for translocation of recycled AT_1R to the inner nuclear membrane and Ang II to the intranuclear membrane space. Recall that *newly synthesized* AT_1R may also, in theory, traffic in a retrograde fashion to the nuclear membranes. But delivery of Ang II (which, in the conventional pathway, is processed from the preproprotein, preangiotensinogen) to the inner nuclear membrane space requires a more complex model such as that we have presented here.

If Ang II does not traffic via endosomes from the plasma membrane to the perinuclear area, how might this peptide gain access to the intranuclear membrane space to activate its cognate receptor in an intact cell? What other models support activation of nuclear membrane-associated receptor? Unfortunately, there are few alternative

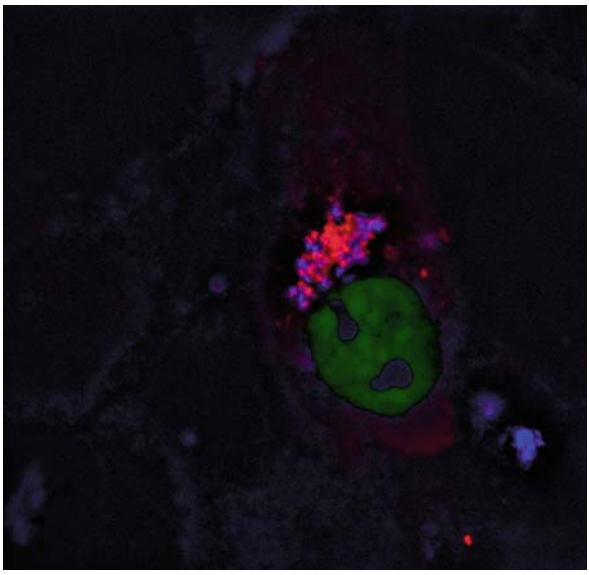

Fig. 4.6 The PNRC shares membranes with the Golgi. COS-7 cells, 48 h post-transfection with *pECFP-Golgi* (*blue*) and *pHcRed/Rab11* (*red*). *Magenta color* indicates colocalized proteins

biologically sound proposals. Certainly, other models can be offered to explain the existence of intracellular Ang II, e.g., uptake and release from endosomes, or intracellular synthesis from internalized angiotensinogen, or intracellular synthesis from a hypothetical variant form of non-secreted intracellular angiotensinogen. But, to our knowledge, trafficking of a peptide to the intranuclear membrane space requires retrograde movement through subcellular membrane pathways. One notable possibility is that Ang II could be generated from angiotensinogen, within the secretory pathway, by way of mature enzymatically active forms of renin and ACE/chymase. To our knowledge, there is no data to support this notion. But, by way of example, some growth factors are known to interact with their receptors within the secretory pathway and to mediate downstream effects. For example, v-sis interacts with the PDGF receptor within the ER and Golgi and stimulates transformation[19]. A fully transformed phenotype is still obtained following modification of the v-sis protein with a KDEL sequence, which targets it for retention in the ER/Golgi; plasma membrane ligand:receptor interaction is not required. The secretory pathway can clearly be more than a modification station or conduit to the plasma membrane or extracellular space. For the angiotensinogen protein, this is certainly a testable hypothesis.

Assuming an intranuclear membrane interaction between AT_1R and Ang II, how might signals be transduced into the nucleus? While there are considerable data to support the concept of signaling at the level of the nuclear membrane, it is a new enough area that it remains controversial. Many of the signaling molecules, including inositol phospholipids, phospholipase, diacylglycerol (DAG) and inositol triphosphate, protein kinase C and calcium, traditionally thought of as plasma membrane-associated and/or cytosolic, are alternatively associated with nuclear

membranes and nucleosol[12, 20–25]. Evidence also exists that these signaling intermediates are functional in the context of the nucleus. For example, there is strong evidence that nuclear DAG is a key molecule in the mitogenic effect of IGF-1 on 3T3 fibroblasts. Treatment of 3T3 cells with IGF-1 stimulates accumulation of nuclear DAG kinase activity, which in turn increases nuclear PKC. PKC is retained in the nuclear membrane in association with lamins A, B, and C, and nucleolin even after DAG levels return to normal. Moreover, DAG kinase inhibitors, which block the IGF-1-dependent increase in nuclear DAG kinase activity, leading to continued elevated DAG levels, also potentiate mitogenic effects[26]. Other studies support nuclear functions for phosphatidylinositol 3, 4, 5-triphosphate, phosphatidylinositol 4, 5-biphosphate, and phospholipase C in nuclear function and abnormal cell growth[27, 28]. Moreover, it has been suggested that immediate short-term effects are mediated by cell surface receptors while long-term responses may be mediated by intracellular receptors, many of which are localized at the cell nucleus[29].

Nucleosolic AT_1 Receptor

Our data support the existence of intranuclear, nucleosolic AT_1 receptors as well as nuclear membrane receptors[30, 31]. The AT_1R/EYFP fusion protein is not present in the nucleus when expressed from single plasmid transfections (*pAT1R/EYFP*). However, when cells are cotransfected with *pAT1R/EYFP* and *pECFP/AII* [encodes Ang II fused downstream of enhanced cyan fluorescent protein (ECFP)] or when *pAT1 R/EYFP*-transfected cells are treated with exogenous Ang II, yellow fluorescence accumulates in the nucleus. Our studies of the rat AT_1R (1) genetically labeled at the C-terminus with EYFP, (2) labeled at the C-terminus with EYFP and the N-terminus with ECFP (ECFP/AT_1R/EYFP), and (3) overexpressed native protein show that a population of the receptor is cleaved with trafficking of the C-terminus to the nucleus and trafficking of the N-terminus to the extracellular space[31].

Several transmembrane receptors are known to accumulate in nuclei, some as holoreceptors and others as cleaved receptor products[13, 32–36]. Image analyses of the double-labeled protein in COS-7 cells, CCF-STTG1 glial cells (Fig. 4.7), and A10 vascular smooth muscle cells show the two fluorescent moieties to be largely spatially colocalized in untreated cells. Ang II treatment, however, leads to a separation of the fluorescent moieties with yellow fluorescence accumulating in more than 30% of cellular nuclei. Immunoblot analyses of extracts and conditioned media from transfected cells indicate that the ECFP domain fused to the extracellular amino-terminal AT_1R domain is cleaved from the membrane and that the EYFP domain, together with the intracellular cytoplasmic carboxy terminus of the AT_1R, is also cleaved from the membrane-bound receptor. The carboxy terminus of the AT_1R is essential for cleavage; cleavage does not occur in protein deleted with respect to this region. Overexpressed native AT_1R (nonfusion) is also cleaved; the intracellular 6-kDa cytoplasmic domain product accumulates to a significantly higher level following Ang II treatment[31].

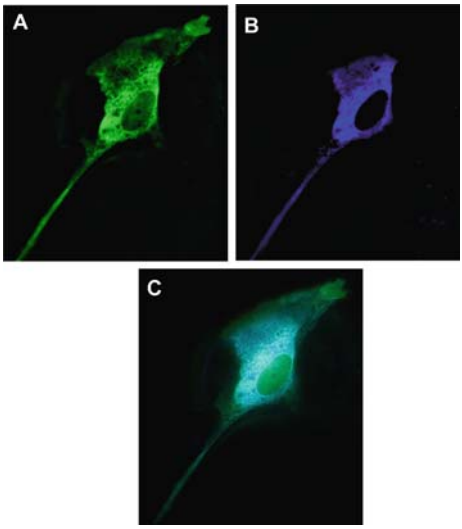

Fig. 4.7 Double-fluorescent protein ECFP/AT_1R/EYFP is cleaved. Glial cell transfected with *pECFP/AT_1R/EYFP* and imaged at 48 h post-transfection. (**a**) *Yellow* filter image, (**b**) *blue* filter image, (**c**) merged images of (**a**) and (**b**). Note accumulation of *yellow fluorescence* in the nucleus and loss of *blue fluorescence* at the cell perimeter

Since efficient trafficking of the C-terminus is dependent on Ang II, either extracellular or intracellular, it implies that intracellular (possibly cytoplasmic) Ang II is involved in nuclear trafficking of the receptor fragment. Obviously, the AT_1R C-terminus does not possess the conventional ligand-binding pocket, suggesting that Ang II may bind indirectly to the receptor fragment through other proteins in a complex and act as a cofactor for nuclear transport. The cleavage fragment (CF) consists of the entire cytoplasmic C-terminus and a portion of the seventh transmembrane domain of the AT_1R. A nuclear localization consensus sequence (NLS, KKFKK) has been identified in the membrane-proximal C-terminal domain by competitive peptide and mutational studies[37,38] and, indeed, we have verified that mutation of this sequence significantly reduces nuclear accumulation of the CF (unpublished). The small size of the CF (<8 kD) suggests that it can be translocated into the nucleus by passive diffusion and does not require a NLS. The presence of a functional NLS indicates that nuclear transport proteins, such as alpha and beta importins, bind and traffic the CF to the nucleus via microtubule pathways, perhaps in order to increase the efficiency and velocity of transport. Assuming that cytoplasmic Ang II is critical for transport of the CF to the nucleus, we must propose a model which explains the existence of cytoplasmic Ang II. As we mentioned earlier, it is well known that contents of endosomes, including peptides like Ang II, can leak into the cytosol. While endosomal escape is not a new concept, it is poorly understood. There exists a general consensus that polycation-DNA complexes enter cells via endocytotic pathways, escape the endosomes into the cytoplasm, and are then transported to the nucleus where the DNA may be expressed, but the finer points are far from understood[11]. Ligands derived from internalized receptor-ligand complexes could similarly escape endosomes and enter the nucleus via nuclear pores; indeed, mechanisms by which this may occur have been discussed at length (for review[13,14,36]).

One other possibility that we have not yet addressed is the idea that Ang II could be retrotranslocated from the ER into the cytoplasm via the ERAD (ER-associated degradation) system. This system could function following PNRC delivery to extract Ang II from the lumenal space of the trafficking pathway into the cytoplasm via the Sec61 translocon[39,40].

Recent studies in human vascular smooth muscle cells (VSMC) and endocardial endothelial cells support our findings of nuclear AT_1R localization. Bkaily and colleagues[11] show that hAT_1R/GFP is induced by exogenous Ang II to undergo rapid nuclear translocation in cultured human VSMC and increases free cytosolic and nuclear calcium. They find a diffuse, punctate nuclear accumulation of the AT_1R upon Ang II stimulation. More often, we observe nuclear receptor fluorescence as very fine and diffuse but occasionally we observe a discrete and non-uniform distribution suggesting association with the matrix or chromatin. On occasion, we also see the nuclear receptor as being clearly associated with nucleoli. Collectively, this suggests that the precise nuclear localization may be cell-type specific, developmentally regulated, and/or cell-cycle or stage-dependent. A related recent publication[11] investigates the localization of native (non-fusion) proteins of Ang II, AT_1R, and AT_2R in fetal human endocardial endothelial cells. By immunofluorescence and confocal microscopy, Jacques and colleagues[11] find Ang II to be present in nucleus and cytoplasm with high concentration in cytoplasm. AT_1R is present in cytoplasm and nucleus with preferential localization in nucleus and AT_2R is present solely in nucleus. They also suggest that the AT_1R, which is distributed in a non-random manner in the nucleus, could be associated with nucleoli. As in the Bkaily[11] study, they find that exogenous Ang II induces free cytosolic and nuclear calcium levels. Other recent studies demonstrate the presence of AT_1 receptors in rat renal cortical nuclei in control and Ang II-infused animals[41] and the existence of AT_1 receptors in nuclei of naive ventricular cardiac cells[27].

We have made some recent advances in understanding the differential effects of intracellular and extracellular Ang II as well as plasma membrane versus nuclear AT_1R[30, 31]. Both intracellular and extracellular Ang II stimulate CREB phosphorylation and cell proliferation. But CREB, in each situation, is phosphorylated through different kinase pathways. At the plasma membrane, the AT_1R is cleaved in an Ang II-dependent manner; either intracellular or extracellular Ang II is required for nuclear trafficking of the C-terminus. We are launching studies designed to determine the effect of the intracellular CF; preliminary results suggest that it is linked to apoptosis.

Nuclear Ang II

The Baker laboratory has reported that intracellular Ang II can cause cellular proliferation independent of the AT_1R. In CHO-K1 cells (which are deficient in AT_1R), intracellular Ang II, synthesized as a peptide from a simple (non-fusion) expression construct, enhances cellular proliferation. Intracellular Ang II enhances proliferation

to a greater extent in CHO-K1 cells stably transfected with AT_1R. This group has also reported that intracellular Ang II synthesis is stimulated in neonatal rat ventricular myocytes and that the Ang II may accumulate in cellular nuclei depending on the activating stimulus. High glucose stimulates Ang II retention and translocation to the nucleus; isoproterenol stimulates Ang II secretion.

Baker and coworkers believe that Ang II is generated from intracellular angiotensinogen by renin and ACE or chymase, respectively, in cardiac fibroblasts and myocytes. Collectively, their data support the idea of intracellular synthesis of Ang II, which behaves in a manner independent of the receptor.

The location of Ang II within the nucleus of myocytes following glucose stimulation is not clear, from the published studies. Ang II appears to be diffuse within the nucleus, consistent with a largely nucleosolic location. Subnuclear position, however, is likely to change during the cell cycle and nucleosolic proteins may become associated with chromatin during mitosis. Re and colleagues showed that Ang II binds to specific chromatin sites and that it broadly upregulates RNA synthesis[42,43] while Peter Eggena and coworkers, in the course of demonstrating Ang II-mediated upregulation of renin, angiotensinogen, PDGF, and c-myc in isolated nuclei, also found that a portion (10%) of Ang II-specific binding was associated with chromatin.

The Next Decade: Challenges and Opportunities

The challenge over the next few years will be to determine how intracellular Ang II, nuclear membrane-associated AT_1R, and nucleosolic AT_1R cleavage fragment mediate their cellular effects. Certainly, significant data support the concept of intracellular synthesis of Ang II. Assuming this occurs, might angiotensinogen be synthesized as a non-secreted variant and retained within cells[44], or is it secreted and taken up from the tissue culture media or extracellular space? By what mechanism and in what subcellular site is angiotensinogen cleaved by renin and ACE/chymase? Determining the intermediates in the processing pathways and transport events that lead to the accumulation of intracellular Ang II and the relative roles of intracellular nuclear Ang II, nuclear membrane-associated AT_1R, and nucleosolic AT_1R is vital. Ultimately, moreover, we would hope to determine whether these various intracellular systems are independent of one another or whether they converge toward a mutual end-point.

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Chapter 5
Pathophysiologic Implications of Cell Swelling in the Failing Heart: Influence of Intracrine and Extracellular Renin–Angiotensin Systems

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Abstract The regulation of cell volume is of fundamental importance to heart cell function and is involved on genetic expression, growth and activation of ionic channels including the swelling-activate chloride channel which reduces the action potential duration and facilitates the generation of cardiac arrhythmias. Extracellular renin or angiotensin II cause heart cell swelling through inhibition of the sodium pump and activation of the Na-K-2 Cl cotransporter while intracellular Ang II reduces cell volume through an activation of the electrogenic sodium pump. Ang (1-7) also reduces cell volume by activation of the sodium pump. The implications of these findings to cardiac arrhythmia during ischemia reperfusion are discussed.

Under normal conditions the osmolarity of body fluids is well preserved but marked changes can occur during pathological conditions like myocardial ischemia, diabetic coma, and septic shock in which changes in cell volume are produced.

Preservation of cell volume is fundamental to normal cellular function. Indeed, variations of cell volume activate stretch-sensitive ion channels and are an important contributor to metabolism, gene expression, and protein synthesis[1,2] (Fig. 5.1). Cardiac hypotonic stress induced by ischemia, for instance, leads to accumulation of metabolites intracellularly with consequent cell swelling due to water entering the cells. The harmful influences of cell swelling are many including the release of hormones, neurotransmitters, and ATP as well as the activation of plasma membrane receptors and integrins which also participate in the regulation of cell volume[3]. The process of cell volume regulation activated following cell swelling involves the efflux of ions through activation of K^+ channels and/or anion channels and parallel activation of K^+/H^+ exchange and Cl/HCO3 exchange, while cell shrinkage involves accumulation of ions through different mechanisms including activation of the Na–K–2Cl cotransporter and Na^+/H^+ exchange[4,5]. Variations of cell volume

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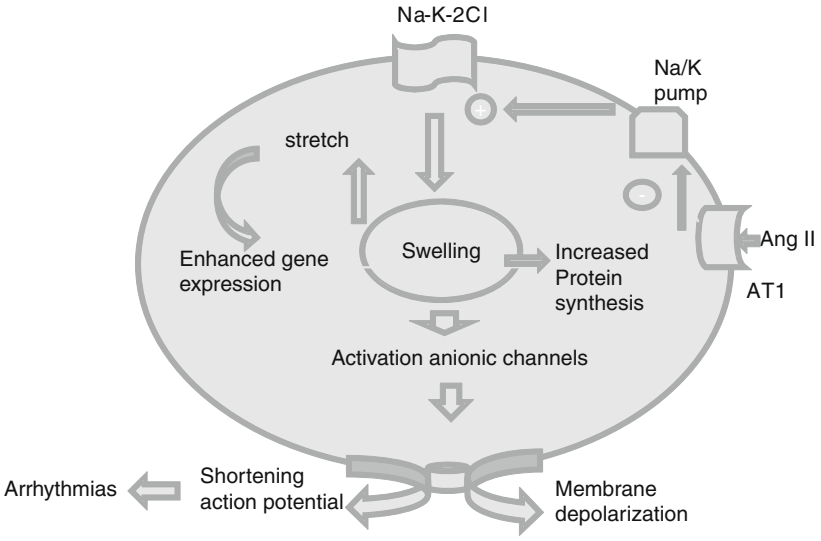

Fig. 5.1 Diagram showing the increase of heart cell volume caused by extracellular Ang II and elicited by Na/K pump inhibition and Na–K–2Cl cotransporter activation. Other consequences of cell swelling like enhanced gene expression, increased protein synthesis, and activation of anionic channels are also shown

regulation contribute to several diseases such as diabetic ketoacidosis, liver insufficiency, sickle cell anemia, and infection[4].

Cell volume is also involved in gene expression. Cell swelling, for instance, increases the expression of proteins like β-actin, tubulin, cyclooxygenase-2, extracellular signal-regulated kinases ERK1 and ERK2, JNK, the transcription factors c-Jun and c-Fos, ornithine decarboxylase, and tissue plasminogen activator[3]. However, our knowledge of the mechanisms involved in the changes in gene expression is meager[8]. Cell shrinkage, on the other hand, increases the expression of heat shock proteins and of other proteins such as P-glycoprotein, ClC–K1, and Na^+–K^+–ATPase α_1-subunit, cyclooxygenase-2, the GTPase-activating protein for Rac α_1-chimerin, the immediate early gene transcription factors Egr1-1 and c-Fos, vasopressin, phospho*enol*pyruvate carboxykinase, tyrosine aminotransferase, tyrosine hydroxylase, dopamine β-hydroxylase, matrix metalloproteinase 9, and several matrix proteins[3].

A major consequence of cell swelling is mechanical stress which stimulates protein kinase C[6,7] and increases tyrosine phosphorylation of several proteins[3], a finding particularly relevant because it is known that Ang II changes the inward calcium current in the heart through the activation of PKC and tyrosine kinases[8]. Membrane stretch and ionic channel activation might involve (a) the release of fatty acids from the membrane and activation of stretch-sensitive channels and (b) stretch activation of some component of the cytoskeleton such as spectrin [see 3]. Furthermore, it is known that stretching depolarizes the heart cell membrane during diastole, changes

the action potential, and produces arrhythmias mediated by stretch-activated ion channels' activation[9]. Interestingly, mechanical stress activates angiotensin II AT1 receptors independently of angiotensin II[10] suggesting that the AT1 receptor is a mechanical sensor by itself or it is associated with stretch sensors such as integrins[10]. Because chronic deformation of surface cell membrane like that seen in heart failure, essential hypertension, and myocardial ischemia causes mechanical stress, alteration of genetic expression and changes in heart cell excitability are likely events which might be in part associated with AT1 receptor activation[11]. Stretching of cardiac muscle produced by myocardial ischemia and heart failure also activates mechanosensitive channels, which are able to translate mechanical energy into electrochemical signals. The activation of these channels which are involved in the regulation of cell volume and cell contractility[12], are responsible for the generation of cardiac arrhythmias[12]. Agents that modulate mechanosensitive channels may not even bind to the channel but alter the properties of tensor-sensing lipid bilayer or of the cytoskeleton[13].

A major consequence of cell swelling is the activation of swelling-activated Cl current ($I_{Cl\ swell}$), which is broadly distributed throughout the heart. Shortening of the action potential, depolarization of the cell membrane, and the generation of cardiac arrhythmias are possible consequences of cell swelling[5]. Moreover, swelling enhances membrane tension and mechanical stretch[5]. Because there is evidence that inhibition of RAS is beneficial during myocardial ischemia and that activation of Ang II AT1 receptors has adverse effect on myocardium after myocardial infarction (MI)[14–16], it is reasonable to think that part of the harmful effect of Ang II during MI is related to the cell swelling induced by the peptide as shown below, with consequent activation of ionic channels and the generation of cardiac arrhythmias.

I(to,fast), which is an important determinant of the early repolarization of the cardiac action potential, plays an important role in the adaptive remodelling of cardiac myocytes, during myocardial ischaemia, hypertrophy, and heart failure[17]. Recent studies indicate that hyposmotic cell swelling caused a marked increase in densities of the peak I(to,fast) and a significant shortening in phase 1 repolarization of the action potential duration and that the voltage-dependent gating properties of I(to,fast) were, however, not altered by changes in cell volume[17]. It is well known that the shortening of the action potential enhances the probability of cardiac arrhythmias.

Recent observations[18] indicated that the renin–angiotensin–aldosterone system is involved in the regulation of cell volume in normal as well as in the failing heart. In cells isolated from the failing ventricle and exposed to renin (128 pmol Ang I/ml) plus angiotensinogen (110 pmol Ang I generated by renin by exhaustion) an increase of cell volume was seen concurrently with the inhibition of the sodium pump[18] (Table 5.1). The effect of renin is related to the formation of Ang II because it was abolished by losartan. In addition, ouabain inhibited the effect of both renin and Ang II. The increase of cell volume elicited by extracellular renin plus Ao as well as Ang II is related to the activation of the Na–K–2Cl cotransporter elicited by sodium pump inhibition. Indeed, because bumetanide, a specific inhibitor of the cotransporter, abolished their effect[18].

Table 5.1 Effect of extracellular and intracellular Ang II (10^{-8} M) on heart cell volume (μm^3) of cardiomyopathic hamsters (TO2)

Control	Extracellular Ang II	Intracellular Ang II
29060 ± 1100 (n = 25) (5 animals) P < 0.05	35200 ± 1320 (n = 26) (4 animals) P < 0.05	20987 ± 1278 (n = 28) (5 animals)

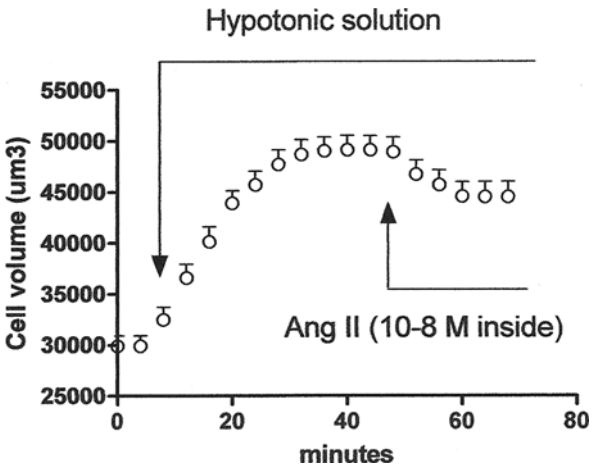

Fig. 5.2 Increase in heart cell volume elicited by hypotonic solution on cardiomyocytes isolated from cardiomyopathic hamsters (4-month-old) and its partial reversion caused by intracellular Ang II administration. Each point is the average from 25 cells (4 animals). Vertical line at each point SEM. P < 0.05

Intracellular Ang II, on the other hand, activates the sodium pump and reverses the cell swelling caused by hypotonic solution (see Fig. 5.2) raising the possibility that the activation of the intracrine RAS might play a protective role during myocardial ischemia by reducing cell volume[18]. These observations indicate that an important function of the renin–angiotensin system is the regulation of cell volume with extracellular renin and Ang II increasing the cell volume while the intracrine RAS reducing it (Fig. 5.3). Because extracellular Ang II causes cell swelling in the failing heart the question remains if the peptide increases $I_{Clswell}$ as occurs with hypotonic solution. Recent experiments indicated that Ang II (10^{-8} M) added to the bath increased the $I_{Clswell}$ in the failing heart of cardiomyopathic hamsters at an advanced stage of the disease (De Mello, unpublished).

These observations have important implications to cardiology because the activation of the circulating renin–angiotensin system is harmful to the ischemic heart by enhancing the cell swelling already elicited by the ischemic process. Cell swelling, for instance, might represent an important cause of heart edema with possible compression of coronary vessels with decrease of coronary flow and further deterioration of the ischemic myocardium. Furthermore, it can generate cardiac arrhythmias. These factors might be involved in the beneficial effects of ACE inhibitors and AT1-receptor blockers seen during myocardial ischemia[15]. On the other hand, the decline

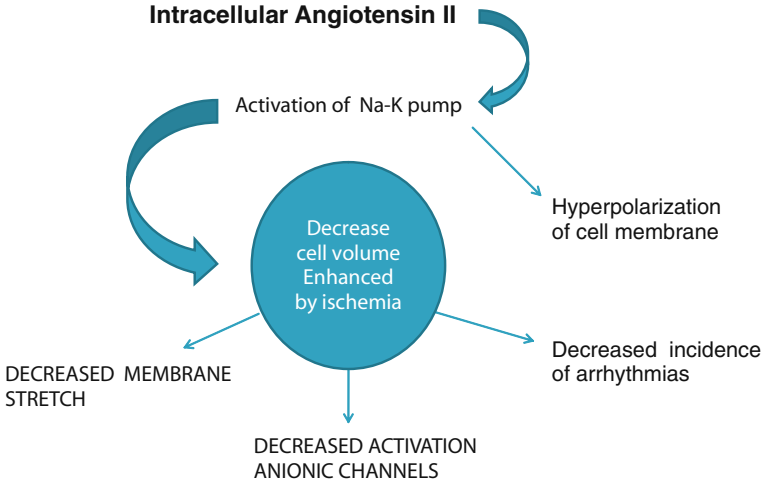

Fig. 5.3 Diagram showing the possible beneficial effects of intracrine RAS activation on cell volume regulation during myocardial ischemia

in cell volume caused by intracellular Ang II[18] might ameliorate coronary perfusion and decrease the incidence of cardiac arrthythmias caused by cell swelling during myocardial ischemia (see Fig. 5.3).

$I_{Clswell}$ is persistently activated in ventricular myocytes from canine-pacing-induced dilated cardiomyopathic model[19]. Indeed, even in isotonic solutions a large outwardly rectifying Cl^- was found currently in the failing heart but not in normal controls[19]. Similar results were described in other models of heart failure[20]. Although the mechanism by which $I_{Clswell}$ is persistently activated is not known, it is quite possible that the enhancement of cell volume caused by hypertrophy as well as membrane stretch elicited by dilation is involved in this process[21]. The possibility that the activation of the renin–angiotensin system and consequent generation of Ang II leads to hypertrophy[22] and activation of $I_{Clswell}$ must be seriously considered. Indeed, our results indicated that Ang II increases $I_{Clswell}$ in myocytes from the failing heart of cardiomyopathic hamsters. In addition, angiotensin II also causes oxidative stress[23] and recently it was found that NADPH oxidase is intimately coupled to $I_{Clswell}$[24], opening the possibility that oxidative stress is one of the mechanisms involved in the regulation of this channel.

Conclusion

The renin–angiotensin system is involved in the regulation of heart cell volume in normal and in the failing heart. The pathophysiological implications of cell swelling include the generation of cardiac arrhythmias as well as alterations in gene expression and growth.

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Chapter 6
Exploiting Rat Genetics to Investigate Hypertensive End-Organ Damage

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Abstract Transgenic animal models are important tools, which have been extensively used to gain insight into the molecular mechanisms of human diseases. We have created two transgenic consomic rat strains, in which malignant hypertension can be induced by administration of a nontoxic xenobiotic. Studies combining quantitative trait locus (QTL) analysis, pathological examination and molecular biological investigation have demonstrated that the renal renin–angiotensin system (RAS), especially angiotensin converting enzyme (Ace), is a key factor affecting the degree of hypertensive kidney damage in these strains.

Introduction

Transgenic animal models are important tools that aid in the understanding of pathological mechanisms and identify the genetic basis of complex cardiovascular diseases[1]. The transgenic rat model, TGR(mRen2)27, created by inserting the mouse *Ren2* renin gene into the Hanover Sprague-Dawley rat genome, provides an excellent model of malignant hypertension (MH), exhibiting many phenotypic similarities with the human disease[2]. This model remains one of the widely used experimental models of hypertension and has been extensively reviewed[3]. Studies using the model revealed that on certain genetic backgrounds the animals showed a transition to MH with a high mortality[4,5]. By genome-wide screening and QTL analysis, we identified a modifier locus on rat chromosome 10, at or near the *Ace* gene, which contributes to the lethal MH phenotype. Inheritance of a Fischer modifier allele reduced life span by 7.3 ± 1.6 days and increased the probability of MH by 4.2-fold compared with animals inheriting a Lewis allele. Further pharmacological studies suggested that Ace might be the modifier of MH phenotype[6,7].

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The TGR (mRen2)27 strain, maintained on an outbred Sprague-Dawley genetic background, exhibited spontaneous and rapid (24–48 h) development of MH between 50 and 90 days of age, and was therefore not ideally suited to further investigate the candidate MH-modifier gene(s). To overcome the shortcomings of the model, an inducible transgenic hypertensive rat model, TGR (Cyp1a1.Ren2.F344 or Ren2.F), was developed[8]. In this model, the mouse *Ren2* transgene, controlled under the inducible promoter cytochrome p-4501a1, was introduced onto the inbred Fischer F344 background, integrating into the Y chromosome. Transgene expression and development of MH can be induced by the administration of a nontoxic xenobiotic such as indole-3-carbinol (I3C). Following chronic induction, animals exhibit the classic signs of MH such as the appearance of fibrinoid necrosis in small arteries of tissues such as the kidney. The inducible nature of the transgenic model enables researchers to precisely monitor the progress and development of MH and accurately dissect the molecular factors involved in the development of the phenotypes in a controlled manner. To facilitate the fine mapping of the MH modifier QTL region, previously identified, and more accurately identify the candidate gene or genes of the modifier, a consomic strain, TGR (Cyp1a1.Ren2.Lewis or Ren2.L) was derived, in which the Y chromosome carrying the mouse *Ren2* transgene was transferred from Ren2.F to Lewis by continuous backcross for 12 generations.

Our recent studies on the transgenic consomic strains indicate that they exhibit significant differences in the severity of hypertensive end-organ damage under the same induction conditions, despite the fact that plasma-active renin concentration and the mouse renin transgene expression in liver are comparable between the two strains. Although serum Ace activity is significantly different between the two strains no difference is observed in lung, which is the main source of serum Ace. This implies that local RAS might contribute to the MH phenotypic difference. The objective of the current study therefore is to (1) further characterise the newly established consomic strain Ren2.L, (2) investigate the possible contribution of renal Ace activity and expression to the MH phenotypic differences between the two consomic strains and (3) examine changes in the expression of other components of renal RAS during development of malignant hypertension.

Materials and Methods

Animals

Animals from Ren2.F and the consomic strain Ren2.L were used in this study. The animal studies were undertaken under UK Home Office licence, following review by local ethics committee. The consomic strain of inducible, transgenic, hypertensive rat, Ren2.L, was derived by backcrossing the inducible transgenic Ren2.F males with Lewis females. Males were genotyped for markers spaced approximately every 15 cM. Optimal males from each backcross (BC) generation were chosen and mated with Lewis females for 12 generations. Animals from Ren2.F and Ren2.L are

assumed to have an identical Y chromosome (which carries the transgene), as there is little opportunity for Y chromosome recombination during the BC protocol.

Treatment

To monitor blood pressure change in animals of the Ren2.L strain under induction, 14 rats (10–12 weeks old) were randomly divided into two groups (group 1 and group 2). A further seven age-matched non-transgenic Lewis males were used as control (group 3). A radiotelemetry transmitter (Data Sciences International, St. Paul, MN) was surgically implanted into each animal and at least a week was given to allow the animals to recover from the surgery. All the rats were given free access to water and standard commercial rat chow (Special Diet Services, Witham, Essex, UK). The rat diet was changed to standard powder diet 24 h before the experiment. Animals in group 1 were given 200 mg/kg/day of I3C in sesame oil (50 mg/ml) by gastric gavage and animals in groups 2 and 3 were given dietary 0.3% I3C daily for 14 days, respectively.

To examine the pathological changes and RAS gene expression differences between the two consomic strains during induction, animals (10–12 weeks old) from each consomic strain were randomly divided into three groups ($n = 7$). Animals in groups 1 and 2 were given dietary 0.3% I3C for 7 and 14 days, respectively. Control group 3 was given standard powder diet without I3C.

Tissue Collection

At the end of the study, animals were killed by CO_2 inhalation and cervical dislocation. The right kidney was fixed in 10% neutral buffered formalin for pathological examination and immunohistochemical analysis. The left kidney and lung tissues were immediately frozen in dry ice and stored at –80°C until use.

Tissue RNA Extraction, and cDNA Synthesis

Total RNA was extracted using Trozer reagent according to manufacturer's instruction (RNA-Bee kit, AMS Biotechnology UK Ltd., Witney, Oxon, UK), treated with RNase-free DNase (Ambion) and subsequently purified (RNeasy mini kit, Qiagen, Crawley, West Sussex, UK). The RNA was then reverse transcribed using a first-strand cDNA synthesis kit with a random primer (Amersham Pharmacia Biotech UK Limited, Little Chalfont, Bucks, UK).

TaqMan Real-Time RT-PCR Analysis

The mRNA levels of lung Ace, and kidney angiotensinogen (Agt), renin, Ace and Angiontensin II receptor subtype 1 (At1) were quantitatively analysed by TaqMan

Table 6.1 Primer and probe sequences for real-time RT-PCR

Gene	Primer sequence	Probe sequence
Agt	For 5′-CTTGCGCCTAAAACA-3′ Rev 5′-GACCCAAGCTCTCAACAAATGG-3′	5′-VIC-CGTGGTGGGCCTCTTCACT-3′
Renin	For 5′-GAGGCAGTGACCCTCAACATTAC-3′ Rev 5′-CCGGCCTTGCTGATGCT-3′	5′-VIC-AGGGCAACTTTCACTACGT-3′
Ace	For 5′-GATCACAAACCAGGCAACAA-3′ Rev 5′-TCCCATTTGAGGTCTGGATTG-3′	5′VIC-CAGACAACTCACCAATCAACAATCAG-CCAG 3′
At1	For 5′-CCATCGTCCACCCAATGAAG-3′ Rev 5′-GTGACTTTGGCCACCAGCAT-3′	5′-VIC-CTCGCCTTCGCCGCA-3′
Kim1	For 5′-CCACGGCTAACCAGAGTGA-3′ Rev 5′-TAGTTGTGGGCCTTGTGGTT-3′	

real-time RT-PCR. The primers and probes for each of the genes were designed by Primer Express (Applied Biosystems) on the basis of cDNA sequences deposited in the GenBank database and listed in the Table 6.1. The pre-developed TaqMan Assay Reagent Human 18S rRNA (Applied Biosystems) was used as the internal control for each reaction. The TaqMan® MGB probes were 5′-Fluor labelled with VIC and synthesized by Applied Biosystems. The primers were synthesized by MWG (MWG-Biotech AG). The mRNA level of kidney injury molecule 1 (Kim1) was also measured by RT-PCR with Gapdh as internal control (the primers are listed in Table 6.1). All primers were tested for their specificity by conventional PCR and real-time conditions (concentration of primers and probes) were optimised before the quantitative studies. The critical threshold (Ct) value for each gene was obtained from the real-time PCR reactions, and the starting amount of each target mRNA was calculated on the basis of a calibration curve and the Ct value. The relative amount of mRNA was normalised to 18S RNA.

Ace Activity Measurement

Kidney Ace activity was measured by an adaptation of the method described previously[9]. In brief, tissues were homogenised in ice-cold lysis buffer (30 mM KCl, 0.25 M sucrose, 1% Triton X-100), and spun at 5,000 × g for 15 min at 4°C to remove cell debris. The protein concentration of each sample was determined by BCA protein assay kit (Pierce) and adjusted to the same level. Utilising the tri-peptide, Hip-His-Leu (Sigma) as substrate, fluorescence of the di-peptide product was measured in duplicate samples using a 1420 Multilabel HTS Counter (Perkin

Elmer) at an excitation wavelength of 355 nm and an emission wavelength of 460 nm. Ace activity is expressed as mM His-Leu/g protein/h at 37°C.

Pathological Examination and Immunohistochemistry Analysis

For pathological examination, kidney was fixed in 10% formal saline for 24 h and processed to paraffin block, and 3 μm sections were cut and stained using hematoxylin and eosin (H & E) and periodic acid-Schiff (PAS). Sections were examined blinded to the experimental group and time point. The severity of hypertensive microvascular injury was evaluated on a 5-point ordered categorical scale, evaluating the prevalence of reactive myoadventitial changes (score 1–2) and in more severely affected examples, the prevalence of destructive mural lesions that are the hallmark injury of malignant hypertension (score 3–5). For immunohistochemical analysis of Ace protein, dewaxed and rehydrated paraffin 3 μm sections underwent antigen retrieval in a microwaveable pressure cooker for 2.5 min. The sections were then incubated with mouse-anti-Ace monoclonal antibody in 1:125 dilution (MAB3502, Chemicon International) for 30 min at room temperature. The bound antibody was labelled and visualised using the DAKO Envision + System HRP kit.

Microarray Analysis

Gene expression profiles from kidney RNA of Ren2.F and Ren2.L were analysed at 0, 7 and 10 days after induction. Four biological replicates were used for each condition. Kidney total RNA was extracted (TRIzol reagent; Invitrogen, Carlsband, CA, USA), purified (RNeasy Mini kit; Qiagen, Crawley, UK) and DNase I treated (RNase-Free DNase Set; Qiagen), according to the manufacturer's protocols. RNA concentration and purity was determined with a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA). RNA samples were immediately frozen and stored (–80°C). Biotin-labeled targets for the microarray experiment were prepared using 1 μg of total RNA. Ribosomal RNA was removed with the RiboMinus Human/Mouse Transcriptome Isolation kit (Invitrogen), and cDNA was synthesized using the GeneChip® WT (Whole Transcript) Sense Target Labeling and Control Reagents kit (Affymetrix, Santa Clara, CA, USA). Five μg of biotinylated target was hybridized with the GeneChip® Rat Exon 1.0 ST array (Affymetrix) at 45°C for 16 h. After washing, specifically bound target was detected using the GeneChip Hybridization, Wash and Stain kit, and the GeneChip Fluidics Station 450 (Affymetrix). Arrays were scanned using the GeneChip Scanner 3000 7G (Affymetrix) and CEL intensity files were produced using GeneChip Operating Software version 1.4 (Affymetrix). CEL files were analysed in Bioconductor using the OneChannelGUI package. RMA-sketch was used to generate probe summaries using the extended probe set for the gene and exon level. Limma was used to generate raw p-values and the Benjamini & Hochberg (BH) procedure was

used for multiple testing corrections. Genes with a corrected p-value less than 0.05 were classed as differentially expressed.

Statistical Analysis

Data are given as mean ± S.E. Differences between groups were evaluated by analysis of variance followed by post hoc test of Bonferroni. Only p values less than 0.05 were accepted to indicate a significant difference.

Results

Blood Pressure and Body Weight

The average daily food intake for a 10- to 12-week-old rat (average body weight 300 g) was 21.3 ± 4.35 g, so the average intake of I3C (0.3% of the diet) is 200 mg/kg/day. Animals were therefore induced with either 200 mg/kg/day I3C by gastric gavage or with dietary 0.3% I3C, to determine whether they responded similarly. Under induction, blood pressure increased rapidly in both groups 1 and 2 by 24 h, while the BP of nontransgenic control animals remained unchanged. However, the increase in blood pressure for group 1 was much higher than that for group 2 (Fig. 6.1a). Body weight increased in control animals, but decreased in groups 1 and 2, the decline being much steeper for group 1 than group 2 (Fig. 6.1b). Animals in group 1 also showed significant clinical symptoms of MH such as polyuria, lethargy and seizure. Since weight loss was over 10% by day 7 of induction, this group was terminated.

Pathological Changes in Kidney

Pathological changes in microvasculature of kidney were scored for prevalence and degree of reactive myoadventitial changes and destructive mural lesions in the two consomic strains, following induction. Typical histological sections are shown (Fig. 6.2a). The reactive myoadventitial changes were characterised by myoadventitial expansion with mononuclear cells and myofibroblasts, together with medial changes of myocyte enlargement, amphophilic cytoplasmic staining, nuclear enlargement, occasional mitoses and in the most severe examples, apparent myocyte disarray. The more severe vascular destructive changes that are the pathologic hallmark injury of MH were normally found in vessels also showing the reactive myoadventitial changes, and were characterised by evidence of intramural contiguous cell death (necrosis) and/or fibrinoid change. Ren2.L cosomic animals showed delayed, less severe acute renal vascular injury than those of Ren2.F animals, as judged by the mean severity score of the groups at 7 days after induction ($p = 0.0005$, 2-tailed T test).

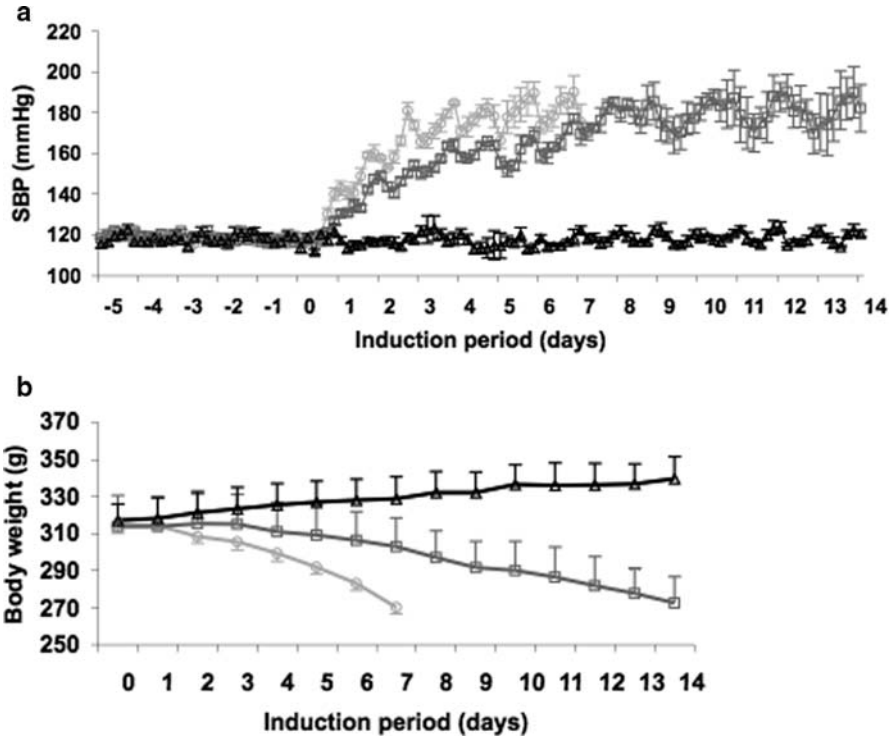

Fig. 6.1 Systolic blood pressure and body weight changes during induction. (**a**) Systolic blood pressure 5 days prior to induction and during the 14-day induction period. (**b**) Body weight changes during the induction period

In a separate study, where I3C (100 mg/kg/day) was administered by gastric gavage, microarray analysis of kidney samples collected from the consomic animals prior to induction and following 7 and 10 days of I3C induction were analysed to identify markers of kidney damage. Kidney injury molecule-1 (Kim1; also known as Havcr1) is an epithelial cell adhesion molecule upregulated in renal cells after injury[10–12]. Microarray data (Table 6.2) suggested that Kim1 was significantly upregulated following induction in these animals, and to a higher level in Ren2.F than Ren2.L rats. Microarray analysis failed to demonstrate significant changes in p38 MAP kinase, PDGFRβ or GRK2, all of which have been reported to show increased activation in TGR(mRen2)27 rats[13], but their increased activation may reflect subtleties in phosphorylation status, rather than protein levels per se.

The severity of kidney injury during 14-day induction by dietary I3C administration was confirmed by RT-PCR analysis of Kim1 expression (Fig. 6.2b). Kim1 was not detectable in control untreated animals, but was expressed after 7 days induction, and expression was further increased by day 14 of induction, the expression level being higher in Ren2.F than that in Ren2.L animals at these later time-points.

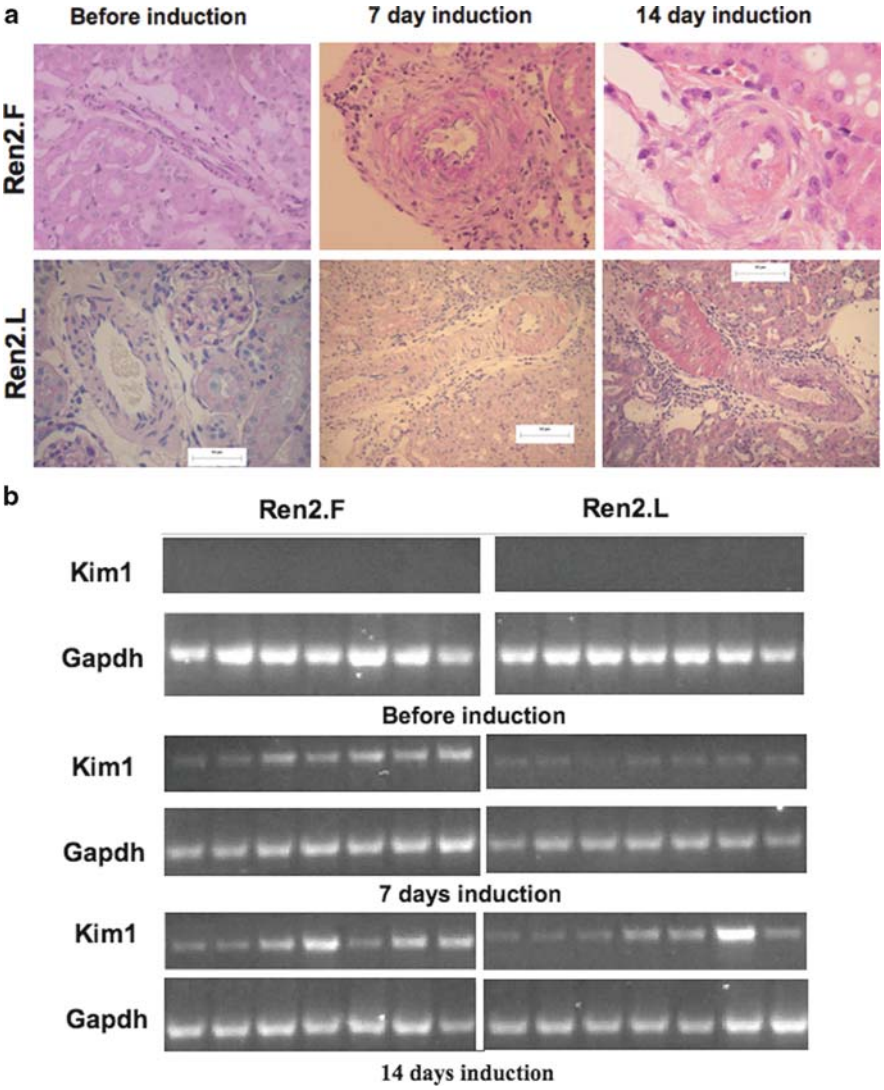

Fig. 6.2 Assessment of kidney injury between the two strains during induction of MH. (**a**) Time course of hypertensive pathological changes of kidney before and after induction for 7 and 14 days. (**b**) Kidney injury biomarker Kim1 expression analysed by RT-PCR (7 animals in each group). The PCR products were separated by electrophoresis on a 2% agarose gel, visualized by ethidium bromide staining

Expression of RAS Genes in Kidney

Renal Ace activity was significantly different between the two strains across all induction time-points (Fig. 6.3a). Renal *Ace* mRNA was also significantly differentially expressed between Ren2.F and Ren2.L before and after induction

Table 6.2 Microarray analysis of Kim1 expression during induction (mean of 4 replicates ± standard deviation; normalized intensity units)

	Ren2.F	Ren2.L
0 day	14 ± 1	15 ± 3
7 day	138 ± 64	16 ± 3
14 day	135 ± 69	68 ± 57

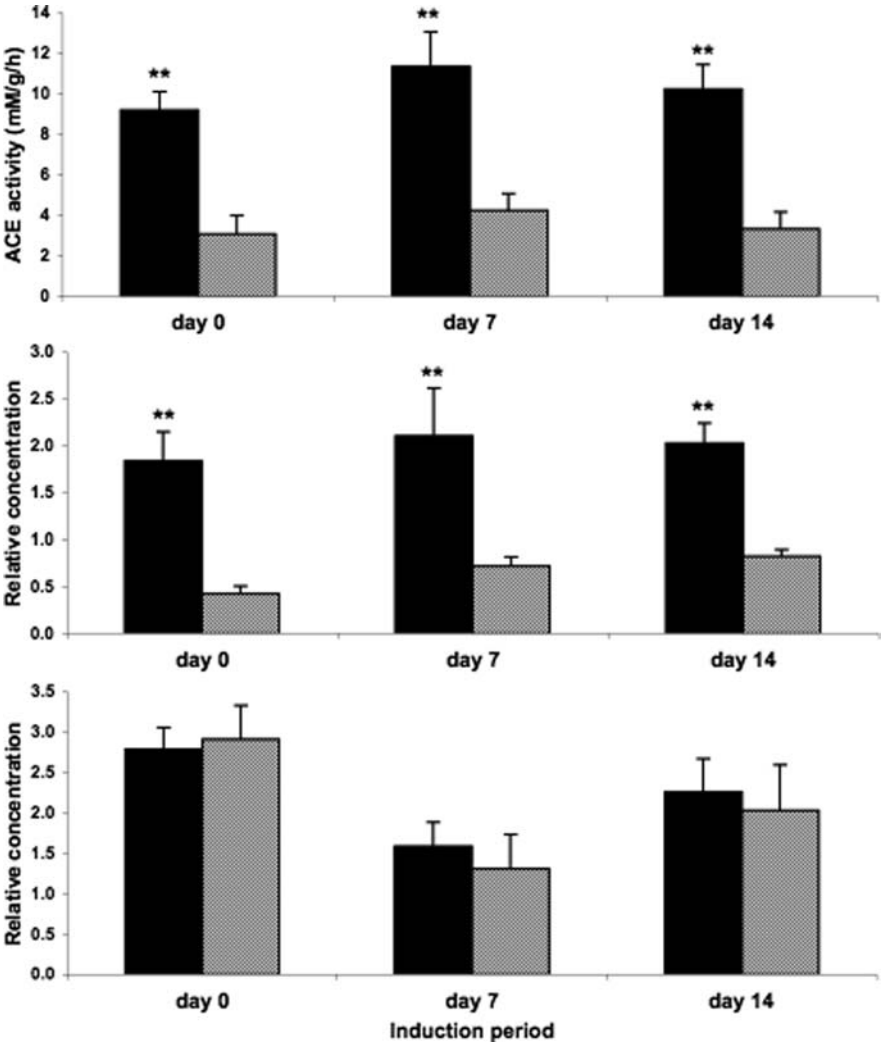

Fig. 6.3 Comparison of tissue Ace expression and function between strains during induction. (**a**) Assessment of kidney Ace activity, (**b**) measurement of kidney and (**c**) lung *Ace* gene expression using Taqman real-time PCR. (*Dark grey*—Ren2.F; *light grey*—Ren2.L; ∗∗, difference between two strains, $p < 0.01$)

(Fig. 6.3b). Since lung is the main source of systemic Ace, its expression level in lung was also checked, but no difference was found between the two strains (Fig. 6.3c). The expression levels of other components of renal RAS including AGT, renin and At1 were also examined but no significant difference was found between the two strains in either the uninduced state or following 7 or 14 days of induction (Fig. 6.4a–c). Kidney renin expression level was significantly decreased

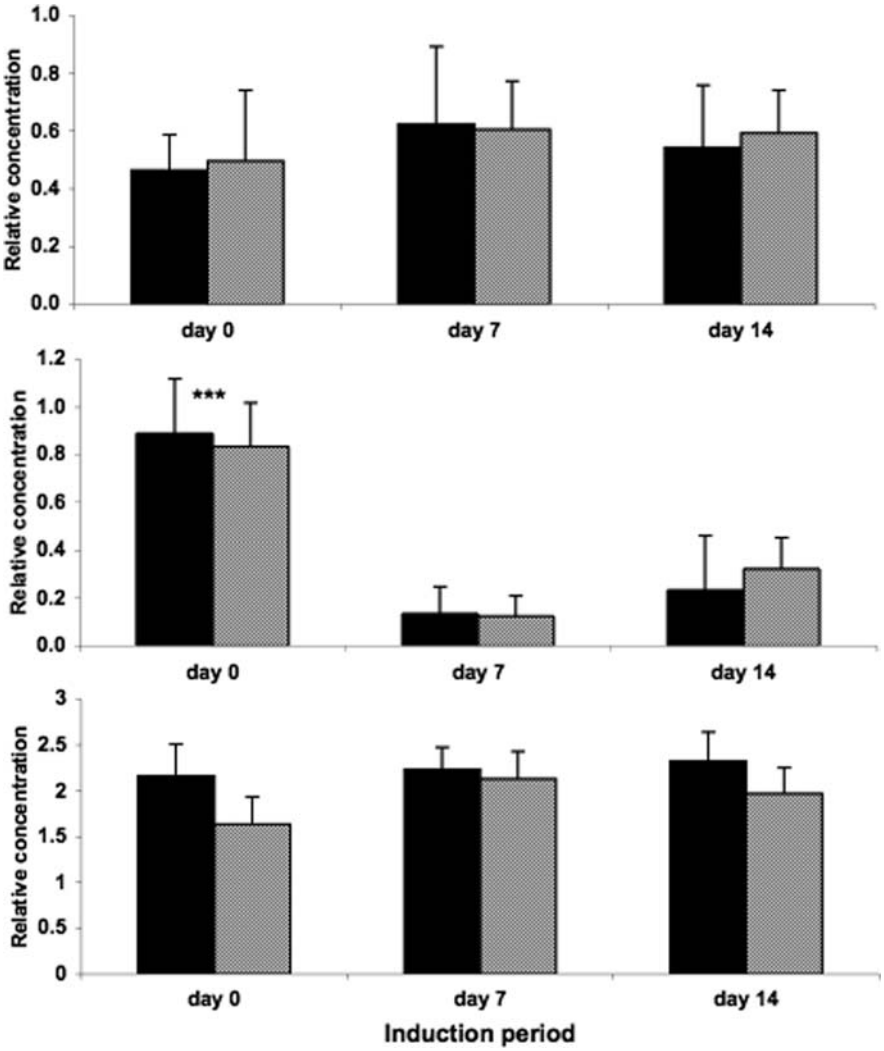

Fig. 6.4 Comparison of kidney expression of RAS components between strains during induction. Assessment of (**a**) Agt, (**b**) renin and (**c**) At1 mRNA levels using TaqMan real-time PCR (*Dark grey*—Ren2.F; *light grey*—Ren2.L; ***, difference between control (day 0) and induction groups, $p < 0.001$)

after induction because the steady-state transgene expression suppressed endogenous renal renin expression. Immunohistochemical staining of Ace was seen at the distal-proximal tubular brush border in Ren2.F but not in Ren2.L rats (Fig. 6.5), confirming that Ace was differentially expressed in kidney between the two strains. The Ace tubular immunopositivity is a feature of Fischer strain (but not Lewis) whether induced or not.

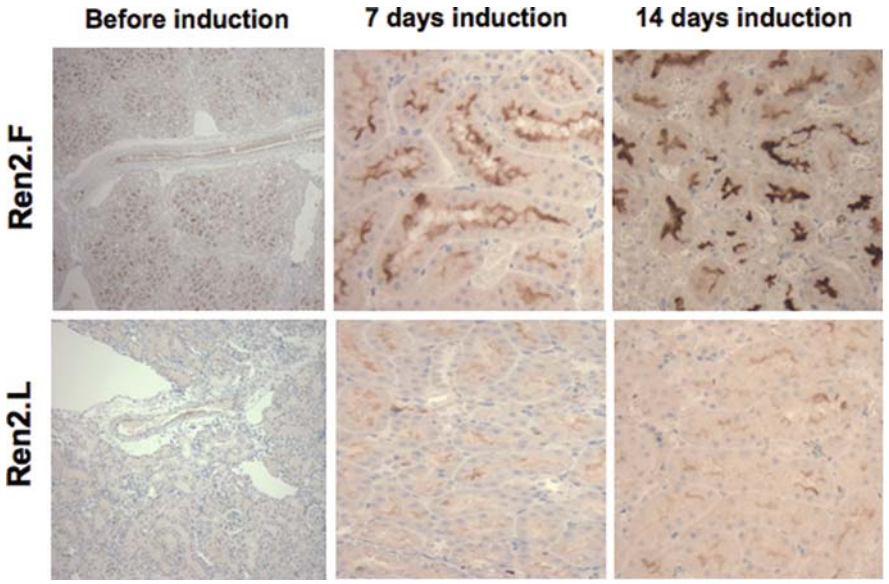

Fig. 6.5 Renal Ace immunostaining at 0, 7 and 14 days of induction in the two consomic strains

Discussion and Conclusion

Transgene expression in this model is tightly controlled by the inducible Cyp1a1 promoter in a dose-dependent manner[8]. Animals induced by gastric gavage or dietary ingestion of I3C showed significantly different blood pressure and body weight changes despite being given apparently equivalent amounts of I3C inducer. Two possible reasons have to be considered. Firstly, administration of I3C by gastric gavage rapidly achieves a high concentration of inducer in the body, triggering high levels of transgene expression and rapidly increasing blood pressure. However, animals induced by dietary supplement, build up I3C levels more gradually, so transgene expression increases less rapidly and blood pressure rises more slowly. Additionally, animals receiving I3C by gastric gavage, according to their body weight, receive a full daily dose. Animals receiving dietary I3C significantly reduced their food intake as hypertension developed, presumably due to loss of appetite, and therefore received less I3C (data not shown).

When the Ren2.F rats were induced with dietary 0.3% I3C, severe hypertension developed, reaching a systolic blood pressure of 200 mmHg within 1 week of treatment, which was sustained throughout the period of administration. Under the same induction conditions, blood pressure of Ren2.L rats raised relatively slower, achieving a peak blood pressure of about 180 mmHg. Assessment of pathological changes indicated that Ren2.L rat also exhibited milder microvascular damage in comparison to Ren2.F rats.

Kim1 is an epithelial cell adhesion molecule, expressed in proliferating bromo deoxyuridine-positive and dedifferentiated vimentin-positive epithelial cells in regenerating proximal tubules and is markedly upregulated in the proximal tubule of post-ischemic human and rat kidney[10–12]. Kim1 has been suggested as an acute proximal tubule injury biomarker. In the present study, Kim1 expression was not detected by RT-PCR in control animals, but it increased with development of kidney injury, and the expression level was higher in Ren2.F than in Ren2.L rats. The p38 MAP kinase has been suggested to potentiate shedding of Kim1 from damaged tubular epithelial cells[14] and blockade of both Ace and p38 kinase has been shown to attenuate induction of Kim1[15]. Though microarray analysis failed to demonstrate increases in p38MAP kinase expression following induction, the increased activation observed in TGR(mRen)27 rats may reflect subtle differences in phosphorylation, rather than protein levels, or may reflect chronic exposure to high renin levels.

Our QTL mapping and pharmacological studies suggested that Ace might be the candidate modifier of MH phenotype[6]. Therefore we looked at the RAS in detail. It is indisputable that the RAS is the major regulator of blood pressure and body electrolyte balance. The classic RAS enzymatic cascade, involving cleavage of the decapeptide angiotensin I (Ang-I) from angiotensinogen, by renin, and its subsequent cleavage, by Ace, to yield the physiologically active octapeptide hormone, Ang-II, leads ultimately to receptor-mediated actions on target cells. Though two pharmacologically distinct subtypes of Ang-II cell surface receptors, At1 and At2[16,17], have been identified by ligand binding studies, the widely distributed At1 receptor subtype is the receptor subtype responsible for most of the cardiovascular and haemodynamic effects of Ang-II, whilst less is known of the functions of the At2 receptor.

With the identification of RAS components in a variety of tissues, the physiological importance of local paracrine RAS is widely recognised[18]. Components of the RAS are found in brain, heart, adrenal, kidney, placenta, testis, adipose tissue, eye and blood vessels[19–28]. Renin is acknowledged to be the rate-limiting step in Ang-II production, which then elicits negative feedback on both primary renin synthesis and renin secretion, to effect tight autoregulation[29]. Tissue Ace represents another key element of the paracrine RAS and a number of studies have shown that it is tissue Ace that determines the long-term response to Ace inhibition, rather than its plasma counterpart[30–32]. The inhibition of Ace activity in certain critical tissues rather than inhibition of plasma enzyme activity has been found to correlate with the magnitude and duration of blood pressure reduction.

The role of tissue as opposed to circulating Ace was investigated pharmacologically in the TGR(mRen2)27-Edinburgh Sprague Dawley rat[7]. Administration of low-dose Ace inhibition to these TGR(mRen2)27 rats, during the transition to MH, reduced mortality from 63% in the untreated population to only 4% in the low-dose Ace inhibitor-treated group, despite exposure to similar blood pressure levels. Plasma Ace activity was not significantly different, but there was a significant reduction of tissue Ace in the treated animals, in blood vessels, left ventricle, right ventricle and kidney tissue, where Ace levels fell to between 25 and 40% of control levels. Untreated animals showed evidence for malignant hypertension, with fibrinoid necrosis and proliferative endarteritis on histopathological examination, while treated animals showed only hypertrophy of resistance vessels as an adaptive response to blood pressure elevation, with no evidence of fibrinoid necrosis, proliferative endarteritis, or blood pressure-dependent tissue injury. This study demonstrated that inhibition of tissue Ace activity prevented the transition to malignant phase hypertension, implying that tissue Ace activity is critical in the pathophysiological development of the disease.

Other transgenic animal models also strongly support the concept that tissue-bound Ace is essential to the control of blood pressure and kidney function. Mice expressing a modified form of Ace (which lacked the COOH-terminal half of the molecule and was catalytically active but not retained by cells) had significant plasma Ace activity but no tissue-bound enzyme[33]. These animals exhibited low blood pressure, renal vascular thickening and a urine-concentrating defect. Though less severe than the renal pathology seen with complete Ace-deficiency[34], these studies implicate tissue-bound Ace in the control of blood pressure and the structure and function of the kidney.

In comparison with Ren2.F rats[8], the blood pressure rose more slowly and the peak pressure was much lower in Ren2.L rats even under the same induction conditions. The kidney injury resulting from hypertension was also less severe in Ren2.L than in Ren2.F. Though plasma Ace activity was slightly higher in Ren2.F than in Ren2.L, both at baseline and after induction (data not shown), as demonstrated in previous studies[35,36], it is unlikely to be a causative factor, differentially modifying the MH phenotype in the two consomic strains under induction[7,34]. Generally, the lung produces abundant Ace, but neither *Ace* mRNA nor Ace activity was different between the two strains throughout the induction period. The important role of intrarenal RAS has been reviewed recently[37]. Given the demonstration of increased renal angiotensinogen in Ang-II-dependent hypertension [38,39] we anticipated an increase in renal angiotensinogen following induction, but this was not confirmed by RT-PCR. Recent evidence in mice suggests that a rapid increase in renal AngII levels does not elicit an increase in renal angiotensinogen[40]. Among the remaining components of RAS in kidney, Ace is the only element that showed a difference between Ren2.F and Ren2.L rats. Ace mRNA expression and enzyme activity tended to be increased during induction, but not to a significant level. Immunohistochemical analysis, however, indicated an increased immunopositive staining of Ace during induction, particularly in the brush borders in Ren2.F rats.

In conclusion, analysis of the consomic strains suggest that kidney Ace may be a key RAS factor responsible for the difference in MH phenotype between the strains. Congenic strains, in which the original mapped QTL has been reciprocally introduced onto each consomic background, have now been generated. The use of these newly derived strains will further clarify the role of kidney Ace in hypertensive end-organ damage.

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Chapter 7
Alternative Renin Transcripts and Functions of Cytoplasmatic Renin

Jörg Peters and Heike Wanka

Abstract Renin is commonly known as a secretory glycoprotein which is expressed, stored, and secreted in a regulated manner by the kidney. Recently additional renin transcripts have been identified lacking exon 1 and thus the coding sequence for a cotranslational transport to the endoplasmatic reticulum. From these transcripts a non-secretory cytoplasmatic renin is translated. In the rat heart, exclusively cytoplasmatic renin is expressed. Here the expression of cytosolic renin transcripts increases markedly after myocardial infarction, indicating a role for cytosolic renin during and after ischemia. In H9c2-cardiomyoblasts cytosolic renin is targeted at mitochondria, where it stimulates the rate of apoptosis but decreases rate of necrosis. In contrast secretory renin increased the rate of necrosis. In the adrenal gland both secretory and cytosolic renin are expressed. Whereas secretory renin may be part of a secretory intra-adrenal angiotensin amplification system, cytosolic renin is targeted to mitochondria, where it may stimulate aldosterone production by as yet unknown intracellular pathway. In support of this, increased expression of cytosolic renin is associated with increased aldosterone production in transgenic rats.

Keywords Tissue renin–angiotensin systems · Renin sorting · Mitochondrial renin · H9c2 cells · Mitochondrial apoptosis · Aldosterone · Renin transgenic rats

Introduction

Until recently renin was known exclusively as a secretory protein. The targeting of proteins to secretory pathways requires the cotranslational transport of the protein to the endoplasmatic reticulum (ER). The transport signal for renin to the ER is encoded by a sequence derived from exon 1 of the renin gene. We have

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isolated and characterized a second renin transcript in the rat, termed *exon(1A-9) renin*. Exon(1A-9)renin lacks exon 1 and thus the signal for the transport to the ER. This transcript codes for a cytosolic protein that cannot be secreted[1]. Similar transcripts have been found in other species as well, including humans[2,3]. In rats, exon 2 of the *exon(1A-9)renin* transcript is preceded by a short sequence of 80 base pairs derived from intron A[1]. The intron A sequence is non-coding and therefore can only have regulatory functions. The *exon(1A-9)renin* transcript is translated into a truncated cytosolic prorenin using the first in-frame ATG in exon 2 as translation start site.

Most tissues express both renin transcripts, but the rat kidney expresses exclusively the exon(1-9)renin transcript encoding for secretory renin whereas the heart expresses exclusively the exon(1A-9)renin transcript encoding for cytosolic renin[4]. In the rat heart, transcript levels for cytosolic renin but not for secretory renin increase markedly after myocardial infarction[4], indicating that cytosolic renin may play a role in post-ischemic repair processes. In the rat adrenal gland cytosolic renin is expressed, targeted to[1] and found within[5,6] mitochondria, where it may stimulate aldosterone production. This is supported by the observation that under some conditions stimulation of aldosterone production is associated with increased levels of mitochondrial renin[6]. In the mouse brain, cytosolic renin was suggested to regulate blood pressure. Mice overexpressing human cytosolic renin together with human angiotensinogen exhibit increased blood pressure levels[7].

Aims of our recent studies were (a) to investigate the functions of cytosolic renin in the heart, (b) to test the hypothesis that overexpression of cytosolic renin in the adrenal cortex increases aldosterone production, and (c) to test the hypothesis that overexpression of cytosolic renin in the brain increases blood pressure.

Overexpression of Cytosolic Renin in Rat H9c2 Cardiomyoblasts

To investigate the effects of cytosolic renin on the heart in vitro we overexpressed either secretory or cytosolic renin in rat H9c2 cardiomoyblasts. These cells were chosen because they were cardiac cells in origin, do not express secretory renin transcripts, and exhibit low levels of cytosolic renin transcripts.

In H9c2 cells renin activity coprecipitates with mitochondrial fractions and renin immunoreactivity was colocalized with mitochondria as determined by confocal microscopy[8]. Overexpression of cytosolic renin led to an increase of renin activity within the mitochondrial fractions when compared with corresponding fractions of control vector transfected cells. In contrast, overexpression of secretory renin led to an increase in renin concentrations within a low-density fraction representing light vesicles. Thus the proteins derived from different renin transcripts are sorted differentially. Supporting the hypothesis that only secretory renin can be secreted, inactive renin (prorenin) concentrations and to a lesser degree also active renin concentrations were increased only in the medium of cells overexpressing secretory renin. In

contrast, control cells and cells overexpressing cytosolic renin did not secrete renin into the medium.

Cells transfected with the expression vector encoding for secretory renin exhibited signs of hypertrophy. They were greater in size and their protein content was higher when compared with cells transfected with the control vector. In contrast, such an effect was not obvious in cells transfected with expression vector encoding for cytosolic renin.

Cells transfected with secretory renin exhibited a markedly increased rate of necrosis as indicated by an increased LDH release/content ratio, whereas cells transfected with cytosolic renin were even protected from necrotic death, as indicated by a decreased LDH ratio[8].

Furthermore, when compared with control cells or cells overexpressing secretory renin the cells overexpressing cytosolic renin showed increased rate of apoptosis as indicated by (a) an enhanced number of shrunken cells, (b) an increase in caspase activation, (c) an increased Fas receptor expression, and (d) an increased phosphatidylserin translocation[8]. The increase in translocation of phosphatidylserin to the outer cell membrane further indicates that the apoptosis induced by cytosolic renin is primary mitochondrial-derived.

Overexpression of Cytosolic Renin in the Adrenal Gland, Heart, and Brain in Transgenic Rats

To investigate the functions of cytosolic renin in vivo we generated CXexon (2-9)renin transgenic rats overexpressing cytosolic renin under control of the CXCMV promoter[9]. Cytosolic renin was overexpressed in several tissues including the adrenal gland, kidney, heart, and brain of the transgenic rats. Plasma renin levels were not elevated supporting the concept that cytosolic renin cannot be secreted. All lines, which exhibit marked overexpression of cytosolic renin in the adrenal gland (three out of four independently generated transgenic lines), exhibited an elevated plasma aldosterone-to-renin ratio (blood was obtained under light ether anesthesia), indicating increased plasma renin independent aldosterone production.

In the heart, we did not observe any detrimental effect of cytosolic renin although the transgene was expressed and the renin content was elevated, particularly within the cytosolic and mitochondrial fractions in cardiac tissue[9]. In contrast to previous studies with transgenic mice[7], we did not observe any changes in blood pressure despite expression of cytosolic renin in the brain.

Discussion

Recent studies demonstrate that (pro)renin is sorted to different intracellular compartments dependent on the presence or absence of exon 1. Cells overexpressing

secretory renin transport the encoded prorenin to light vesicles and release renin into the medium. In contrast, cells overexpressing cytosolic renin transport the encoded protein to mitochondria, where it can be detected by means of immunocytochemistry/electron microscopy in the adrenal[6] and by confocal microscopy in cardiac H9c2 cells[8]. Supporting the hypothesis that cytosolic renin is targeted to mitochondria in vitro experiments indicate that cytosolic renin but not secretory prorenin can be imported into mitochondria[1].

Are the functions of secretory and cytosolic renin different from each other? Our studies indicate that the two renin transcripts have significantly different functions in the heart. Whereas secretory renin promotes cellular hypertrophy and induces necrotic cell death the cytosolic/mitochondrial renin protects from necrosis and induces apoptosis. The induction of apoptosis by cytosolic renin, although certainly undesired, may be considered to be a protective mechanism under such instances where it prevents necrosis. Necrotic cell death results in the release of subcellular membrane constituents that are capable of triggering the complement cascade[10] recruiting neutrophils and monocytes into the injured myocardium[11]. Immigration and activation of leukocytes then promote fibrosis and cardiac remodeling (for review see: 12,13). These responses are prevented by apoptosis.

Although we do not yet have any proof for such a protective function of cytosolic renin in the heart, the fact that transgenic rats overexpressing the cytoplasmatic renin variant do not exhibit any cardiac damage despite fivefold elevation of cytosolic renin levels in cardiac tissue[9] argues at least against detrimental effects under healthy conditions.

In the rat adrenal gland both exon(1-9)renin and exon(1A-9)renin transcripts are expressed. It has been suggested that there is a functionally active adrenal RAS in the zona glomerulosa. This assumption was based on the fact that under some conditions, such as potassium load or bilateral nephrectomy, aldosterone levels correlate better with adrenal renin levels than with circulating renin levels. The functional significance of intra-adrenal renin has meanwhile been demonstrated in vitro and in vivo and there is considerable evidence that there is a local secretory renin system in the adrenal cortex, which stimulates aldosterone production by means of amplifying the circulating angiotensin signal (for review see[14–16]).

Indirect evidence further suggested that the adrenal cortex might harbor additionally an intracellular renin system. Renin was detected immunohistochemically and enzymatically within adrenocortical mitochondria[5,6] and bilateral nephrectomy markedly increased mitochondrial-renin activity (about 20-fold) as well as aldosterone production[6]. First data with transgenic rats support the hypothesis that adrenal expression of cytosolic renin stimulates aldosterone production. Overexpression of cytoslic renin is associated with increased aldosterone-to-renin ratio[9]. However, since blood was obtained under ether anesthesia this finding still needs to be confirmed in conscious rats.

The circulating RAS, determined by secretory exon(1-9)renin, is known to increase blood pressure. In the present study overexpression of non secretory, cytoplasmatic exon(2-9)renin was not associated with an increase in blood pressure when compared with WT controls. Recently, astrocyte-specific overexpression of

human cytosolic renin together with human angiotensinogen has been reported to lead to increased blood pressure in double transgenic mice[7]. The discrepant findings between these studies may be explained by the different species (mice vs. rats) or different constructs used (astrocyte-specific promoter vs. nonspecific promoter). However, in the double transgenic mouse model plasma renin activity was (unexpectedly) increased[7] whereas it remained normal when cytosolic renin was overexpressed in rats.

To date, little information is available on the potential mechanisms of action of cytosolic renin. Like conventional renin, cytosolic renin may generate ANGI from angiotensinogen. Intracellular actions of ANGII have been proposed[17–19]. Furthermore, intra-cytoplasmatically applied ANGII produced effects without the need to bind ANG receptors from the extracellular space[19–21]. In addition, overexpression of cytoplasmatic angiotensinogen in a hepatoma cell line expressing cytosolic but not secretory renin increased mitosis and proliferation rates[22]. Since H9c2 cells express neither ACE nor angiotensinogen, however, we assume that the effects of cytosolic renin in H9c2 cells are angiotensin-independent.

Are there targets for cytosolic renin other than angiotensinogen? A renin receptor has been found, which exerts angiotensin-dependent and -independent mechanisms[23,24]. (Pro)renin binds to this receptor and activates signal transduction cascades (Erk1/2) independently of angiotensin generation[23]. Furthermore, secretory prorenin induces intracellular signaling in cardiomyocytes (p38 MAPK, HSP27, and TIMP1) angiotensin-independently[25]. These factors are known to promote hypertrophy. H9c2 cells transfected with secretory prorenin became hypertrophic in our studies. So far, however, we could not detect a prohypertrophic effect of cytosolic renin and do not yet have any evidence that cytosolic renin interacts with the (pro)renin receptor.

Another possible intracellular target for *exon(1A-9)renin*-derived cytosolic renin is the renin-binding protein[26]. Renin-binding protein is identical to the enzyme *N*-acetyl-D-glucosamine 2-epimerase[27]. The epimerase inhibits renin activity[28] and is itself inhibited by renin[29]. Renin-binding protein does not have access to secretory prorenin, because it is located within the cytosol, whereas secretory prorenin is not. On the other hand, renin-binding protein may well interact with *exon(2-9)renin*-derived cytosolic renin, both sharing the same location.

In summary, accumulating evidence indicate that in several species including human different renin transcripts are expressed from the same renin gene. These transcripts encode for renin proteins that are differentially sorted. The classically known secretory renin exerts most of its known functions by its enzymatic capability to generate angiotensin I from angiotensinogen. Additional functions of secretory renin are mediated by the prorenin receptor. In our hands, overexpression of secretory renin in H9c2 cardiomyoblasts induces hypertrophy and increases the rate of necrosis, both probably angiotensin-independently. The other transcript of the renin gene encodes for a cytosolic protein that can be imported into mitochondria. In cardiac cells this protein under certain circumstances protects from necrosis but increases the rate of apoptosis by as yet unknown mechanism.

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Chapter 8
Novel Aspects of the Cardiac Renin–Angiotensin System

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Abstract Involvement of the renin–angiotensin system (RAS) in human pathophysiology has expanded to include several diseases beyond a traditional role in salt and water homeostasis. In diabetes, there is significant overactivity of the RAS, which is inhibited by treatment with RAS blockers, thus decreasing diabetic complications. Activation of the RAS in diabetes includes several unique aspects, such as elevation of circulating prorenin levels and angiotensin (Ang) II-independent effects, mediated through interaction of pro(renin), with the pro(renin) receptor. Ang II-independent RAS actions suggest that efficacy of angiotensin receptor blockers (ARBs) and ACE inhibitors would have limitations in the treatment of diabetic patients. Recent meta-analyses of clinical trials have suggested that currently used RAS blockers may not provide additional benefits in diabetics compared to non-diabetics. We recently reported another novel aspect of the RAS, the intracellular system, which is dramatically activated in hyperglycemic conditions. In cardiac myocytes and fibroblasts, we demonstrated the presence of RAS components and synthesis of Ang II intracellularly. Hyperglycemia selectively upregulated the intracellular system in cardiac myocytes, vascular smooth muscle cells (VSMC), and renal mesangial cells where Ang II synthesis was largely catalyzed by chymase, not ACE. We also demonstrated elevation of intracellular Ang II (iAng II) levels in diabetic rat hearts, which resulted in increased cardiac myocyte apoptosis, oxidative stress, and cardiac fibrosis, suggesting a significant role of iAng II in diabetic cardiomyopathy. Others and we have previously reported that iAng II elicits multiple biological effects, some of which are not blocked by ARBs. Using Chinese hamster ovary (CHO) cells that do not express AT_1 receptor, we confirmed that the latter are not required for intracellular actions of Ang II. The AT_1-independent effects of iAng II are likely mediated by novel interactions between Ang II and intracellular proteins. The mechanism of RAS activation and intracellular accumulation of

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components by cardiac myocytes in high glucose (HG) conditions is not known. There is a possibility that an increased influx of glucose into the hexosamine biosynthesis pathway (HBP) and resultant O-glycosylation of proteins/transcription factors is responsible for the activation of the RAS and intracellular synthesis of Ang II.

Introduction

The RAS is classically viewed as a circulating system and as a major determinant of blood pressure and electrolyte homeostasis through vasoconstrictor and aldosterone-stimulating effects. Others and we have demonstrated the presence and regulated synthesis of components of the RAS in many tissues including the heart, indicating the existence of local Ang II-generating systems[1–5]. Local Ang II directly mediates cell growth, regulates gene expression, and activates multiple intracellular signaling pathways in cardiovascular cells[6–11]. The effects of Ang II are attributed to interaction with at least two specific, high-affinity, plasma membrane receptors, AT_1 and AT_2[10,12–14]. Most effects of Ang II, such as cell growth, vasoconstriction, and electrolyte homeostasis, are mediated by AT_1, which internalizes upon binding to Ang II. The function of the AT_2 receptor, though less well understood, is often antagonistic to that of AT_1[14–17]. In addition to these receptors, iAng II-binding sites or receptors, some of which are AT_1 like, have been reported by others and us[18–23]. A new non-AT_1, non-AT_2 Ang II receptor has been characterized in the brain[24]. Physiological significance and identity of intracellular receptors remains unknown.

Left ventricular hypertrophy (LVH) is characterized by an increase in myocyte size, re-expression of the fetal gene program, and increased accumulation of extracellular matrix proteins such as collagen and fibronectin[25,26]. Hemodynamic stress is fundamental to the development of LVH; however, non-hemodynamic factors such as the RAS have been strongly implicated in the hypertrophic response[7]. Significant clinical evidence supporting the latter is provided by efficacy of ACE inhibitors and AT_1 antagonists in blocking cardiac hypertrophy and remodeling[27]. Experimentally, Ang II infusion in rats induces cardiac hypertrophy through direct cellular effects, independent of blood pressure elevating effects[12]. Ang II-stimulated hypertrophy has been demonstrated in cultured NRVM, where Ang II induces expression of early genes (e.g., c-fos, c-jun, jun B, Egr-1, c-myb, and c-myc), genes for growth factors (e.g., TGF-β), and the hypertrophy-marker genes, α-actin and ANP[28]. Ang II also stimulates proliferation of neonatal rat cardiac fibroblasts. The mitogenic effects of Ang II are blocked by AT_1 but not by AT_2 antagonists[29]. It has been reported that Ang II secreted from myocytes has a central role in stretch-induced hypertrophy, functioning in an autocrine manner in the neonatal rat myocyte primary culture system[30]. The significance of Ang II in cardiac hypertrophy is also supported by studies utilizing transgenic animal models. Overexpression of the AGT gene in cardiac myocytes results in increased cardiac Ang II and both right and left ventricular hypertrophy. The latter is not accompanied by hypertension, indicating

a direct effect of Ang II[31,32]. When AT_1 expression is targeted to cardiac ventricles in transgenic rats, the hypertrophic response to pressure overload is increased compared to control animals, suggesting synergism between mechanical load and AT_1 activation in inducing cardiac growth[33]. Recently, there has been a debate over a direct role of Ang II in cardiac hypertrophy[34,35]. It appears that locally produced rather than circulating Ang II is an important determinant of cardiac effects[35,36]. Our studies indicate that local RAS activity is regulated by iAng II[37]. The absence of direct cardiac effects of Ang II in some studies could be explained by the lack of activation of iAng II[36,38,39].

The Intracellular Renin–Angiotensin System

The widely accepted mode of action of Ang II is through extracellular binding of the peptide to AT_1 receptor. However, recent reports have demonstrated that Ang II also elicits biological effects from an intracellular location, referred to as "intracrine actions"[40–45]. Ang II undergoes AT_1-mediated internalization, largely considered a mechanism of receptor downregulation and degradation of the agonist[46]. However, a portion of the internalized Ang II was localized to perinuclear and nuclear regions, suggesting Ang II binding sites at the level of the nucleus[47–49]. We were the first to demonstrate AT_1-like Ang II binding sites on the nuclear envelope of rat hepatocytes[18]. Others have recently reported similar sites in renal cortex and medulla[50]. This AT_1-like nuclear receptor was functionally active, as there was coupling to gene transcription[19]. AT_1-like Ang II receptors were also detected in sarcolemma, T-tubules, and nuclei of rat cardiac myocytes, using electron microscopic and immunofluorescence techniques[51]. Additionally, Ang II colocalized with transcriptionally active euchromatin in endothelial and granule cells[47]. Ang II binding to solubilized rat liver chromatin fragments and the existence of a discrete Ang II-binding nucleoprotein particle suggested the presence of novel Ang II receptors on chromatin[21,52]. Another line of evidence for intracrine effects of Ang II was provided by studies in which Ang II synthesis was modulated intracellularly. Renin dialysis into isolated cardiac myocytes of cardiomyopathic hamsters, reduced gap junction conductance, which was inhibited by the ACE inhibitor, enalaprilat[53]. Intracellular administration of enalaprilat showed a significant increase in cell coupling in cardiac myocytes of 6-month (hypertrophic phase) and 11-month (dilatation phase) old hamsters[43]. These studies suggested that endogenous iAng II is involved in the regulation of cell coupling at advanced stages of hypertrophy, when the cardiac RAS is activated. The most convincing evidence came from direct demonstration of the intracrine effects by others and us, by generating iAng II using recombinant approaches, in vitro and in vivo, or by microinjection of Ang II into isolated cells. We demonstrated that iAng II induced cell growth in cultured NRVM and cardiac hypertrophy in adult mice[40]. In vascular smooth muscle cells, microinjection of Ang II led to a rapid increase in intracellular calcium and tyrosine phosphorylation[44,54]. In cardiomyopathic hamsters, at advanced stages, intracellular dialysis of Ang II abolished cell coupling in cardiac myocytes[55]. In CHO-K1 and hepatoma

cells, intracellularly generated recombinant Ang II induced cell proliferation[41,56,57]. There is now substantial evidence demonstrating intracrine effects of Ang II in multiple cell types and organs including the heart[58].

Physiological Functions of Intracellular Ang II

As described above, others and we have demonstrated effects of iAng II on several cellular functions including the following: cardiac hypertrophy and myocyte cell growth; cell proliferation in vascular smooth muscle cells, hepatocytes, CHO, and COS cells; and heart cell communication and inward calcium current in myocytes[59]. In addition, our recent studies show that iAng II has a major role in regulation of the local RAS, which is an important determinant of cardiac effects[36,60]. A physiological role for iAng II has been indicated by animal studies, which showed increased Ang II inside cardiac myocytes in various conditions. For example, transgenic animals overexpressing AGT, which developed biventricular hypertrophy, showed increased iAng II immunostaining in heart sections[31,32]. Similarly, rats that were made diabetic by streptozotocin and developed cardiomyopathy showed enhanced iAng II staining in cardiac myocytes[61,62]. Significantly, in humans, iAng II levels in cardiac myocytes were 3.4-fold higher in diabetic patients compared to non-diabetics and an additional 2-fold higher in diabetic hypertensive patients compared to diabetic non-hypertensive patients[63]. The latter observations in diabetic rats and humans support the significance of intracrine Ang II.

Source of Intracellular Ang II

A conundrum for intracrine effects of the peptide relates to the source of iAng II in physiological or pathophysiological conditions. The prevailing paradigm is that Ang II is synthesized in the interstitial space, not intracellularly, in the heart. This raised an important question as to the source of iAng II. Addressing this question, there were two likely possibilities:

(i) *Receptor-mediated internalization*: Binding of Ang II to plasma membrane AT_1 receptor results in internalization of the Ang II-AT_1 complex through clathrin-coated pits[13,64]. While a significant amount of internalized Ang II is degraded, a regulated portion is targeted to intracellular locations (such as the nucleus), where it could function as an intracrine mediator[47–49].

(ii) *Intracellular synthesis*: For intracellular synthesis, all RAS components, AGT, renin, ACE (or alternative enzymes, such as chymase), need to be present inside the cell. AGT and renin are generally secreted from cells. The third component, ACE, is a membrane-bound enzyme with catalytic domains on the extracellular surface. The likelihood of intracellular synthesis of Ang II became more tenable with several studies suggesting intracellular distribution of RAS components in certain physiological conditions or cell types. AGT has a signal sequence which is required for endoplasmic reticulum (ER) translocation, but

does not necessitate extracellular secretion. The destination of proteins in the ER is further determined by glycosylation and the presence of other targeting sequences. Significantly, brain astrocytes produce a non-glycosylated form of AGT, which is not secreted and is targeted to the nucleus[49]. Cardiac cells produce multiple, differentially glycosylated forms of AGT[65], suggesting a complex distribution pattern, including intracellular, depending on the cellular state. The second component of the RAS, renin, is internalized by cardiac myocytes through mannose-6-phosphate and/or other unidentified receptors[66–68]. A non-secretory intracellular renin has been shown to be upregulated in the left ventricle following myocardial infarction[69] and contributes to intracellular Ang I generation from AGT[69]. Additionally, certain pathological conditions such as hyperglycemia decrease renin secretion and increase intracellular renin activity, resulting in an increase of Ang II generation in rat mesangial cells[70]. Ang I to Ang II conversion can occur by ACE or chymase, both of which are expressed in the heart[71,72]. Though ACE is mainly membrane bound or soluble, it has also been detected inside cardiac myocytes and fibroblasts by immunohistochemical analysis[73]. Recently, active N-domain forms of ACE have been demonstrated intracellularly in rat mesangial cells[74]. Chymase is an intracellular enzyme, which was recently demonstrated to generate vascular and renal Ang II in diabetic patients[75,76]. The intracellular presence of all components of the RAS, under certain conditions, strongly supports iAng II synthesis. Additional evidence for iAng II synthesis included release of Ang II following mechanical stretch in cultured NRVM[30,77], and increased levels of iAng II following incubation of rat myocytes with exogenous, non-glycosylated proreninrref[78]

Recently, we have reported the pathways for iAng II generation and intracellular localization in NRVM and fibroblasts under physiological settings[37]. We observed that the site of Ang II production in cardiac cells is dependent on the cell type and nature of the stimulus. In HG conditions, cardiac myocytes activate and retain the RAS components, AGT and renin, intracellularly[37]. This results in a significant increase in intracellular generation of Ang II, which is directed to the nucleus instead of being secreted. In contrast, fibroblasts secrete intracellularly synthesized Ang II, in addition to extracellular synthesis. In HG conditions, iAng II synthesis by cardiac myocytes is renin and chymase, not ACE, dependent. A renin inhibitor (aliskiren) that is efficiently internalized by NRVM completely blocks iAng II synthesis[37]. The observation of only an intracellular increase in Ang II in cardiac myocytes in HG conditions suggests a major role for intracrine Ang II in diabetes. Recently, our observations have been substantiated by other investigators, who similarly reported chymase-mediated iAng II synthesis by HG in rat mesangial and vascular smooth muscle cells[79,80]. The significance of HG-induced intracellular retention of AGT and renin by cardiac myocytes is further evidenced by studies demonstrating increased expression of kallikrein-like prorenin converting enzyme (PRECE) in diabetic mouse heart and an intracellular renin receptor that couples to signal transduction[81,82]. It will be important to identify the mechanism(s) of HG-induced RAS activation in cardiac myocytes.

Hyperglycemia and the RAS

Poor glycemic control is associated with a high incidence of heart failure in diabetic patients. The metabolic derangement together with stimulation of the local RAS results in unfavorable cardiac remodeling and myocardial dysfunction[83,84]. An interesting aspect of the RAS in diabetes is that while circulating levels of active renin and Ang II are reduced, tissue levels are increased[63,85,86]. Several studies have demonstrated tissue activation of the RAS; however, the precise location of RAS components, i.e., extracellular, as is generally assumed for AGT and renin, or intracellular, was not determined until recently[87–89]. HG was shown to decrease renin secretion but increase intracellular renin activity, resulting in increased Ang II generation in rat mesangial cells[70]. It is likely that various cell types within a single tissue respond differently to hyperglycemic conditions, some contributing to the extracellular RAS, others retaining components intracellularly. Our observations under HG conditions in cardiac fibroblasts and myocytes, with both responding by increasing Ang II production, are that one secretes RAS components and the other retains components intracellularly, respectively[37,90].

Regulation of the Cardiac RAS by High Glucose

A strong association has been established between diabetes and Ang II-activated oxidative stress and signaling pathways, such as p38 MAPK, Jak2, and p53[62,91–93]. However, the mechanism of cardiac RAS activation in hyperglycemia is not completely understood. Several cellular metabolic events are associated with hyperglycemia as follows: (1) increased influx of glucose through the hexosamine biosynthesis pathway (HBP); (2) increased O-glycosylation of proteins; (3) increased activity of the polyol pathway; and (4) oxidative stress[94]. The effect of these events on gene expression and cellular function has been reported[95]. However, information on the regulation of the RAS, particularly the cardiac RAS, is very limited.

Hexosamine Biosynthesis Pathway

Glucose is normally metabolized by glycolysis to produce pyruvate, which enters the TCA cycle, resulting in energy production. Small amounts of glucose (1–3%) enter the HBP acting as a nutrient sensor (Fig. 8.1)[96]. Increased glucose flux into the HBP has been implicated in the development of insulin resistance as well as the vascular complications of diabetes[97,98]. Activation of the HBP has also been associated with glucose-induced transcriptional upregulation of AGT[87], TGFα[99], TGFβ[100], leptin[101], plasminogen activator inhibitor-1 (PAI-1)[102], and decreased phosphorylation of Akt and GSK3[103]. The end product of HBP is uridine diphospho-*N*-acetylglucosamine (UDP-GlcNAc), which is a substrate for the enzymatic O-glycosylation of proteins (Fig. 8.1). O-glycosylation is a dynamic, reversible, and ubiquitous post-translational modification that is an important

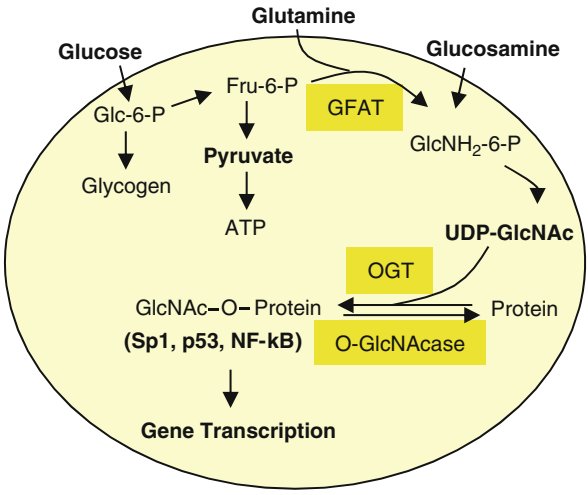

Fig. 8.1 Cellular glucose utilization, hexosamine biosynthesis, and protein O-glycosylation pathways. In cardiac myocytes, O-glycosylation of transcription factors Sp1 and p53 increases the transcriptional activity, which is associated with diabetic complications

regulatory mechanism involved in signal transduction in higher eukaryotes[95,97,104]. Several different classes of proteins; kinases, phosphatases, transcription factors, metabolic enzymes, chaperons, and cytoskeletal proteins are modified by O-glycosylation[95]. The transcription factor p53 was shown to initially undergo O-glycosylation in response to hyperglycemia followed by phosphorylation in adult cardiac myocytes[86]. Both glycosylation and phosphorylation activated p53 and increased expression of p53-dependent genes[86]. Similarly, Sp1 was modified by O-glycosylation in NRVM, which was accompanied by reduced expression of sarcoendoplasmic reticulum $Ca^{(2+)}$-ATPase 2a (SERCA2a) and myocytes enhancer factor-2A (MEF-2A)[105]. In mesangial cells, increased O-glycosylation of the p65 subunit of NF-κB by HG was reported, which resulted in enhanced activity of human VCAM-1 promoter. It was also reported that O-glycosylation of p53 had a significant role in AGT transcription[86]. However, expression of renin, a rate-limiting enzyme in Ang II synthesis and ACE were not controlled by p53. Other than p53, the transcription factors Sp1 and NF-κB regulate expression of more than one RAS component; i.e., the expression of AGT, ACE, and renin is modified by Sp1; and NF-κB contributes to the transcriptional control of AGT and renin[106–109]. Whether HG-induced activation of RAS components is controlled by Sp1 and NF-κB is not known.

High Glucose-Induced Oxidative Stress

Several studies have provided evidence that hyperglycemia stimulates oxidative stress, which contributes to the pathological effects of diabetes[110]. Hyperglycemia-induced reactive oxygen species (ROS) are generated by an increased influx of glucose into metabolic pathways, i.e., glycolysis, polyol pathway, and HBP[111]. Using

antioxidants and overexpression of uncoupling protein-1 and manganese superoxide dismutase, the central role of ROS in hyperglycemic damage has been confirmed[110]. In addition to causing oxidative damage to lipids, proteins, and DNA, ROS act as a second messenger in several signaling pathways such as PKC and MAPK, modulating the activity of transcription factors, including NF-κB and activator protein-1 (AP-1)[112]. Oxidative stress has been shown to increase expression of AGT through activation of NF-κB[113]. Thus, a role of NF-κB in HG-induced activation of the RAS is likely.

Mechanism of iAng II Actions

Having demonstrated intracrine effects and intracellular synthesis of Ang II the next important question is regarding the iAng II receptor. AT_1 receptor that internalizes from the plasma membrane or intracellular AT_1-like receptors detected on nuclear membranes and other organelles are believed to be possible mediators of the intracrine effects of Ang II. However, data from different labs are equivocal in this regard[114]. Involvement of AT_1 receptor seems to depend on the cell type, species, and the function being studied[114]. In our laboratory, intracrine Ang II-induced cardiac myocyte cell growth and hypertrophy were not inhibited by an AT_1 receptor blocker (ARB)[40]. Though there is no direct evidence of cellular internalization, intracellular efficacy of ARBs is indicated by the effect on PPARγ activation[115]. However, certain other intracrine functions of Ang II such as inward Ca^{2+} current and smooth muscle cell growth were not blocked, even by intracellular delivery of ARBs[55,114]. Significantly, iAng II induced cell proliferation in CHO cells that do not express AT_1 receptor[56]. The weight of evidence indicates that discrete functions, including hypertrophy in cardiac myocytes, are mediated by interaction of iAng II with novel intracellular binding proteins or receptors.

Discovery of Ang II Receptors

In the late 1970s, like other peptide hormones, Ang II was postulated to act on a receptor located on the plasma membrane (not intracellularly) of target cells. The initial search for an Ang II-binding protein was limited to the particulate (membrane) fractions of cells. Binding studies in rat liver and kidney cortex led to discovery of two Ang II receptors that were classified as AT_1 and AT_2[116]. In addition to plasma membrane receptors, the likelihood of a nuclear Ang II receptor was suggested by studies that showed internalization and perinuclear localization of radiolabeled Ang II[117]. Ang II binding to rat hepatocyte nuclei identified an "AT_1-like" receptor, which differed from the plasma membrane receptor in pH sensitivity, time course for binding, affinity for Ang I, II, and III, and rate of dissociation[18]. This nuclear receptor has not been cloned and thus, the identity of this AT_1-like receptor is unknown. A similar AT_1-like Ang II receptor was recently described in

renal cortex and medulla[118]. Additionally, Ang II binding to solubilized rat liver chromatin fragments and the existence of a discrete Ang II-binding nucleoprotein particle suggested the existence of novel Ang II receptors on chromatin[21,52]. In other studies, a soluble Ang II-binding protein was purified from rabbit hepatic particles that bound Ang II with high affinity in a receptor-like manner (saturable, reversible, high affinity)[119]. This protein was later identified to be an endopeptidase and was not explored further, as it was believed that a peptidase could not be a receptor[120]. There is now evidence that some peptidases can act in a receptor-like manner. For example, the Ang IV receptor is a peptidase (insulin-regulated aminopeptidase, IRAP)[121]. Recently, another peptidase, prostate-specific membrane antigen, has been shown to induce signal transduction and to regulate angiogenesis[122]. Some peptidases of the RAS such as ACE and ACE2 also act as receptors[123,124]. Now that an intracellular role of Ang II has been identified, it will be important to determine the cytosolic partners and nuclear binding of Ang II. It is likely that the nuclear AT_1-like receptor is distinct from the plasma membrane AT_1 receptor. This is indicated by our observation demonstrating intracrine effects of Ang II in CHO cells, which do not express significant levels of membrane AT_1 receptor[56]. The iAng II might also directly bind to transcription factors in the cytoplasm or nucleus, as recently described for the intracellular renin receptor and intracrine FGF-2[82,125]. A new Ang II receptor, which is non-AT_1, non-AT_2, has recently been identified in brain, suggesting that the search for more Ang II receptors is not over[24].

Significance

Our findings of iAng II synthesis and demonstration of an AT_1-independent mechanism of iAng II effects are important for understanding the mechanism of Ang II actions and effectiveness of current therapeutic strategies. We observed that iAng II synthesis, under conditions that cause nuclear translocation of Ang II, is chymase-dependent and not blocked by ACE inhibitors. AT_1-independent intracrine effects of Ang II are not inhibited by ARBs. Thus, current therapeutic modalities utilizing ACE inhibitors and ARBs may only be partially effective under pathological conditions where iAng II is produced. The benefits of ACE inhibitors and ARBs during and after myocardial infarction (MI) have been found to be greater in diabetics than non-diabetics and activation of the RAS has been implicated in diabetes[126]. However, following MI the incidence of heart failure and mortality rates are increased twofold in patients with diabetes compared to non-diabetics[127,128]. There is growing consensus that inhibition of the RAS using ARBs and ACE inhibitors has not provided as much cardiovascular benefit as anticipated[27,129]. Mechanistically, these observations are likely related to activation of the intracellular RAS. Understanding the regulation of the intracellular RAS, identification of intracellular receptors or binding proteins for iAng II, and determining the mechanisms of iAng II actions will provide additional targets for therapeutic intervention.

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Chapter 9
Intracrine Function from Angiotensin to Stem Cells

Richard N. Re and Julia L. Cook

Abstract Intracrine action is increasingly being appreciated as a physiologically relevant signaling mechanism. Growing out of the study of angiotensin biology, intracrine physiology is becoming better understood and general principles of intracrine action have been proposed. Here the field will be briefly reviewed and some predictions of intracrine theory discussed to illustrate these principles of intracrine action. The potential relevance of these ideas to the working of the local renin–angiotensin systems and to diverse other biological processes such as differentiation and neoplasia is discussed.

Background

Intracrine action, the intracellular functioning of extracellular-signaling peptides, has been a long-term interest of our laboratory. We have proposed ideas regarding the evolutionary basis of these factors and their roles in differentiation and tissues responsiveness and have suggested ways in which intracrine physiology can aid in the development of novel pharmacological approaches to diseases. It is particularly heartening to see great progress being made in the area of intracrine renin–angiotensin system function. Arguably, angiotensin II was the first intracrine to be identified and, as many of the papers at this symposium attest, the physiological relevance of its intracrine function is great. But it must also be remembered that the intracrine hypothesis transcends the renin–angiotensin system. The intracrine paradigm can be applied in the cases of hormones, growth factors, deoxyribonucleic acid (DNA) binding proteins, and even enzymes[1–11].

As mentioned, angiotensin II was the first hormone for which an intracrine action was proposed when the injection of tritiated angiotensin II was found to

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be associated with rapid accumulation of tracer in the nuclei and mitochondria of rodent cardiomyocytes as well as with the stimulation of ribonucleic acid (RNA) synthesis. High-affinity nuclear binding sites for angiotensin II were subsequently identified on isolated nuclei and binding of angiotensin II to these sites upregulated RNA synthesis, including the upregulation of renin and angiotensinogen expression. Later parathyroid hormone-related protein was shown to be internalized by target cells and traffic to nucleus. Moreover, an isoform of the PTHrP was found to traffic directly to nucleus without the intermediary of secretion. The nucleolar protein nucleolin was also shown to serve as a cell surface protein capable of shuttling extracellular factors such as midkine from the extracellular space to the nucleus. Indeed, many angiogenic and anti-angiogenic peptides were shown to traffic from the extracellular space to nucleus. Inhibition of ribosomal gene expression appeared to be associated with inhibition of cell growth. Moreover, dynorphin, an endogenous opiod, was found to bind to isolated nuclei and upregulate its precursor prepro-dynorphin—a circumstance similar to that observed in the case of angiotensin II stimulation of angiotensinogen expression. The instillation of angiotensin II directly into cardiac myocytes could be shown to influence intracellular calcium transients and intercellular conductance. These and other results led us to a wider appreciation of intracrine action[1–37].

Early on we proposed the term intracrine for the intracellular action of an extracellular signaling peptide, and we proposed that many peptide factors could act as intracrines. We also suggested that intracellular renin–angiotensin systems likely existed[1–7]. However, 10 years ago we began to develop a hypothesis that unified the observations regarding intracrine functionality to that time into one hypothesis[3–8]. We went on to suggest that intracrines grew out of earlier systems linking ribosomal function with cell growth; that intracrine intracellular regulatory loops of various sources existed; that intracrine functionality could be associated with various molecular entities including hormones, growth factors, DNA binding proteins, and enzymes among others; and that finite gain positive feedback regulatory loops could produce forms of cellular and tissue differentiation or regulate hormone responsiveness[3–8]. Although originally met with some skepticism, these notions related to intracrine action have come to be more widely accepted of late. One of the most contentious issues was the idea that finite gain positive feedback loops could produce differentiation. We have discussed this issue elsewhere but a recent finding may make this notion more palatable. PAX5 is a transcription factor that generates a finite gain positive intracellular regulatory loop, which actively maintains the differentiated state in B-lymphocytes. Knock down of PAX5 in B-lymphocytes results in their dedifferentiating to uncommitted bone marrow progenitor cells from which they may even emerge as T-lymphocytes or as neoplastic lymphoma cells[32]. Although we have no evidence that PAX5 is an intracrine, its actions in B-lymphocytes are an example of the active form of differentiation, which we postulated. The function of the so-called read-through form of acetylcholinesterase provides yet another example of active differentiation in a more clearly intracrine context. These and other issues related to the intracrine hypothesis are discussed in detail elsewhere[1–7].

The intracrine hypothesis, therefore, has much to say about a variety of biological processes and, indeed, can make predictions on various levels. In evaluating the validity of a hypothesis, it is usual to both look to its ability to explain known facts as well as investigate the validity of predictions that follow from its tenets. Both forms of validation are important. Although there has been little time for many of the predictions of intracrine theory to be experimentally tested, some have been, and, therefore, rather than again review the known facts explained by the theory, we will here examine the correctness of the predictions which have been tested to date. Next the intracrine renin–angiotensin system (iRAS) will be discussed in the context of the intracrine hypothesis. New suggestions based on the intracrine hypothesis applied to stem cell biology will then be touched on.

Relevant Experimental Observations

Vascular endothelial growth factor (VEGF) is a potent angiogenic factor and, as already noted, many angiogenic factors are intracrines which traffic to nucleus. Also, like many other intracrines VEGF is synthesized in multiple isoforms. These characteristics coupled with the potent angiogenic effects which the transfection of ischemic limbs with VEGF-containing plasmids produced—effects apparently disproportionate to the number of successfully transfected target cells—led us to postulate that VEGF was an intracrine which produced active differentiation through the establishment of intracellular positive loops with subsequent secretion to affect nearby cells[33]. At almost the same time the nuclear trafficking of VEGF after tissue wounding was reported. About two years later, as proposed, an intracellular VEGF-driven intracrine positive feedback loop was identified and shown to produce cellular differentiation[34,35]. These initial reports were in hematological stem cells but the same VEGF intracrine action was later discovered in certain neoplastic cells such as myeloma cells. Still later it was shown that similar albeit more complex loops involving VEGF and other intracrine angiogenic factors such as angiogenin were operative in endothelial cells where they participate in angiogenesis as originally suggested[36].

Beginning in the 1990s, evidence has grown to indicate that homeodomain transcription factors can function as local extracellular signaling molecules—that is, can function as intracrines[37,38]. It was later shown that the administration of glucagon-like-peptide I to hepatic duct cells led to the upregulation of the homeodomain protein PDX1 and this set up a cascade of transcriptional events leading to the differentiation of the duct cells along the beta islet cell line resulting in insulin expression. Because of the intracrine nature of homeoproteins, we hypothesized that transfection would not be required to mimic endogenous PDX1 action in this setting—as an intracrine PDX1 should behave as an extracellular signaling molecule so that the administration of the peptide to the duct cells should result in internalization, upregulation of PDX1 itself, and movement of the cells along the beta cell line[7]. About

a year later this experiment was conducted and the predicted result was obtained—upregulation of PDX1 was found and subsequent insulin synthesis was observed[39]. This was a striking confirmation of Prochiantz's findings regarding the secretion and uptake of homeoproteins and of the notion that intracrine physiology is important in some forms of differentiation[37].

Heregulin, neuregulin, and EGF are intracrines and are involved through interactions with tyrosine kinase receptors of the EGF receptor (EGFR) family in stimulating cellular proliferation. The HER2 receptor in this family plays an important role in the stimulation of breast cancer cell proliferation. A monoclonal antibody (Herceptin) to the HER2 is clinically useful in cancer therapy, although resistance to this agent not infrequently develops. This resistance has been ascribed to the development of constitutively active receptor which are unaffected by antibody. Because of the intracrine nature of heregulin, neureglin, and EGF we hypothesized that EGFR-ligand produced proliferation not only by acting at the cell surface but also by acting in the intracellular space and more particularly at the nucleus. We suggested that intracellular EGF receptor–ligand loops could effectively drive the proliferation of tumor cells in a fashion unaffected by externally administered agents directed at cell surface receptors and these intracrine loops could thereby produce a second kind of antibody resistance[39]. Recently, this kind of intracellular growth factor upregulation was confirmed. In some cases resistance to Herceptin was found to be associated with upregulation of heparin-binding EGFR ligands, including heparin EGF and heregulin[40]. Moreover, resistance to the HER1 tyrosine kinase inhibitor gefitinib (Iressa) was associated not only with upregulation of EGFR ligands but also with their enhanced trafficking to nucleus. The authors of the latter report felt that this previously unrecognized intracrine feedback warranted further study as a potentially remediable form of resistance[41,42].

The important role of nucleolin in intracrine physiology was recognized early on. This pleurifunctional protein is integral to ribosomal synthesis on the one hand and to the internalization and nuclear trafficking of intracrine factors, and in particular angiogenic intracrines, on the other. We therefore argued that nucleolin plays an important role in intracrine physiology[5,40,43]. Later, an anti-tumor antibody was found to bind to surface nucleolin on both tumor cells and angiogenically active endothelial cells. Because of this and other findings related to intracellular intracrine trafficking, we suggested that inhibition of nucleolin trafficking or function could have important effects on inhibiting angiogenesis and cancer cell proliferation. The extension to tumor cells was suggested by what we called "intracrine reciprocity," the utilization of similar intracrine systems by both tumor endothelial cells and tumor cells themselves with crosstalk occurring between these systems. For these and other reasons, we suggested that inhibiting nucleolin function and trafficking could be an important therapeutic target[5,40,43]. Some time thereafter it was reported that the potent anti-angiogenic factor endostatin—a peptide whose mechanism of action was at that time controversial—bound to cell surface nucleolin was internalized and trafficked to nucleus where it had the effect of inhibiting nucleolin function by inhibiting its phosphorylation[44]. This action was critical to its effects on angiogenesis. Moreover, it has recently been reported

that a pseudopeptide which binds to the carboxy terminus of cell surface nucleolin downregulates cell surface expression of the protein, apparently because the complex is internalized but cannot enter nucleus. This pseudopeptide inhibits nucleolin trafficking to nucleus and thereby inhibits both angiogenesis and tumor cell growth as predicted[45]. Another example is provided by AS1411, a quadruplex-forming oligonucleotide aptamer that targets nucleolin. It is currently in clinical oncology trials and appears to act by disrupting the function of nucleolin-containing complexes[46].

The Intracrine Renin–Angiotensin System (iRAS)

As noted above, angiotensin was the first described intracrine hormone and its intracellular action suggested to us the existence of the iRAS[1–7,47,48]. In recent years an increasing amount of research has been carried out to better define the intracrine actions of this peptide. Much of this work is reviewed in this symposium. After nuclear binding sites were identified and characterized along with the genes they regulated, the next phase of research dealt with artificially upregulating intracellular angiotensin and its receptors, resulting in activation of the iRAS and cellular proliferation[1–11,15]. Extension of these studies to cardiac myocytes led to the striking demonstration of cardiac hypertrophy in animals whose myocytes were made to express angiotensin II intracellularly. Upregulation of the iRAS in cardiac myocytes by hyperglycemia was next demonstrated and, consistent with intracrine principles, a positive feedback loop was shown to exist such that intracellular angiotensin II upregulated the synthesis of angiotensinogen and renin. Inhibition of intracellular renin downregulated angiotensinogen expression[12–14]. In still more recent studies the intracellular generation of angiotensin II as opposed to its generation in the extracellular space was shown to reduce cardiac myocyte cell volume, suggesting that the iRAS could play a role in off-setting cell swelling after ischemic insult[7]. Also the effects of intracellular angiotensin II on calcium fluxes were shown to be inhibited by the mineralocorticoid blocker eplerenone, suggesting a role for aldosterone in the functioning of the iRAS[49]. Thus, research into the functioning of the iRAS is expanding and is producing unexpected and important results. The availability of pharmaceuticals such as the renin inhibitor aliskiren that are capable of inhibiting the iRAS and the development of new agents should provide important therapeutic advances[50,51]. Finally, the trafficking and fate of the AT-1 receptor after ligand binding at the cell surface or in the intracellular compartment is being defined and this not only is providing an improved understanding of the workings of the iRAS but also identifies new therapeutic targets[10,52,53].

At the same time, however, it is important to note that other components of the RAS appear to be intracrines and are therefore worthy of investigation in this regard[47]. For example, based on its homology to serpins, among which are anti-angiogenic intracrines, its own anti-angiogenic action as well as its potential to act as a signaling molecule, we suggested that angiotensinogen would likely be found

to be an intracrine[7,54]. It was later shown that depending on its phosphorylation state angiotensinogen can be retained in glial cells and traffic to nucleus[55]. Also, angiotensin converting enzyme immunoreactivity has been detected in mesangial cell nuclei along with angiotensin (1-7) immunoreactivity[56]. We have noted that renin fulfilled the definition of an intracrine but was somewhat atypical in that it had not been identified in nucleus[47]. Recent studies demonstrate the nuclear renin immunoreactivity in mesangial cells (D. Casarini; personal oral communication; May 2007). The (pro)renin receptor is a homologue of the vacuolar ATPase associated protein and is therefore widely expressed in the intracellular space[57,58]. Although renin internalization by the mannose-6-phosphate/IGF II receptor leads to the degradation of the enzyme, there appears to be another receptor which can internalize non-glycosylated renin (which can be found in plasma) permitting it to function within the cell[59–61]. Taken together, these findings strongly suggest that renin, angiotensin converting enzyme, and angiotensin[1–7], like angiotensin II, are intracrines. We have only begun to scratch the surface of the functioning of the iRAS and it is likely that these and other intracrine components of the RAS likely participate in intracrine regulatory feedback loops of their own with important implications for cell function.

Intracrine Stem Cell Physiology

As predicted by the intracrine hypothesis, intracrines play an important role in tissue and cellular differentiation[1–8]. The role of dynorphin in cardiac development, the role of homeodomain transcription factors such as Oct-3/4 and Nanong in the differentiation of embryonal stem cells, and the role of intracrines such as VEGF and high-mobility group box 1 (HMGB1) in tissue progenitor cell recruitment or development, among other instances of intracrine involvement in development and stem cell regulation, have been discussed in detail elsewhere[1–8]. Some of these observations may have potential implications for therapy. For example, the possibility suggests itself that in the process of generating pleuripotent stem-like cells from somatic cells the retroviral transfer of *Oct-3/4* or *Nanong* may be replaced by the simple external application of the proteins because these factors are homeoproteins and likely, therefore, intracrines[6]. If this is the case, a reduction in the oncological potential of the induced stem cells could be expected by virtue of reducing their exposure to retrovirus. Indeed, there is also reason to speculate that other factors required for the generation of induced stem cells, for example *Sox2*, could act in an intracrine fashion[6,62].

There is one proposal we have made regarding the participation of intracrines in development, which although unorthodox is testable and potentially important. Cancer is usually thought to result from the uncontrolled proliferation of disordered cells, each one of which is capable of tumor propagation. Some recent evidence, however, challenges this notion. These data suggest that in many tumors there exists a small population of slowly growing “tumor stem cells,” which asymmetrically

divide to produce stem cells as well as faster proliferating daughter cells which then form the bulk of the tumor mass but which are not immortal and cannot reproduce tumor in recipient animals. Therapies designed to eliminate rapidly proliferating cells could kill a large fraction of the daughter cells but leave untouched the more slowly dividing stem cells, resulting in recurrence of tumor. If this is the case in some tumors, one can ask if intracrine biology could play a role in the differentiation and growth regulation of tumor stem cells as it does in normal stem cells. Indeed, some data indicate low levels of expression of Oct-3/4 in scattered cells of a variety of tumors. Although this finding does not fit with the theory of intracrine differentiation in that one would expect upregulation of intracrines through the tumor, it could be explained if some tumor cells lacked the ability to upregulate a given intracrine but nonetheless required it to maintain their stemness. The expression and secretion of intracrines such as Oct-3/4 by a few cells could then rescue cells unable to upregulate it—cells we have termed "conditional stem cells"—and thereby maintain their stemness[6,62]. This process is analogous to "intracrine reciprocity" which we have described in tumor angiogenesis. Although this suggestion of conditional stem cells is not a part of the standard intracrine hypothesis, it is consistent with it. It again leads to the suggestion, as yet untested, that the interruption of intracrine loops could play a role in cancer therapy.

Conclusion

In this chapter recent advances in the field of intracrine biology have been explored, albeit in a somewhat cursory manner. The scope of intracrine physiology is large and much about it is still not understood. However, the picture is becoming clear and one can expect continued rapid progress and therapeutic application in the years to come.

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Chapter 10
Renin Cell Identity and Homeostasis

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Abstract Renin-expressing cells are precursors for vascular smooth muscle cells within the kidney, which in turn are capable of dedifferentiating and synthesize renin in response to challenges to homeostasis. The mechanisms that determine the identity of renin cells are the subject of many studies. Using a cell model of renal arteriolar smooth muscle cells dually labeled with fluorescent markers we showed that in vitro the cells can reexpress renin upon cAMP stimulation. In addition, reexpression of renin through the cAMP pathway involves chromatin remodeling by acetylation of histone 4. Further experiments confirmed that histone acetyl transferases play an essential role in the maintenance of the renin cell identity.

The renin–angiotensin system (RAS) regulates blood pressure and fluid and electrolyte homeostasis[5,8]. Clinical and experimental evidence has also accumulated demonstrating the morphogenetic and growth actions of the RAS within and outside the kidney[5].

A key event in the RAS is the exquisite regulation of renin synthesis and release by the kidney juxtaglomerular cells. Juxtaglomerular (JG) cells have been considered for a long time as terminally differentiated because they synthesize a hormone, are few in numbers (about 0.1–0.01% of the total kidney cell mass), and have a specific restricted localization. However, renin cells are neither terminally differentiated nor do they reside solely in the juxtaglomerular apparatus. In fact, recent work from our laboratory showed that renin cells are precursor cells that give rise to other cell types in the kidney and extrarenal organs[15]. In addition, the localization of renin cells changes markedly during mammalian development and during phylogeny[4,11,20,21]. During early development in the vertebrate kidney, renin cells are broadly distributed in the large kidney vessels and as maturation ensues the number of renin cells in the kidney vasculature diminishes as they become restricted to the "classical" juxtaglomerular localization found in the adult animal. This devel-

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opmental pattern of renin distribution has been shown in all mammals examined including humans[20]. In addition, the same developmental pattern has been observed throughout the phylogenetic scale. In primitive fish, renin or renin granules are found in the large arterial vessels, presumably to regulate blood pressure and extracellular fluid composition although other roles cannot be excluded[11]. Thus, the presence and distribution of renin in the mammalian kidney has maintained the ancestral phylogenetic pattern, which is replicated throughout kidney ontogeny. The reasons for this are unknown but it has been speculated that the early functions of the renin–angiotensin system are to control growth and morphogenetic processes locally[5,6]. As animals evolved, additional functions were added, the most recent perhaps, the classical endocrine system which in mammals is intertwined functionally with the local RAS to regulate not only blood pressure and fluid and electrolyte homeostasis but also growth processes. How the local and systemic systems are coordinated remains to be determined.

Renin Cells Are Precursor Cells

The developmental pattern of renin distribution mentioned above and additional experiments showing that embryonic granulated cells expressed renin before they expressed smooth muscle markers suggested that contrary to existent views renin cells did not derive from smooth muscle cells as previously thought, suggesting instead that renin cells may actually give rise to arteriolar smooth muscle cells of the kidney[16]. To address this issue, we generated a *knock in* mouse that expresses cre recombinase under the control of the *Ren 1d* locus and subsequently crossed this mouse with reporter mice (R26R[19], Z/EG[12]) which express either β-galactosidase or green fluorescent protein respectively after cre-mediated recombination[15]. Because this strategy allows the introduction of an inheritable, inerasable marker, all cells that expressed renin as well as their descendants will be blue (beta-galactosidase positive) or GFP positive regardless of whether renin expression has ceased, thus marking the renin lineage. We showed that in addition to JG cells, renin-expressing cells differentiate into non-renin-expressing cells such as arteriolar smooth muscle cells, mesangial cells, a subset of epithelial cells, and other extra renal cells including cells from the adrenal gland. In the kidney and in the adrenal gland these differentiated cells retain the capability to synthesize renin when additional hormone is required to reestablish homeostasis[9,15]. Thus, a population of differentiated cells derived from the renin lineage is held in reserve to respond repeatedly by de-differentiating and expressing renin in response to homeostatic threats, and re-differentiating when the crisis passes[15]. These experiments confirmed the hypothesis that the "recruitment" of renin-expressing cells is determined by the developmental history of the cells, which retain the memory to re-express the renin gene under physiological stress. Overall the studies suggested that the range of responses in an adult animal is determined and at the same time constrained by the developmental history of our cells. A model for the lineage of the renin cell is shown in Fig. 10.1.

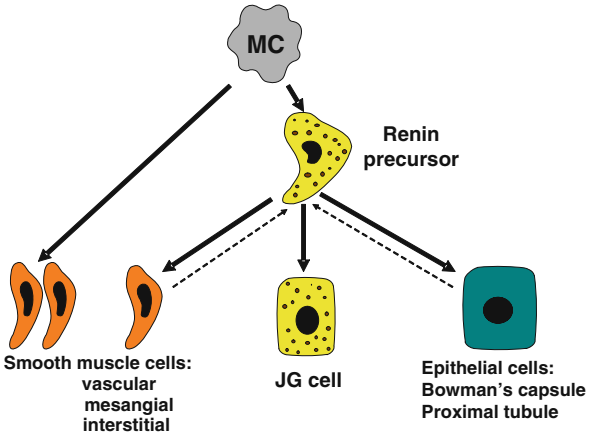

Fig. 10.1 Lineage of the renin cell. Mesenchymal cells give rise to renin precursor cells and to a population of smooth muscle cells. During ontogeny, the renin precursor cell expresses renin before it acquires any smooth muscle markers[16]. This cell in turn gives rise to the renin expressing JG cell in the adult and to a subset of vascular smooth muscle cells, and mesangial and interstitial cells. In addition, the renin precursor cells also give rise to epithelial cells of the Bowman's capsule and some proximal tubule cells. A threat to homeostasis results in an increase in renin. This increase is accomplished by re-expression of renin by these descendants of the renin precursor cell[15]. (Modified from[16])

Understanding the Molecular Events in Renin Cells Differentiation and Recruitment

Many questions regarding the molecular events regulating renin synthesis and release would be more readily addressed using a suitable in vitro cell culture model. However, maintaining renin cells in culture has been a major roadblock to the field of renin cell physiology. When freshly purified juxtaglomerular cells are placed in culture they stop synthesizing renin and differentiate into other cell types or die[7,14]. In some situations, available tumoral cells can be used successfully for certain studies[17]. However, some of those cells either express renin constitutively and therefore cannot be used to study renin cell recruitment as in the case of the As4.1 cell line[17], or they derive from a human non-kidney source such the Calu 6 cells that originated from a lung tumor[18].

To overcome these issues, we first generated a mouse in which cells from the renin lineage are marked with cyan fluorescent protein (CFP) and cells currently expressing renin mRNA are labeled with yellow fluorescent protein (YFP)[13]. Both markers accurately report the in vivo behavior expected from cells of the renin lineage: they present the appropriate distribution of renin, YFP, and CFP during development. Further, in the adult animal CFP cells respond to physiological challenges with expression of renin and YFP along the vasculature, that is, they recruit appropriately as in the wild type animal.

Having verified that those dually labeled cells were regulated as expected for renin cells we proceeded to isolate arterial smooth muscle cells and culture them to investigate the molecular events underlying their ability to switch on and off the renin gene.

It has been known that cAMP plays an important role in the regulation of renin synthesis and release[2,3,10,18]. Intracellular generation of cAMP may be a final common pathway for renin release in response to a variety of physiological demands. The cAMP stimulates renin release and also regulates renin gene expression at multiple levels to replace renin stores[1]. Therefore, we hypothesized that cAMP may be a key molecule regulating the ability of differentiated cells from the renin lineage to regain the capability to synthesize renin when the physiological conditions indicate a threat to homeostasis.

To address this question, we studied these cultured smooth muscle cells. As expected, they do not express renin in the basal state (without stimulation). However, they expressed consistently for over 30 passages, many of the markers of differentiated smooth muscle cells such as SM myosin heavy chain (SM-MHC) and alpha smooth muscle actin (α-SM actin). These cells also expressed constitutively many of the components of the RAS including angiotensinogen, angiotensin converting enzyme, and the angiotensin II type1 receptor (AT1) indicating the possibility of an intracrine RAS operating in these cells[13]. As we describe below, they were also capable of expressing renin when stimulated appropriately. Furthermore, the cells fully expressed the components of the Creb complex (Creb1, ATF, and the coactivators CBP and p300) involved in the regulation of gene transcription by the cAMP pathway. To test whether the CREB complex was active we treated the cells with either forskolin or cAMP analogues. Upon treatment there was a marked increase in renin mRNA which was accompanied by an increase in the number of cells that became YFP +[13]. Simultaneously, as the cells reverted to an endocrine phenotype, expression of smooth muscle proteins was downregulated. The effects were more pronounced with duration of the treatment. The results indicated that these cultured renal arteriolar smooth cells retained the memory to re-express the renin gene in response to cAMP. Because cAMP activates transcription via phosphorylation of CREB and the subsequent recruitment of the coactivators CBP and/or p300 which possess intrinsic histone acetyl transferase (HAT) activity, we speculated that activation of renin gene transcription was facilitated by the action of CBP and/or p300 which introduced acetylation marks in the histone tails of nucleosomes near the cAMP responsive element (CRE) located in the enhancer region in the renin promoter where Creb binds.

Experiments showed that in fact, activation of renin and YFP transcription by cAMP was accompanied by chromatin remodeling, more specifically, H4 acetylation at the cAMP responsive element (CRE) of the renin gene[13]. These experiments suggest that the ability to recruit renin cells is governed in large part by the cAMP pathway which in turn may be a common effecter for the regulation of renin cell identity and renin gene expression. Preliminary experiments in vivo suggest that to be the case. A summary of the model whereby cAMP regulates renin cell identity is shown in Fig. 10.2. In this model, intracellular cAMP production triggered by a vari-

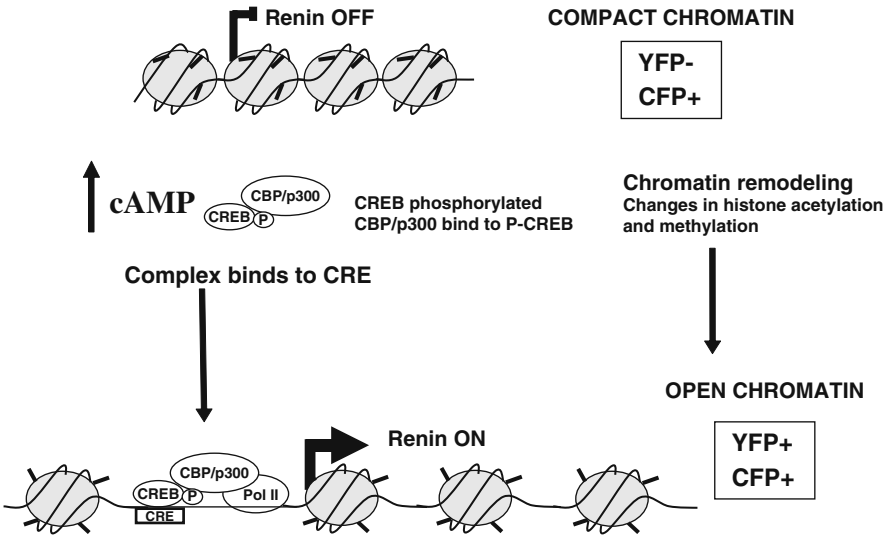

Fig. 10.2 Renin transcription is regulated by cAMP and histone acetylation. In the unstimulated state, chromatin is compact, the histones are not acetylated, and renin is not expressed. The cultured cells of the renin lineage express CFP but since they are not expressing renin, they are YFP–. Upon stimulation of the cAMP pathway, CREB is phosphorylated and the co-activators CBP and p300 associate with the phosphorylated CREB. The CREB complex binds to the CRE element in the renin promoter and associates with Pol II to initiate transcription. In addition, the HAT activity of CBP and p300 results in acetylation of histones in the region of the CRE, which results in an open chromatin configuration, and renin is transcribed. The CFP+ cells that are now making renin become YFP+

ety of stimuli results in the eventual generation of phospho-Creb and the recruitment of HATs, histone acetyl transferases (CBP/p300), which induce chromatin remodeling (acetylation of histone 4) at the CRE of the renin gene and transcription of renin. Ongoing experiments in our laboratory indicate that HATs are fundamental in this response.

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Chapter 11
Role of Renal Aminopeptidases and Angiotensin Type-2 (AT_2) Receptors in Sodium Excretion and Hypertension

Robert M. Carey, Shetal H. Padia, John J. Gildea, and Susanna R. Keller

Abstract The renin–angiotensin system (RAS) is a coordinated hormonal cascade of major critical importance to the regulation of sodium (Na^+) excretion and blood pressure. Recent studies from our laboratory have provided evidence for a critical role of angiotensin type-2 receptors (AT_2Rs) in the control of renal Na^+ excretion in response to different stimuli. In the normal rat, natriuretic responses to intrarenal angiotensin type-1 receptor (AT_1R) blockade are abolished by concurrent intrarenal AT_2R blockade. Surprisingly, angiotensin II (Ang II) is probably not the major effector of AT_2R-mediated natriuresis. We have recently demonstrated that, in the presence of systemic AT_1R blockade, intrarenal administration of the heptapeptide derivative of Ang II [des-aspartyl^1-Ang II] (Ang III) increases renal Na^+ excretion. This effect is also abolished with concurrent AT_2R inhibition. In contrast, intrarenal Ang II administration in molar equivalent and higher concentrations did not increase urinary Na^+ excretion. These observations suggest that renal AT_2Rs mediate natriuresis and that Ang III, not Ang II, is the preferred agonist. This conclusion is bolstered by our observation that intrarenal inhibition of aminopeptidase N (APN), the enzyme that metabolizes Ang III to Ang IV, induces a marked augmentation in natriuretic responses to Ang III in the AT_1R-blocked rat. In addition, natriuretic responses to intrarenal administration of dopamine (DA) D_1-like receptor (D_1R) agonist fenoldopam (FEN) are accompanied by recruitment of AT_2Rs to the apical plasma membranes of RPT cells, and intrarenal AT_2R blockade with PD abolishes FEN-induced natriuresis. Recent studies also have shown that intrarenal Ang III administration translocates AT_2Rs to RPT apical plasma membranes and induces natriuresis in normal Wistar Kyoto rats (WKY) but not in 12-week-old spontaneously hypertensive rats (SHR). These studies suggest that defects in AT_2R-mediated natriuresis and cellular trafficking may be important in the pathogenesis of hypertension.

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Introduction

The renin–angiotensin system (RAS) is a hormonal cascade of crucial importance to the regulation of blood pressure (BP). Two major receptors, AT_1 and AT_2, mediate the actions of angiotensin II (Ang II), the most important effector peptide of the RAS[1]. AT_1 receptors (AT_1Rs) are responsible for the majority of the known actions of Ang II, including vasoconstriction and antinatriuresis[1]. In contrast, much less is known about the functions of AT_2 receptors (AT_2Rs). The majority of available studies have demonstrated that AT_2R activation induces responses, which oppose those mediated by AT_1Rs. For example, in opposition to AT_1Rs, AT_2Rs dilate both small resistance arteries as well as large capacitance vessels and inhibit cellular proliferation and growth[2,3].

As demonstrated by Guyton and colleagues[4,5], renal sodium (Na^+) excretion and BP are intimately linked. In experimental animal models, the kidneys are necessary both to initiate and to sustain a hypertensive process and renal Na^+ handling appears to be preeminent[6,7]. Recent cell-specific renal cross-transplantation studies in mice have shown that the renal proximal tubule (RPT) is the major nephron site whereby Ang II-induced Na^+ reabsorption leads to sustained hypertension during Ang II infusion[8]. In both spontaneously hypertensive rats (SHR) and hypertensive humans, increased RPT Na^+ reabsorption has been demonstrated and is thought to be an early event in the pathophysiology of hypertension[9–15]. However, the mechanisms of increased RPT Na^+ reabsorption that lead to hypertension are presently unknown.

This article focuses on recent studies from our laboratory demonstrating that (1) renal AT_2Rs mediate Na^+ excretion, opposing the actions of renal AT_1Rs to enhance Na^+ reabsorption and induce volume expansion; (2) des-aspartyl[1]-angiotensin II (angiotensin III; Ang III), not Ang II, is the preferred agonist in mediating AT_2R-induced natriuresis; (3) renal dopamine D_1-like receptor (D_1R)-induced natriuresis involves activation of AT_2Rs; (4) renal aminopeptidase A (APA) and aminopeptidase N (APN), acting in concert, are likely to regulate the amount of Ang III formation that governs these responses; and (5) AT_2R-induced natriuresis is defective in hypertensive SHR. These studies shed light on the mechanisms for defective natriuresis that ultimately lead to the initiation of hypertension.

Renal AT_2R Expression

Within the adult kidney, AT_2R mRNA and protein are distributed throughout the tubular and vascular segments of renal cortex and medulla[16,17]. AT_2Rs are present in the main renal artery and renal arcuate and interlobular arteries[16,17]. In particular, AT_2Rs are highly expressed in the proximal tubule[16,18–22]. AT_2Rs are decreased in kidneys from the stroke-prone spontaneously hypertensive rat (SHR-SP) compared with the WKY control rat, and growth-factor-dependent induction of AT_2Rs occurs in cultured mesangial cells from WKY but not from SHR-SP[23].

In addition, marked overexpression of AT_2Rs is observed in rats with renal failure[24].

Past Studies on the Role of AT_2Rs in Renal Na^+ Excretion

Regarding a potential role of AT_2Rs in the control of renal Na^+ excretion, very little information has been available. The only published in vitro study showed that bicarbonate reabsorption in RPT cells is opposed by AT_2Rs[25]. Most of the past knowledge derives from AT_2R-null mice, which have exaggerated antinatriuretic responses to Ang II infusion and a shift to the right (less sensitive) in their pressure–natriuresis relationships[26,27]. However, difficulty exists in interpreting studies in AT_2R-null mice because AT_1Rs are chronically upregulated in this model and it is uncertain whether increased AT_1R expression accounts for some or all of the antinatriuretic hypersensitivity in this model[28].

AT_2Rs have been implicated in the natriuresis of both obese Zucker rats and streptozotocin-induced type-1 diabetic rats[29–31]. In obese Zucker rats, AT_1R blockade induces natriuresis to a greater degree than in lean rats and the natriuresis is abolished with AT_2R blocker PD. In streptozotocin-induced diabetes mellitus, the rise in fractional Na^+ excretion is inhibited by administration of PD in the absence of exogenous hormone, suggesting a tubular action of AT_2R blockade[30]. In both models, AT_2Rs are markedly upregulated in RPT cell basolateral and brush border membranes compared to lean or non-diabetic controls. In normal rat RPTs, the AT_2R selective peptide agonist CGP-42112 decreased Na^+/K^+ATPase activity in a NO and cGMP-dependent manner[31].

AT_2Rs Mediate Natriuresis in Response to Intrarenal AT_1R Blockade

We investigated the effects of specific intrarenal AT_1R blockade with insurmountable AT_1R antagonist candesartan (CAND) with and without specific AT_2R blockade with PD-123319 (PD) on urinary Na^+ excretion ($U_{Na}V$) in Sprague-Dawley rats[32]. Figure 11.1 demonstrates the increase in $U_{Na}V$ of uninephrectomized anesthetized rats ($N = 8$ per group) during normal Na^+ intake in response to direct RI infusion of CAND (black bars). As shown by the white bars, PD abolished the natriuretic response to CAND. Time control data for vehicle infusion (cross-hatched bars) were unchanged throughout the experiment. RI CAND infusion reduced mean arterial pressure (MAP) during Periods 1 and 2, indicating some escape of CAND from the kidney during RI infusion. In Periods 3 and 4, MAP returned to control levels. Addition of PD to the RI infusion of CAND reversed the reduction of MAP during Periods 1 and 2. These results indicate that the natriuresis engendered by intrarenal AT_1R blockade is mediated by AT_2R activation and that CAND, because of its long half-life, spilled over into the systemic circulation to reduce BP in this experimental model.

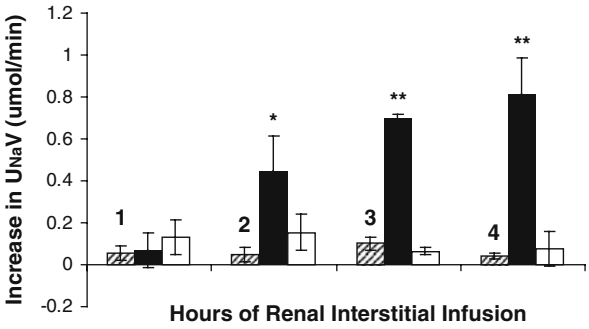

Fig. 11.1 Increase in $U_{Na}V$ of uninephrectomized anesthetized rats (N = 8 per group) in response to RI AT_1R and AT_1R + AT_2R blockade during normal Na^+ intake. Black bars, $U_{Na}V$ responses to RI CAND (0.01 mg/kg/min); *white bars*, $U_{Na}V$ responses to RI CAND + PD (10 μg/kg/min) combined infusion; cross-hatched bars, $U_{Na}V$ responses to RI vehicle infusion. Data represent mean ± SE; * $P < 0.05$, ** $P < 0.01$ from vehicle time control. The natriuresis induced by intrarenal AT_1R blockade with CAND was abolished by AT_2R blockade with PD. From Padia et al.[32] with permission

Intrarenal Ang III, but Not Ang II, Activates Renal AT_2Rs and Induces Natriuresis

Next, we explored whether the Ang II heptapeptide derivative, Ang III, is an AT_2R agonist in a normal two-kidney rat model[32]. As shown in Fig. 11.2, Panel A, in the absence of systemic AT_1R blockade unilateral RI infusion of Ang III (3.5–14 nmol/kg/min; black bars) did not significantly affect $U_{Na}V$ from the infused (black bars) or contralateral control (white bars) kidney. In marked contrast (Panel B), in the presence of systemic AT_1R blockade with CAND, renal administration of Ang III into one kidney resulted in a significant natriuresis that was abolished by co-infusion of AT_2R blocker PD (Fig. 11.2, Panel C), indicating that Ang III induces natriuresis via AT_2Rs in the AT_1R-blocked rat. Systemic CAND infusion decreased MAP from 112 ± 4 to 89 ± 6 mm Hg. Unilateral Ang III infusion did not alter MAP at any infusion rate either in the absence or presence of systemic CAND infusion. Similarly, co-infusion of Ang III + PD did not alter MAP. Thus, systemic CAND alone reduced MAP ($P < 0.01$). Therefore, infused Ang III in all likelihood remained within the kidney during the experimental period.

APN Inhibition Augments the Natriuretic Response to Intrarenal Ang III

We were surprised to find that, unlike natriuretic responses to intrarenal Ang III, RI infusion of Ang II, at equimolar (or higher) rates as Ang III, did not induce natriuresis either in the presence or absence of systemic AT_1R blockade[32]. The natriuretic

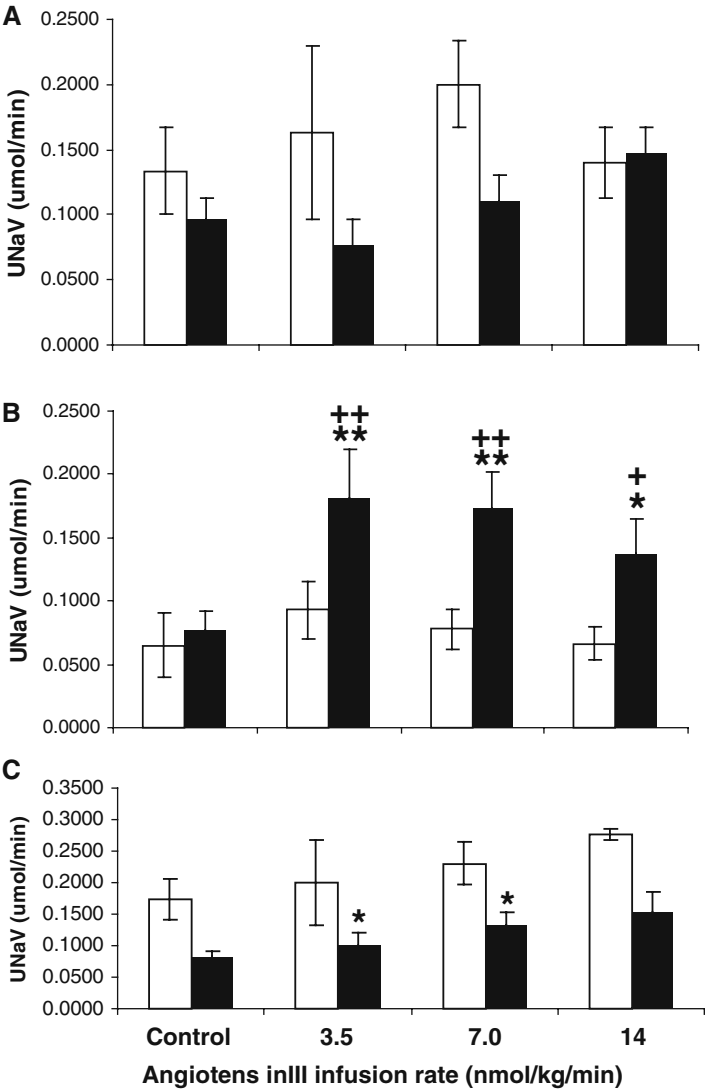

Fig. 11.2 $U_{Na}V$ of anesthetized rats (N = 5 per group) with both kidneys intact in response to unilateral RI Ang III infusion (3.5, 7.0, and 14 nmol/kg/min; *black bars*) after a 1 h pre-control period in which vehicle only was infused (**a**) in the absence of systemic CAND, (**b**) in the presence of systemic CAND (0.01 mg/kg/min) for 24 h, and (**c**) in the presence of systemic CAND for 24 h and RI co-infusion of PD. White bars denote data collected from the contralateral control kidney which received a vehicle infusion. * $P < 0.05$, ** $P < 0.01$, $P < 0.001$ from CAND alone; + $P < 0.05$, ++ $P < 0.01$ from Ang III infused kidney. In the presence of systemic AT_1R blockade, the natriuresis induced with intrarenal infusion of Ang III was abolished by AT_2R blockade with PD. From Padia et al.[32] with permission

responses to Ang III but not to Ang II suggested that Ang III might be a preferred AT_2R agonist to increase renal Na^+ excretion.

APN is the major enzyme metabolizing Ang III to Ang IV. We investigated the role of Ang III as a preferred renal AT_2R agonist in inducing natriuresis by infusing either Ang III alone or Ang III + APN inhibitor PC-18 directly into the RI compartment of AT_1R-blocked Sprague-Dawley rats with normal Na^+ intake[33]. Our hypothesis was that intrarenal APN inhibition would increase the natriuretic response to Ang III. As shown in Fig. 11.3, combined unilateral RI Ang III and PC-18 infusion (black bars) markedly increased $U_{Na}V$ over the dose range of 3.5–28 nmol/kg/min of Ang III. In contrast to Ang III infusion alone (Fig. 11.2, Panel B), the dose–response relationship of Ang III + PC-18 was progressive at all doses of Ang III administered. Interestingly, $U_{Na}V$ responses from the control contralateral kidney (Fig. 11.3; white bars) decreased from pre-control values ($P < 0.01$). In addition, PD abolished the natriuretic response to Ang III in the presence of intrarenal APN inhibion. These observations demonstrate that the natriuretic response to Ang III is enhanced when Ang III metabolism is inhibited and that Ang III-induced natriuresis is due to AT_2R activation. These data substantiated our hypothesis that Ang III is the preferred AT_2R agonist. The data further suggested that renal APN may be an important regulator of renal Na^+ excretion and that distal Ang metabolites such as Ang IV (Ang 3–8) do not account for the natriuretic action of Ang III.

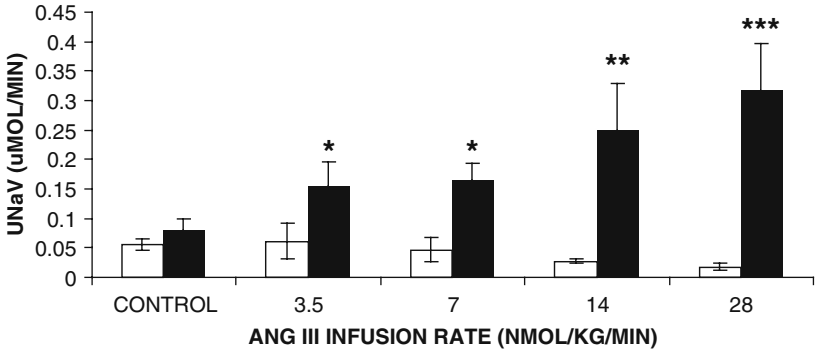

Fig. 11.3 $U_{Na}V$ responses to cumulative RI infusion of Ang III (3.5, 7, 14, and 28 nmol/kg/min, each dose for 30 min) + APN inhibitor PC-18 (25 μg/kg/min) (*black bars*) after a 1 h pre-control period during which vehicle was infused. *White bars*, $U_{Na}V$ when only vehicle was infused into the RI compartment of the control kidney. Data represent mean ± SE. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$ from control kidney (*white bars*). Natriuretic responses to intrarenal Ang III were augmented by aminopeptidase-N (APN) blocker PC-18. From Padia et al.[33] with permission

Conversion of Ang II to Ang III is Critical for AT_2R-Mediated Natriuresis

Because Ang III is a direct metabolite of Ang II via aminopeptidase A (APA), we hypothesized that APN inhibition would also enable intrarenal Ang II administration

to induce natriuresis through metabolism to Ang III[34]. Figure 11.4 demonstrates that Ang II can induce natriuresis, but only when APN is blocked. Combined unilateral RI Ang II and PC-18 infusion in AT_1R blocked rats (N = 8) increased $U_{Na}V$ over the Ang II dose range of 3.5–28 nmol/kg/min. In contrast, RI Ang II was unable to induce natriuresis in the absence of APN inhibition. RI Ang II administration did not change systemic BP (P = NS) at any infusion rate in the presence of PC-18.

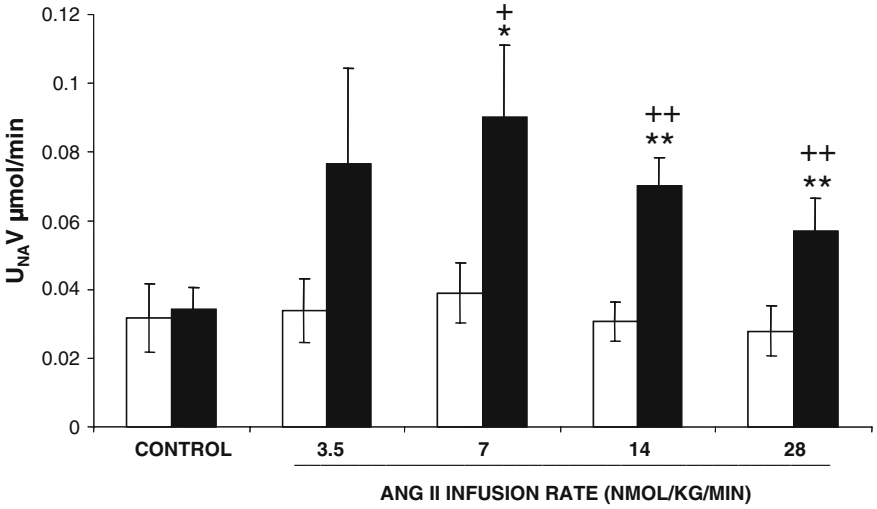

Fig. 11.4 $U_{Na}V$ responses of Sprague-Dawley rats (N = 8) to four cumulative RI infusion rates of Ang II (3.5, 7, 14, and 28 nmol/kg/min) plus PC-18 (25 µg/min; *black bars*) compared with responses from vehicle infused kidneys (*white bars*) in the presence of systemic AT_1R blockade (N = 8). * P < 0.05, ** P < 0.005 from pre-control. + P < 0.05, ++ P < 0.001 from vehicle-infused kidney at the same Ang II infusion rate. Intrarenal aminopeptidase N (APN) inhibition with PC-18 unmasked a natriuretic response to intrarenal Ang II. From Padia et al.[33] with permission

Ang II-induced natriuresis in the presence of PC-18 was abolished by RI co-infusion with PD, demonstrating that this effect is AT_2R-dependent. We further investigated whether Ang II conversion to Ang III is required for AT_2R-mediated natriuresis. Figure 11.5 demonstrates that Ang II-induced natriuresis in the presence of APN inhibitor PC-18 was abolished when the APA inhibitor EC-33 was co-infused. These results strongly suggest that Ang III is the preferred agonist for AT_2R-induced natriuresis and that the regulation of APA and APN activity is probably critical for the natriuretic response.

Renal Dopamine D_1R-Induced Natriuresis is Dependent on AT_2R Activation

Approximately 50% of basal Na^+ excretion is mediated by the paracrine action of DA on RPT cell D_1-like receptors (D_1 and D_5)[35]. DA downregulates AT_1Rs in the RPT, but its effect on AT_2Rs has not been recognized. We explored the effects

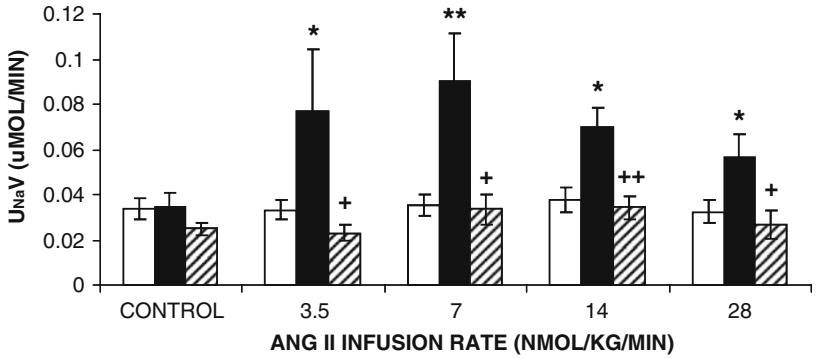

Fig. 11.5 $U_{Na}V$ responses of Sprague-Dawley rats (N = 7) to four cumulative RI infusion rates of Ang II + PC-18 (*black bars*) or Ang II + PC-18 + EC-33 (*cross-hatched bars*), each in the presence of systemic AT_1R blockade with CAND. Time control (*white bars*) denotes RI vehicle infusion. * $P < 0.05$; ** $P < 0.01$ from time control. Conversion of Ang II to Ang III is critical for AT_2R-induced natriuresis. From Padia et al.[34] with permission

of direct RI stimulation of D_1-like receptors with fenoldopam (FEN), a selective D_1-like receptor agonist, on $U_{Na}V$ and AT_2R expression and cellular distribution in uninephrectomized, anesthetized Sprague-Dawley rats (N = 10/group) on 4% NaCl diet for 1 week[36]. Figure 11.6, Panel A, shows a highly significant natriuretic response to intrarenal FEN infusion (1 μg/kg/min for 3 one-hour collection periods; black bars) compared to time-control values (white bars), and Panel B shows a FEN-induced diuresis. Panel C demonstrates that systemic MAP was not influenced by any of the intrarenally infused agents, indicating their likely confinement to the kidney during the infusion period.

To further elucidate the role of the AT_2R in D_1-like receptor-induced natriuresis, we repeated the above-mentioned RI FEN infusion studies (N = 10/group) in the presence of AT_2R blocker PD. As shown in Fig. 11.6, Panels A and B, FEN-induced natriuresis and diuresis were abolished with RI co-infusion of AT_2R blocker PD (2 μg/kg/min; cross-hatched bars) or D_1-like receptor antagonist SCH-23390 (10 μg/kg/min; checkered bars). The results demonstrated that D_1-like receptor-stimulated natriuresis requires the activation of both renal D_1-like receptors and AT_2Rs.

Because D_1-like receptor-induced natriuresis was abolished by AT_2R blockade, we investigated whether RPT AT_2R expression and intracellular distribution were affected by FEN[36]. To demonstrate the effect of FEN on AT_2R trafficking in the RPT cell, we developed a novel streptavidin–biotin lectin pull-down technique for isolating RPT apical membranes using biotinylated *Lotus tetragonolobus* agglutinin (LTA) lectin. As shown in Fig. 11.7, Panel A, there was a significant enrichment of the RPT apical membrane-specific marker villin in apical membranes isolated with the lectin pull-down method. As shown in Fig. 11.7, Panel B, FEN did not significantly increase total renal cortex AT_2R expression but increased RPT apical plasma membrane AT_2R expression by 59% ($P < 0.01$). These results strongly suggested

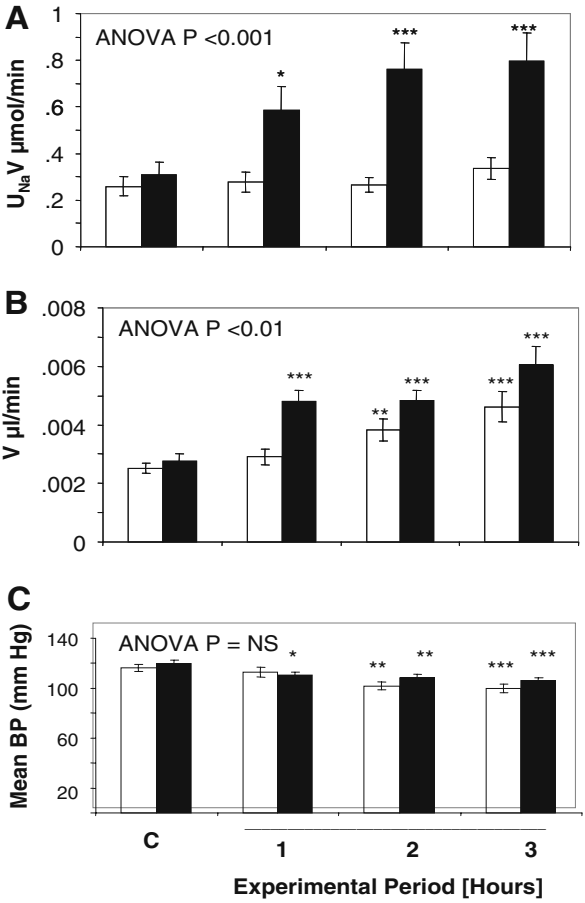

Fig. 11.6 Direct RI D_1-like receptor activation with fenoldopam (1 µg/kg/min) during 3 one-h collection periods induced a sustained natriuresis (**a**) and diuresis (**b**) without change in MAP (**C**) in Na^+-loaded anesthetized, uninephrectomized Sprague-Dawley rats. *Black bars*, fenoldopam-infused rats. *White bars*, vehicle-infused rats. Data represent mean ± SE. * $P < 0.01$, ** $P < 0.001$, *** $P < 0.0001$ from pre-control. From Salomone et al.[36] with permission

that D_1-like receptor activation with FEN results in recruitment of AT_2Rs to the apical plasma membrane of RPT cells. AT_2R recruitment to the plasma membrane thus accompanies D_1-like receptor stimulated natriuresis.

Hypertensive SHR Have Defective AT_2R-Induced Natriuresis

The above results led us to hypothesize that SHR have defective AT_2R-mediated natriuretic responses, possibly leading to hypertension. To explore this possibility, we compared natriuretic responses to intrarenal Ang III infusion in 12-week-old SHR and their genetic Wistar Kyoto (WKY) controls[37]. Figure 11.8 demonstrates a highly significant Ang III-induced natriuresis that was abolished by co-infusion with PD in WKY, but absent natriuretic responses to Ang III in SHR. As shown in the confocal micrographs in Fig. 11.9, in WKY Ang III activation of renal AT_2Rs

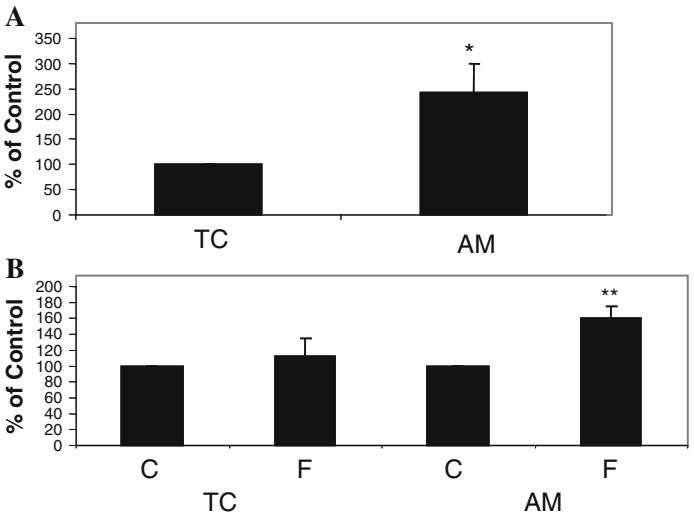

Fig. 11.7 (**a**) Western blot analysis of renal proximal tubule total cell (TC) and outer apical membrane (AM) expression of villin, a specific apical plasma membrane marker, from Na^+-loaded Sprague-Dawley rat kidneys demonstrated a significant enrichment of villin in AM compared with TC; * $P < 0.05$. (**b**) Direct RI D_1R activation with fenoldopam (F) increased AM expression of AT_2R by 59% but did not increase TC expression; ** $P < 0.01$. From Salomone et al.[36] with permission

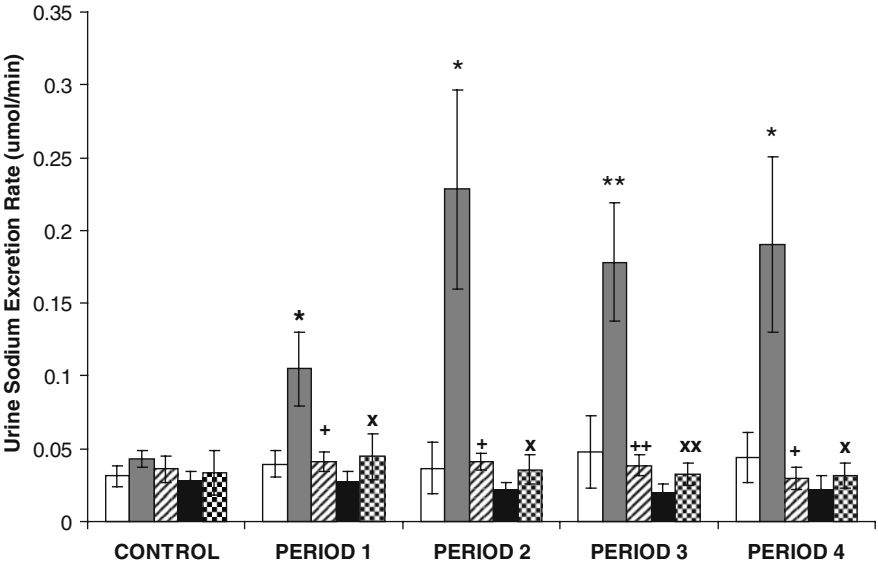

Fig. 11.8 $U_{Na}V$ in the presence of systemic CAND in WKY after RI infusion of D_5W (*white bars*), Ang III (*solid gray bars*), Ang III + PD (*cross-hatched bars*), and in SHR in response to D_5W (*solid black bars*) or Ang III (*checkered bars*), N = 6 per group. During the control period, only D_5W was infused in all groups. Data represent mean ±1SE; *$P < 0.05$, **$P < 0.01$ from respective control period, + $P < 0.05$, ++ $P < 0.01$, X $P < 0.05$, and XX $P < 0.01$ from Ang III-infused kidney in WKY. From Padia et al.[37] with permission

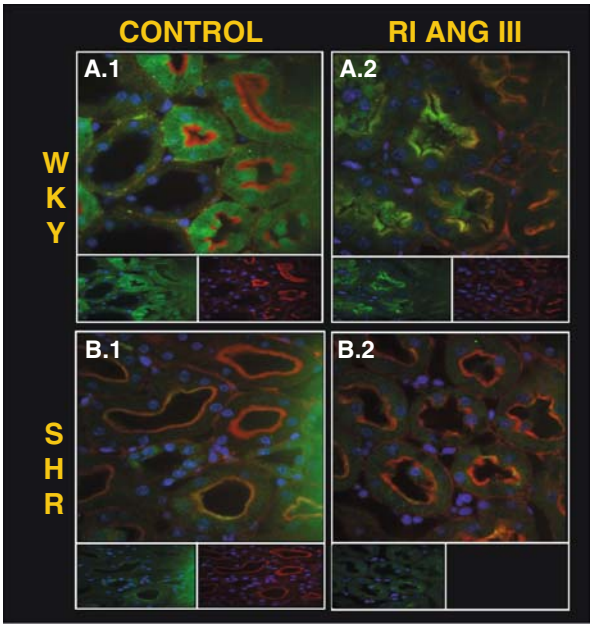

Fig. 11.9 Confocal micrographs (600x) of renal cortical sections (8 μm) obtained from control (D_5W-) and Ang III-infused WKY (**a**) and SHR (**b**) kidneys, in the presence of CAND. In vivo, Ang III induced natriuresis in WKY, but not in SHR. On confocal microscopy of the same Ang III-infused kidneys, RPTC AT_2R (*green*), co-localized with phalloidin (*red*), a marker of proximal tubule brush border, to produce a gold color in WKY (A.2). In SHR (**b**), the RPTC AT_2R (*green*) failed to localize in the brush border (*red*) in response to renal Ang III infusion. *Insets* show individual AT_2R (*left*) and phalloidin (*right*) staining for each condition. From Padia et al.[37] with permission

translocated these receptors to the apical plasma membranes of RPT cells. However, in SHR AT_2Rs remained in the intracellular compartment and were not translocated to apical plasma membranes in response to Ang III. These results were corroborated by Western blot analysis of whole RPT cells and their apical plasma membranes, which demonstrated translocation in WKY but not in SHR. The results suggest that defects in AT_2R-mediated natriuresis and intracellular trafficking may be important in the development of hypertension in SHR.

Summary and Conclusions

We have shown that (1) renal AT_2Rs are natriuretic in normal Sprague-Dawley and WKY rats, and AT_2R-mediated natriuresis is defective in hypertensive SHR; (2) AT_2R-induced natriuretic responses are accompanied by translocation AT_2Rs to the apical plasma membranes of RPT cells in normal rats, but does not occur in SHR; (3) Ang III, not Ang II, is the preferred agonist for AT_2R-mediated natriuresis; (4) natriuresis due to renal D_1R stimulation is dependent upon activation of renal

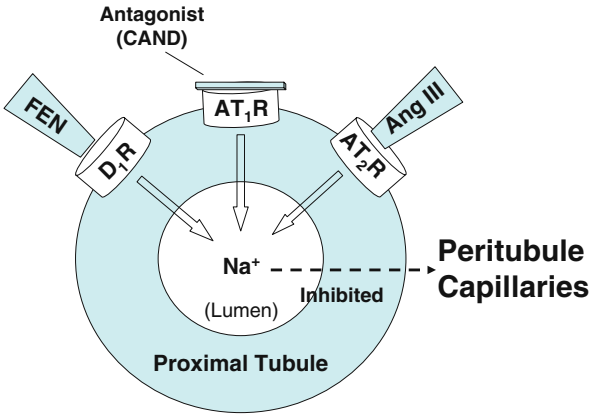

Fig. 11.10 Schematic representation of ligand–receptor interactions mediating natriuresis in the RPT of normal rodents. FEN: fenoldopam, a D_1R agonist; CAND: candesartan, an AT_1R antagonist; Ang III: angiotensin III, an AT_1R and AT_2R agonist; Na^+: sodium

AT_2Rs; and (5) natriuresis due to renal AT_1R blockade is related to AT_2R activation. These results suggest that AT_2Rs, recruited to the apical plasma membranes of RPT cells in response to intrarenal Ang III and D_1R activation or AT_1R blockade (Fig. 11.10), play a major critical role in promoting natriuresis in normal animals and that these mechanisms are defective in SHR. Further studies in young, prehypertensive SHR, which exhibit increased RPT Na^+ reabsorption, will be required to determine whether this mechanism is important in the pathophysiology of hypertension. The results suggest the possibility that the quantity of intrarenal Ang III regulated by the respective activities of APA and APN may be crucial to renal Na^+ handling and hypertension.

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Chapter 12
Intrarenal Angiotensin II Augmentation in Hypertension

Romer A. Gonzalez-Villalobos and L.G. Navar

Abstract Angiotensin (Ang) II-dependent hypertension is characterized by an augmentation in intrarenal Ang II content beyond circulating levels that is associated with functional and morphological derangements in the kidney. This augmentation is due to Ang II sequestration from the general circulation by the Ang II type 1 receptor (AT1R) and to intrarenal Ang II formation by a local renin–angiotensin system (RAS). This review summarizes the evidence in favor of the contribution of these two processes to the observed augmentation of intrarenal Angiotensin II as well as their impact on the regulation of kidney function and blood pressure regulation.

Introduction: Intrarenal Levels of Ang II

Studies in different experimental models have demonstrated that intrarenal Angiotensin (Ang) II content is increased in every form of Ang II-dependent hypertension[9,10,20,37,40]. These high intrarenal Ang II levels are greater than can be explained by equilibration with circulating Ang II concentrations and seem to be regulated by local mechanisms. In addition there is substantial compartmentalization of Ang II within the kidney (Fig. 12.1), with higher Ang II concentrations in the medulla than in the cortex under normal conditions, and they both increase in response to chronic Ang II infusions[22]. Within the cortex, Ang II content in the renal interstitial fluid is much greater than circulating levels[24]. The concentrations of Ang II in the proximal tubule fluid are also several fold higher than circulating Ang II[2] and they are sustained during Ang II-dependent hypertension[4,20]. Micropuncture studies of in vivo microperfused proximal tubule segments demonstrated that Ang II concentrations are also several fold higher in the perfused segments where the contribution from filtered Ang II is prevented indicating that the tubular fluid Ang II concentrations are not derived from the general circulation and that Ang II is either secreted or produced locally[2]. These findings are depicted in Fig. 12.1 and illustrate

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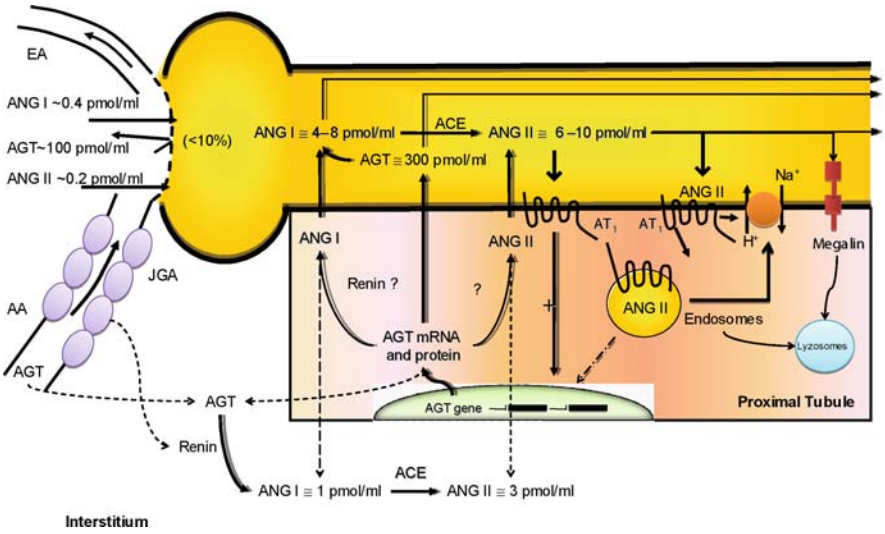

Fig. 12.1 Ang II compartmentalization in the kidney and pathways of formation

the potential role of the intrarenal renin–angiotensin system (RAS) in the regulation of intrarenal Ang II via mechanisms independent from the systemic circulation.

Mechanisms Responsible for the Control of the Intrarenal Ang II Content

Several possible mechanisms have emerged as being responsible for the enhancement of the intrarenal Ang II content in hypertension and can be classified into two groups (Fig. 12.2): AT1 receptor-mediated uptake of Ang II into intracellular compartments and AT1 receptor-mediated stimulation of local Ang II formation. In addition, Ang II degradation via angiotensinases or via ACE2 and other alternative pathways also contribute to the local regulation of Ang II concentrations but their quantitative roles remain unclear. This review focuses on the evidence regarding the mechanisms that increase intrarenal Ang II content during Ang II-dependent hypertension.

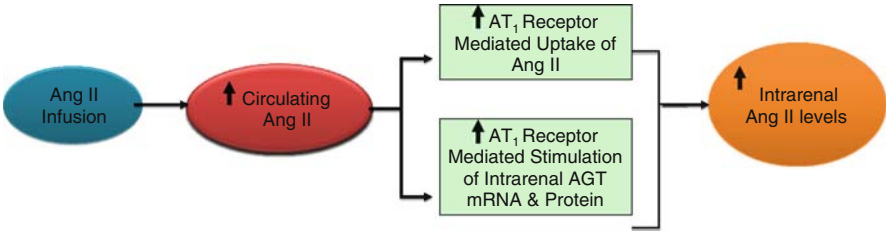

Fig. 12.2 Contribution of Ang II internalization and local Ang II synthesis to the intrarenal Ang II content

Ang II Internalization by the AT1R, Functional Role of Ang II Internalization in the Proximal Tubule and Alternative Uptake Receptors

The demonstration that AT1 receptor blockade prevents the augmentation of intrarenal Ang II that occurs during chronic infusions of Ang II in rats suggested a progressive AT1 receptor-mediated intracellular accumulation of Ang II[40]. These results were confirmed in other models of Ang II-dependent hypertension like 2-Kidney 1-clip (2K1C) hypertension[10], Ren2 transgenetic rats[20], and Ang II-infused mice[9]. These observations were further extended by studies demonstrating that Ang II is accumulated in endosomes in Ang II-infused rats and that this process is prevented by treatment with AT1R blockers[37]. The initial paradigm for Ang II internalization was that this process served to target the peptide to degradation and to downregulate receptor function by decreasing its number at the cell surface. Thus, internalization was part of a protective mechanism to guard the cells against prolonged Ang II stimulation. However, as mentioned, there is substantial intracellular accumulation of Ang II via the AT1R into endosomes that protects it from degradation, presumably extending its half-life (Fig. 12.3). Once in the endosomes, Ang II can be shuttled to the lyzosomes or, at least in theory, be transferred back to the general circulation via transcytosis. In the proximal tubule, Ang II internalization also regulates cell functions. Schelling et al.[32] showed in cell culture studies that apical Ang II-dependent sodium influx is dependent on the internalization process. Becker et al.[1] demonstrated that apical Ang II internalization is associated with phospholipase A activation. Using internalization-deficient mutant forms of the AT1R, Thekkumkara et al.[34] demonstrated that AT1R internalization is required for interaction with G proteins and cAMP signaling. Furthermore, Ang II internalization in rabbit proximal tubule cells induces in vitro transcription of TGF-β1, MCP-1, and NH3[18] and is required for NF-κβ activation[36]. It has also been shown that intracellular microinjections of Ang II induce increases of calcium concentration mediated by the AT1R[38]. In support of these findings, we observed that rat cultured proximal tubule cells exposed to fluorescent Ang II display fluorescence accumulation in perinuclear compartments (Fig. 12.4) via the AT1R[7]. The latter observation suggests that some of the internalized Ang II can be shuttled to the nucleus or the nuclear membrane to exert intracellular effects. In fact, studies in animal preparations have demonstrated the presence of Ang II receptors in nuclei extracted from kidney cells[19,25]. Ang II is now recognized as an intracrine molecule as it is a peptide that acts not only as an extracellular signaling molecule[28], but also acts from intracellular sites of either target cells or the cells in which it was synthesized[28]. In many cases, an intracrine factor can trigger an amplification cascade of its own synthesis. Thus, intracellular Ang II stimulates a variety of systems and this might include the activation of its own precursor angiotensinogen, which in turn could trigger an amplification cascade with synthesis of more Ang II (Fig. 12.3).

There are also other pathways for Ang II internalization. Megalin is an abundant membrane protein expressed in microvilli of proximal tubule cells that is heavily

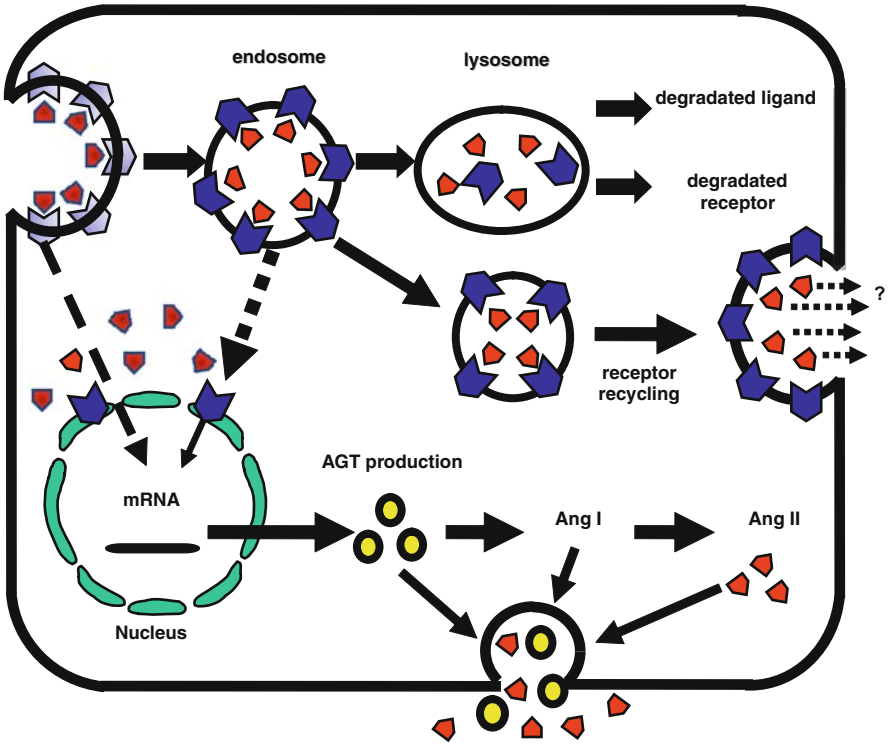

Fig. 12.3 AT1Receptor-mediated Angiotensin II internalization and trafficking

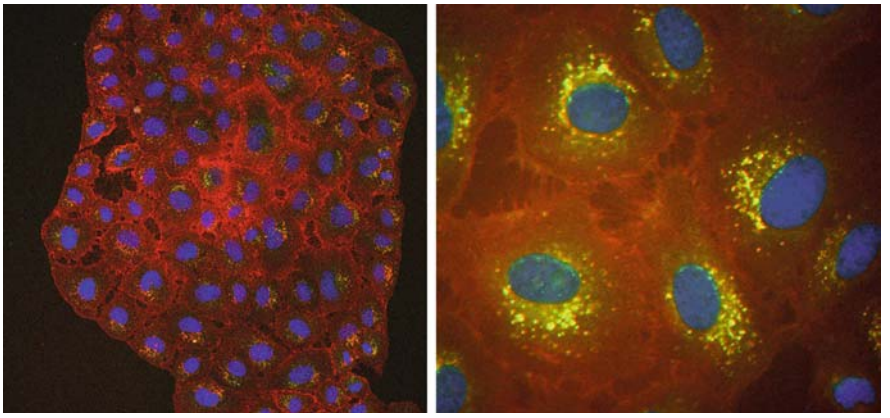

Fig. 12.4 Ang II internalization in proximal tubule cells. Shown in the figures are positive controls (cells treated only with fluorescent Ang II, GREEN). The nuclei were stained with DAPI (BLUE) and the cell membranes with wheat germ agglutinin (RED)

involved in receptor-mediated endocytosis of proteins escaping the glomerular filtration barrier. Using a combination of in vitro and fresh animal preparations, we demonstrated that megalin can bind Ang II and also Ang 1-7[5 – 7]. Because the main function of megalin is to target proteins to the lysosomal pathway for degradation, it has been suggested that megalin can act as a scavenger receptor for Ang peptides (Fig. 12.1). Thus, some of the Ang II that is internalized into proximal tubule cells is via megalin, but it is not clear if this pathway mediates further signaling within the cells.

Endogenous Synthesis of Intrarenal Ang II: Functional Evidence of Its Role During Ang II-Dependent Hypertension

The kidneys express all components of the RAS and can therefore generate angiotensin peptides from locally formed angiotensinogen in proximal tubule cells[11,12,17,21]. Increased Ang II exerts an amplification effect via activation of the AT1R on the expression of its own precursor angiotensinogen mRNA and protein in the kidneys in Ang II-infused rats and mice[8,9,13,14,23]. Proximal tubule angiotensinogen can be secreted into the lumen and, in rats undergoing chronic Ang II infusions, this process translates into an elevation of angiotensinogen excretion in the urine[15]. This stimulatory effect on angiotensinogen has been reported in vitro[12], and it has also been shown that inflammatory cytokines, like interleukin 6, can synergize with Ang II to augment angiotensinogen expression[31].

The presence of substrate in the luminal fluid coupled with the persistent or even augmented expression of renin[26,27] and angiotensin-converting enzyme[11,35] along the nephron during high Ang II-states have served as the basis for the hypothesis that during Ang II-dependent hypertension there is sustained Ang II production that contributes to the increase in intrarenal Ang II, and in turn, the development of hypertension and renal damage. Because internalization of circulating Ang II and stimulation of angiotensinogen expression require activation of the AT1R (Fig. 12.2), the contribution of either factor has been difficult to delineate. Several approaches have been utilized to dissect out the contributions of Ang II internalization from the general circulation versus endogenously and locally produced Ang II.

Double Transgenic Mice Expressing Human Angiotensinogen in the Proximal Tubule and Systemic Renin

Kobori et al.[16] used double-transgenic mice expressing human angiotensinogen in the proximal tubule and human systemic renin to determine if locally produced Ang II from human angiotensinogen could lead to activation of mouse (endogenous) angiotensinogen. Because renin is highly species specific, human renin only cleaves human angiotensinogen. In their model, human angiotensinogen was targeted to the proximal tubule and attached to an androgen-specific promoter. Accordingly,

human angiotensinogen expression increases in the proximal tubule as the male mice mature. This coupled with the presence of systemic renin generated enough Ang I and Ang II that, in turn, augmented mouse angiotensinogen expression and also resulted in intrarenal augmentation of Ang II and the development of high blood pressure levels. This process was restricted to the kidneys and independent of the systemic RAS because Ang II did not increase in the general circulation. Thus, it was demonstrated that the activation of the intrarenal RAS may cause local increments of Ang II in turn are responsible for the development of hypertension. Sachetelli et al.[29] also observed increases in blood pressure and renal damage in mice overexpressing human angiotensinogen and human renin targeted to the proximal tubule of the mouse kidney. In both reports the observed changes were prevented by treatment with RAS blockers (ACE inhibitors and/or AT1R blockers).

Systemic Val^5-Angiotensin II Infusions

Val^5-Ang II is obtained by substituting Valine for Isoleucine in position 5 in the Ang II molecule. The result is a peptide with identical affinity for the AT1R and biological activity as the endogenous Ang II (Ile^5-Ang II). The Val substitution makes possible the separation of both peptides by high performance liquid chromatography (HLPC); both fractions can be collected and analyzed by radioimmunoanalysis. Zou et al.[39] subjected uninephrectomized rats to Val^5-Ang II to demonstrate Val^5-Ang II also induces increases in intrarenal Ang II. Based on HLPC separation, intrarenal Ang II augmentation involved substantial Val^5-Ang II accumulation in the kidneys supporting the role of AT1R-mediated uptake from circulating Ang II. In more recent studies, Shao et al.[33] infused Sprague-Dawley rats with Val^5-Ang II and demonstrated that plasma and kidney endogenous Ang II (Ile^5-Ang II) concentrations were greater in Val^5-Ang II-infused rats that in non-infused control rats. Thus, exogenous Ang II (Val^5-Ang II) induced endogenous Ang II (Ile^5-Ang II) synthesis, which was partially responsible for the increase in total intrarenal and plasma Ang II.

Chronic Ang II Infusions in Mice and Augmentation of Intrarenal Angiotensinogen

Mice represent an attractive model to study the intrarenal mechanisms participating in the regulation of local Ang II due to the availability of knockout and transgenic strains. However, Ang II-induced hypertension in mice has not been explored as comprehensibly as in rats. In recent studies mice have been subjected to chronic Ang II infusions in an effort to characterize the responses of the mouse intrarenal RAS to chronic Ang II infusions. Two different doses of Ang II were evaluated and it was observed that in mice[9], as in rats[13–15], chronic Ang II infusions cause increases in blood pressure and intrarenal Ang II content that are mediated by

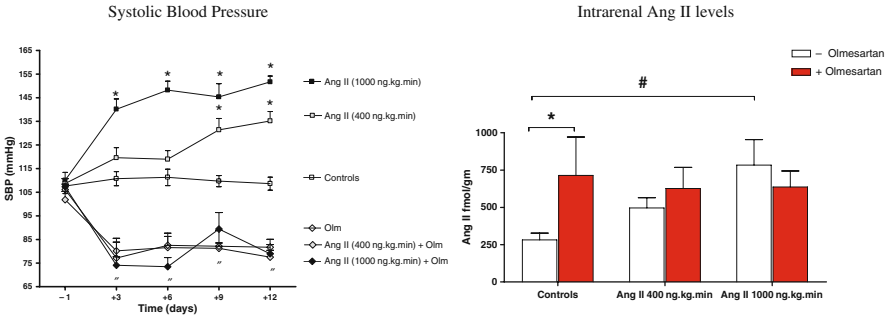

Fig. 12.5 Changes in systolic blood pressure and intrarenal Ang II in Ang II-infused mice[9].Blood pressure and Ang II concentrations determined by tail-cuff method and radioimmunoanalysis, respectively. Ang II = Angiotensin II, Olm = Olmesartan (3 mg/kg/d). ∗ and # = $p < 0.05$

AT1R activation (Fig. 12.5). Importantly, while these changes were concomitant with systemic renin suppression as expected, there was persistence of intrarenal renin activity in mice treated with both doses (Fig. 12.6). Moreover, the low dose of infusion, which caused a slowly progressive increase in blood pressure similar to that observed in rats, induced augmentation of intrarenal angiotensinogen mRNA and protein expression (Fig. 12.6). Interestingly, the high dose failed to

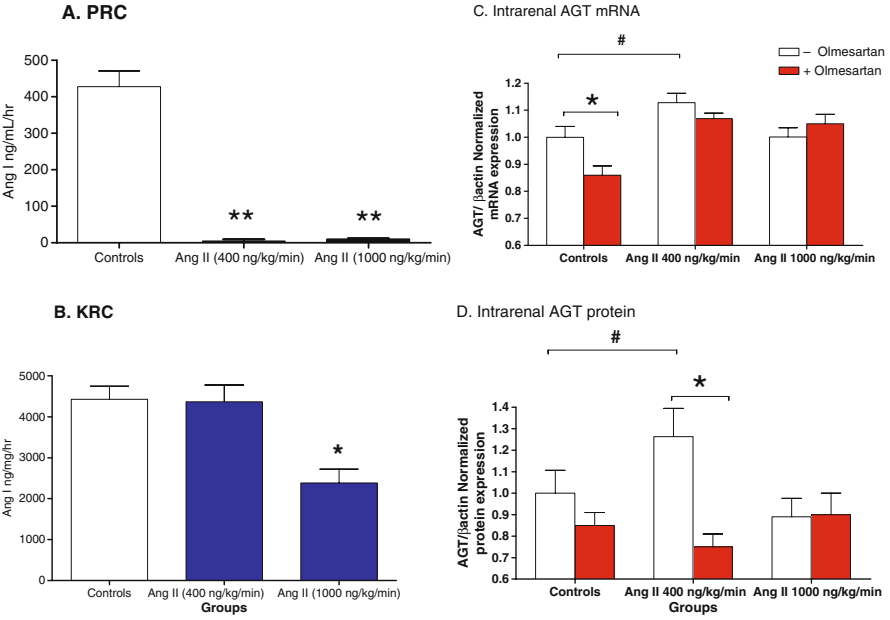

Fig. 12.6 Changes in plasma renin concentration (PRC), kidney renin content (KRC), and intrarenal Angiotensinogen (AGT) mRNA and protein in Ang II-infused mice[9]. PRC and KRC as determined by radioimmunoanalysis. AGT mRNA and protein expression as determined by qRT-PCR and Western blotting, respectively. ∗ and # = $p < 0.05$

significantly stimulate angiotensinogen expression. As a whole these observations indicate that when Ang II induces a slowly progressive increase in blood pressure, there is a sustained activity of the intrarenal RAS characterized by angiotensinogen upregulation and persistent renin activity, indicating that the local generation of Ang peptides plays a significant role in this model. It was also observed that these changes are mediated by Ang II activation of the AT1R as olmesartan treatment completely blocked these responses. The failure of the high dose of Ang II to stimulate angiotensinogen expression indicates the self-limiting nature of the amplification effect that Ang II exerts on intrarenal angiotensinogen and also demonstrates that the increase in angiotensinogen mRNA and protein is not simply the consequence of the elevated arterial pressure.

Role of ACE-Derived Ang II During Ang II-Induced Hypertension in Mice

To address the issue of the importance of ACE-derived endogenous Ang II generation during Ang II-induced hypertension, we subjected mice to chronic Ang II infusions under conditions of systemic ACE inhibition[8]. Because angiotensin-converting enzyme (ACE) is responsible for most Ang I conversion to Ang II in the mouse kidney[3,9], chronically Ang II-infused mice were treated with an ACE inhibitor (Lisinopril) to suppress endogenous generation of Ang II. The rationale was that during chronic Ang II infusions, as a consequence of the reduced capacity for intrarenal Ang II formation due to ACE inhibition, ACE inhibitor-treated mice would display lesser increases in intrarenal Ang II and blood pressure as opposed to Ang II-infused mice not exposed to ACE inhibition. As shown in Fig. 12.7, ACE inhibition significantly ameliorated the increases in 24 h mean arterial pressure caused by chronic Ang II infusions. Intrarenal Ang II was also significantly lower in Ang II-infused mice treated with an ACE inhibitor. These results indicate that besides the exogenous Ang II that is being supplied, ACE activity leading to endoge-

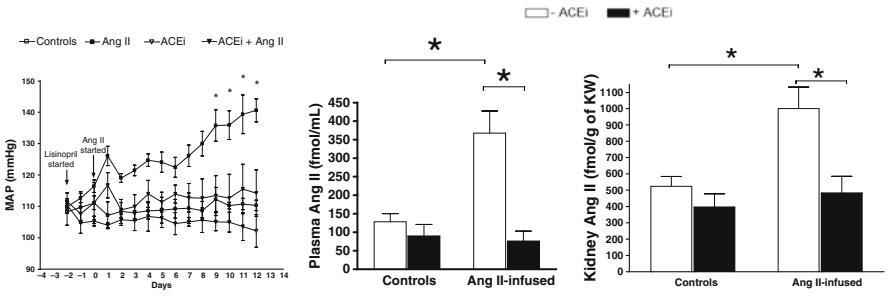

Fig. 12.7 Changes in mean arterial blood pressure and plasma and intrarenal Ang II in Ang II-infused mice with or without an ACE inhibitor[8]. Blood pressure and Ang II concentrations determined by telemetry and radioimmunoanalysis, respectively. Ang II = Angiotensin II (400 ng/kg/min), ACEi = Lisinopril (100 mg/L in the drinking water).*$p < 0.05$ vs. controls by TWO-WAY ANOVA for MAP changes and ONE-WAY ANOVA for Ang II changes

nous Ang II generation is required for the complete development of intrarenal Ang II augmentation as well as the hypertension in the Ang II-induced model. These observations are supported by a report from Sadjadi et al.[30] in which uninephrectomized rats subjected to Ang II infusions and ACE inhibition with enalapril also failed to develop hypertension and increase intrarenal Ang II. Interestingly, ACE inhibition also reduced plasma Ang II concentration in Ang II-infused mice. Because plasma renin activity is suppressed and kidney renin activity is maintained during chronic Ang II-infusions in mice (Fig. 12.6), it is likely that most of the endogenous Ang I and Ang II that accumulate in the kidneys are generated locally in this organ and this process is also responsible for supplying the observed augmentation of Ang II in the systemic circulation.

In summary, sustained intrarenal Ang II formation plays a significant role in mediating the increases of blood pressure, plasma Ang II, and intrarenal Ang II during Ang II-dependent hypertension in mice and in rats. Pharmacological inhibition or genetic manipulation of the intrarenal RAS, and therefore endogenous Ang II formation, significantly alters intrarenal Ang II and blood pressure levels. The results help explain why RAS blockers are effective in patients with essential hypertension even though the vast majority have normal or low plasma renin activity levels and why they also confer renoprotection to an extent greater than can be explained by the reduction in blood pressure. These effects may be explained by modulation of the intrarenal renin–angiotensin system and the prevailing intrarenal Ang II levels. These studies aimed at the mechanisms that govern the intrarenal RAS and consequently local Ang II levels in the kidney contribute to our understanding of hypertension and the development of renal injury.

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Chapter 13
Collecting Duct Renin: A Critical Link in Angiotensin II-Dependent Hypertension

Minolfa C. Prieto and L. Gabriel Navar

Abstract All of the components needed for angiotensin II generation are present within the renal tubular network of the kidney. This brief review is focused on recent evidence demonstrating that inappropriate activation of renin in distal nephron segments by acting on angiotensinogen generated in the proximal tubule cells and delivered to the distal nephron may contribute to increased distal intrarenal angiotensin II formation, sodium retention, and development and progression of hypertension. Moreover, mechanisms regulating collecting duct renin during Ang II-dependent hypertension are discussed.

Introduction

The renin–angiotensin system (RAS) plays a pivotal role in regulating renal electrolyte and water excretion, thereby maintaining body sodium and fluid balance. Renin produced by the kidney cleaves liver-derived angiotensinogen (AGT) to form angiotensin (Ang) I in circulating blood, which is then converted into Ang II by angiotensin converting enzyme (ACE) located at the luminal side of the endothelium in many tissues. An ACE homolog, ACE2, has been shown to cleave a single amino acid from Ang I to form Ang 1-9 and from Ang II to form Ang 1-7[1].

Intrarenal Ang II contributes to the regulation of transport function and renal hemodynamics[2–5]. Angiotensin II levels are greater in the kidney than in the plasma[6,7]. Angiotensin II present in the renal tissues is generated from AGT delivered to the kidney and from AGT locally produced by proximal tubule cells[8]. The presence of AGT in proximal tubule cells[9] provides a source for local generation of Ang I in the early part of the nephron[10,11]. Thus, the localization of

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renin protein and mRNA expression in principal cells of collecting ducts[12–14] along with the presence of AGT in urine[8] and ACE in the distal nephron segments[15,16] suggests that late part of the nephron is a site for intrarenal Ang II formation. In this review, we summarize our current understanding of the independent regulation of the intratubular RAS and discuss the hypothesis that inappropriate activation of renin in distal nephron segments and AGT synthesized and secreted by proximal tubule cells lead to increased formation of angiotensin peptides in the distal nephron segments, and thereby play a role in the development and progression of hypertension.

Synergistic Actions of Proximal Tubule AGT and Collecting Duct Renin During Ang II-Dependent Hypertension

The internalization of Ang II in the proximal tubule can occur via AT1R-mediated uptake into endosomes[17] and intermicrovillar cleft vesicles[18] as well as by a mechanism mediated by megalin[19] which targets Ang II to degradation and protects the cell against Ang II accumulation. The balance between these pathways ultimately determines intracellular Ang II levels[19]. Chronic Ang II infusions lead to increased intrarenal Ang II content in the rat and mouse Ang II-infused models of hypertension[20,21]; however, local de novo Ang II formation due to enhanced intrarenal AGT production also contributes to the overall Ang II levels in the kidneys[8]. In vivo and in vitro studies have shown that Ang II stimulates intrarenal AGT mRNA and protein levels in proximal tubule cells[9,22,23]. Chronic Ang II-infused rats have increases in renal AGT mRNA and protein[9] as well as an enhancement of urinary excretion rate of AGT[24]. This amplification mechanism may be responsible for the sustained or enhanced generation of AGT leading to continued intrarenal production of Ang II under conditions of elevated circulating Ang II concentrations. Intrarenally formed Ang II may exert an additive effect with the Ang II that is internalized via AT1R, both contributing to the overall increased intrarenal Ang II content. Furthermore, the finding of intact AGT in urine suggests its presence throughout the nephron and to the extent that renin and ACE are available along the nephron, there may be continued Ang I generation and Ang II conversion in segments beyond the proximal tubule[12,25,26]. When intrarenal AGT formation is increased, some of the AGT secreted into the tubular fluid spills over into the distal tubule and eventually into the urine[24]. The presence of substrate allows for continued Ang II generation at distal nephron sites dependent on availability of renin and ACE. This issue has raised our interest in a more detailed investigation of renin present at distal nephron segments and on its role in Ang II-dependent hypertension.

Collecting duct renin represents a possible pathway for Ang I generation from proximallydelivered AGT. Although it is well established that plasma renin is primarily derived from the cells of the juxtaglomerular (JG) apparatus, renin gene expression has also been detected in renal tubular segments[12,13,27–29]. Renin is expressed in principal cells of connecting tubules and cortical and medullary collecting ducts of kidneys of normal rats, Ang II-dependent hypertensive rats,

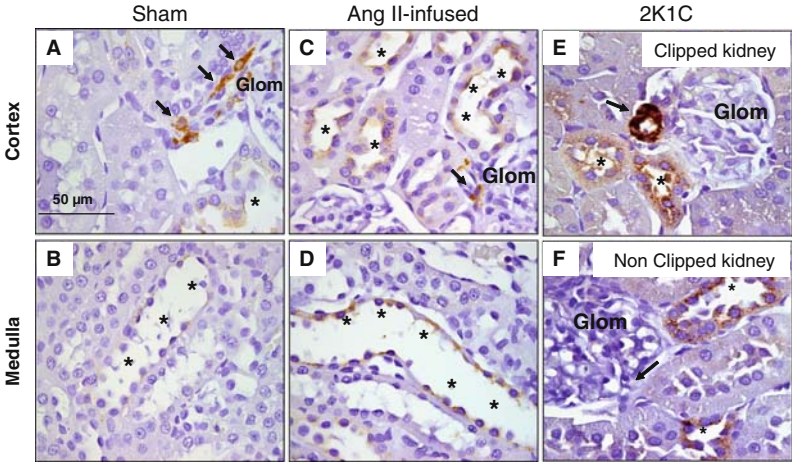

Fig. 13.1. Renin immunoreactivity in cortical and medullary collecting ducts of rat kidneys. **Panels a–f** show the cortex (**Panels a, c,** and **e**) and medulla (**Panels b** and **d**) of kidney sections (3 μm) with specific renin immunostaining in sham rats (**Panels a–b**), chronic Ang II-infused rats (**Panels c–d**) and 2K1C Goldblatt hypertensive rats (**Panels e–f**). *Arrows* show renin immunoreactivity in juxtaglomerular cells localization (DAB chromogen) in a sham (**Panel a**), chronic Ang II-infused rat (**Panel c**) and in the clipped kidney (**Panel e**) and non-clipped kidney (**Panel f**) from Goldblatt rats. Higher renin immunoreactivity (*asterisks*; DAB chromogen) are shown in the collecting ducts of the renal cortex of chronic Ang II-infused rats (**Panel c**) and both, clipped (**Panel e**) and non-clipped (**Panel f**) kidneys relative to sham kidney section (**Panel a**). Glom: Glomerulus

and two-kidney one-clip (2K1C) Goldblatt hypertensive rats (Fig. 13.1). Renin expressed by the tubular segments is differentially regulated from renin synthesized in JG cells[12–14,30,31]. Collecting duct renin is upregulated in chronic Ang II infused rats[13] by an AT1R-mediated mechanism since Ang II-mediated increases are prevented by AT1R blockers (ARB)[14,32].

By using immunohistochemistry, renin has been localized in the principal cells of collecting duct but not in intercalated cells[12,14,32]. Using confocal multiphoton excitation fluorescence microscopy, Peti-Peterdi and associates reported quinacrine-labeled renin granular content in JG cells and collecting duct cells[33,34] and the visualization of renin exocytosis in real time in response to a number of stimuli including Ang II[14]. Rohrwasser et al. suggested in vitro renin secretion by isolated connecting tubules[12] and demonstrated renin and prorenin in urine from mice treated with high salt diet and amiloride[35].

We reported augmented renin transcript levels in the renal medullary tissues of Ang II-dependent hypertensive rats[32,36]. Renal medullary tissues of chronic Ang II-infused rats[32] and 2K1C Goldblatt hypertensive rats[36] exhibit increased renin mRNA levels compared to control rats. Recently, a prorenin receptor -(P)RR- has been cloned and demonstrated in mesangial cells, cortical renal arteries, and distal tubules of the kidney[37–39]. The (P)RR increases Ang II tissue generation by binding either renin or prorenin and increasing the catalytic efficiency[37,38,40]. Along with the increased renin synthesis and secretion from the principal cells of the collecting ducts[14,36], we have found positive (P)RR immunoexpression

in the apical side of the intercalated cells of the collecting ducts[41]. Thus, it is possible that (P)RR located in the distal nephron segments may play a pivotal role to increase the efficiency of intratubular angiotensin peptide generation by anchoring renin or prorenin synthesized and released by principal cells. Nevertheless, according to Nguyen et al.[38] it is still not clear if the affinity of the (P)RR is sufficient for binding renin in picomolar concentration ranges, given the fact that only ≈1% of soluble renin is expected to bind to the receptor[38]. Nguyen et al.[42] described the immunoexpression of this receptor on the basolateral side of distal tubular cells as well as macula densa cells which may be particularly significant in regulating interstitial Ang II levels. Increases in renal interstitial fluid Ang II levels have been reported for two models of hypertension. Siragy and Carey[43] found that renal interstitial Ang II is increased in the wrapped kidney of rats with two-kidney, one-wrap Grollman hypertension. Nishiyama et al.[44] reported that renal interstitial fluid Ang II concentrations are increased in rats infused with Ang II for 2 weeks. Because the renal interstitial values are greater than can be explained on the basis of equilibration with the plasma concentrations, it seems likely that local regulation of Ang II formation in the renal interstitial compartment and enhanced production of interstitial Ang II are due to specialized Ang II-forming pathways or accumulation mechanisms[44]. The presence of (P)RR at the basolateral side of distal tubular segments cells may contribute to the pool of Ang II in this renal compartment.

Regulation of Renin in the Collecting Duct During Ang II-Dependent Hypertension

The mechanisms responsible for the upregulation of renin in the collecting duct during Ang II-dependent hypertension remain unclear. In chronic Ang II-infused rats, renin expressed by principal cells of cortical connecting tubules and cortical and medullary collecting duct is augmented[13]. The increases in collecting duct renin transcript as well as enzymatic activity in the medullary tissues of these rats indicates local synthesis and an adequate source of the enzyme available for cleaving AGT delivered into the tubular fluid from the proximal tubule segment[32,36]. The coexistence of suppressions of JG renin and PRA in the chronic Ang II-infused rat model argues against a recapture effect being the major determinant of augmented renin in medullary regions. Thus, in contrast to the inhibitory effect that Ang II exerts on JG renin, chronic Ang II infusions stimulate renin in the collecting duct cells[13,14].

In our studies using 2K1C Goldblatt hypertensive rat model to dissect the effects on collecting duct renin of high Ang II levels, which are present in both kidneys of 2K1C rats, from the effects of exposure to elevated arterial pressure, which is restricted to the non-clipped kidney (NCK), we found augmented gene expression of renin in the collecting ducts of inner medullary tissues from both kidneys of 2K1C Goldblatt hypertensive rats. These findings indicate that the enhancement of local synthesis and stimulation of renin in the distal nephron segments occurs independently of blood pressure. The observation that the NCK is highly Ang II

dependent even when circulating Ang II levels returned towards normal suggests that there is dissociation between the circulating Ang II levels and the renal Ang II dependency. Importantly, the Ang II content of the NCK is elevated even at a time when JG renin content and its transcript levels are markedly decreased suggesting a unique mechanism responsible for the enhanced intrarenal Ang II in NCK[45–47].

Because plasma Ang II concentrations in 2K1C hypertensive rats return towards control levels after 2–3 weeks of clipping, it is unlikely that circulating Ang II sustains the upregulation of distal nephron renin[47] · However, increases in plasma Ang II concentration occur during the early phases following renal unilateral clipping[6], therefore it is conceivable that this could play a role in initiating the increases in intrarenal Ang II by augmentation of intrarenal AGT[48] and AT1R-mediated uptake[49]. Accordingly, the initial event caused by unilateral renal artery clipping may initiate the cascade responsible for intrarenal Ang II augmentation in the NCK[47,50]. Furthermore, it is also unlikely that circulating renin or prorenin is the stimulus for the upregulation of renin in the collecting duct cells since chronic Ang II-infused rats exhibit stimulation of renin in distal nephron segments in a setting of marked suppression of PRA[13]. It is possible that the local amplification mechanism of intrarenal Ang II on distal nephron renin may allow a moderate increase in Ang II to further augment the intratubular and interstitial Ang II levels in order to achieve rapid homeostatic regulation of sodium balance as needed in a setting of volume depletion. Although this effect appears to be a positive feedback under pathologic conditions, the physiologic consequences of this mechanism would be to prevent or minimize volume and sodium depletion by enhancing sodium reabsorption to re-establish sodium balance and ultimately, to allow renin to return to normal levels. Thus, we have suggested[36] that in physiological conditions, this represents a feed-forward mechanism that anticipates and prevents volume depletion. However, during overactivation of the RAS such as following unilateral renal artery clipping or chronic exogenous infusions of Ang II, this stimulus would be sustained leading to further increases in local Ang II levels through the coordinated actions of AGT in the proximal tubule cells and renin in the distal nephron segments. We recognize that other possible downstream mediators such as distal nephron sodium reabsorption, changes in distal sodium delivery, and aldosterone might also be involved in the regulation of collecting duct renin during Ang II-dependent hypertension.

Potential Role of Enhanced Collecting Duct Renin in Ang II-Dependent Hypertension

Angiotensin II levels are higher in the medullary regions than in the cortical regions in normal rats[51]. These concentrations increase even further in Ang II-infused hypertensive rats. Increased Ang II tissue content in association with high density of AT1 receptors may account for major actions of Ang II in regulating hemodynamics and tubular function in the renal medulla[52,53]. Thus we have hypothesized that the enhancement of renin gene expression as well as its activity in the collecting duct cells is a driving force to increase intrarenal Ang II content in the medullary regions[36].

Angiotensin converting enzyme, which metabolizes Ang II to generate Ang 1-7, is predominantly distributed in the deep renal cortex and outer medulla[54,55]. Recent evidence demonstrating that chronic Ang II infusions upregulate ACE and downregulate ACE2 in vitro and in vivo[56–58] highlights the importance that upregulation of renin in the collecting ducts cells may have to increase formation of Ang I and ultimately Ang II in the distal tubular segments. During Ang II-dependent hypertension, the increases of collecting duct renin contribute to the downregulation of ACE2, which will decrease Ang II degradation and further increase intrarenal content of Ang II in the renal medulla. Therefore, augmented collecting duct renin favors an imbalance in the angiotensin peptide intrarenal content of Ang II and Ang 1-7 and thus contributes further to the progression of high blood pressure during hypertension. More studies using specific renin inhibitors during upregulation of collecting duct renin conditions or specific targeting of the renin gene at the principal cells of the collecting duct will help to elucidate the functional role of renin in the distal nephron segments during Ang II-dependent hypertension.

Conclusions

From a functional perspective, enhanced AGT in urine of Ang II-dependent hypertensive rats reflects spillover of AGT from proximal nephron segments and substrate availability throughout the nephron. Thus, augmented renin in the collecting ducts along with the local presence of the (P)RR may increase Ang I levels in the distal nephron segments. The (P)RR at the surface of the collecting duct cells, by increasing the catalytic activity for Ang I generation and anchoring renin, may

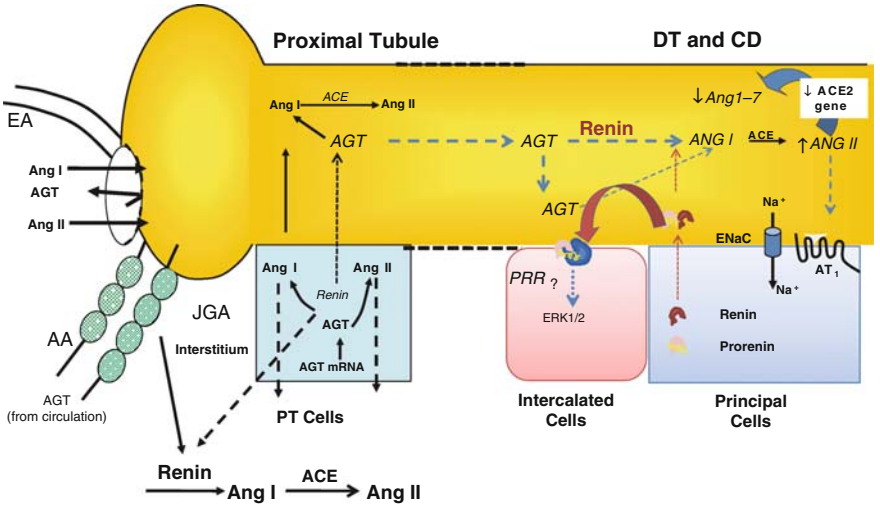

Fig. 13.2. Hypothetical model of intrarenal RAS

reduce washout of prorenin or renin into the urine. The availability of ACE in distal nephron segments along with reduction in ACE2 supports subsequent enhanced formation of Ang II and reduced Ang 1-7 formation. The enhancement of renin in the collecting ducts along with the presence of (P)RR lead to increased intrarenal Ang II formation and consequently decrease its degradation by favoring the downregulation of ACE2. Accordingly, in Ang II-dependent hypertension, renin and ACE in distal nephron segments may provide a critical final mechanism for Ang II formation, which can act on transport systems to stimulate sodium reabsorption and consequently play a major role in the development and maintenance of high blood pressure (Fig. 13.2).

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Chapter 14
Renin–Angiotensin–Aldosterone System and Cardiomyocyte Apoptosis in Hypertensive Heart Disease

Arantxa González, Susana Ravassa, Begoña López, Javier Beaumonta, and Javier Díez

Abstract In the past decade observations have been made showing that cardiomyocyte apoptosis is an integral feature of the structural remodeling of the hypertensive hypertrophied left ventricle. The loss and functional abnormalities of apoptotic cardiomyocytes may be a contributing cause to the transition from left ventricular hypertrophy to heart failure in hypertension. Although the available evidence suggests that apoptosis can be induced in cardiomyocytes by a variety of factors and pathways, a number of findings suggest that the effectors of the renin–angiotensin–aldosterone system can be critically involved in cardiomyocyte apoptosis in hypertension. This review will summarize recent evidence demonstrating the potential contribution of angiotensin II and other components of the system to cardiomyocyte apoptosis in hypertensive heart disease. In addition, some evidence regarding strategies aimed to detect and prevent apoptosis of cardiomyocytes in hypertensive patients will be considered.

Keywords Apoptosis · Cardiomyocytes · Hypertensive heart disease · Heart failure · Renin–angiotensin–aldosterone system

Cardiomyocyte Apoptosis in Hypertensive Heart Disease

Hypertensive heart disease (HHD) is characterized by both the presence of a greater than normal left ventricular mass (i.e., left ventricular hypertrophy or LVH) in the absence of a cause other than arterial hypertension and the development of complex changes in myocardial composition that are responsible for the structural remodeling of the myocardium[1]. Hypertensive myocardial remodeling is the consequence

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of a number of pathologic processes, mediated by mechanical, neurohormonal, and cytokine routes, occurring in the cardiomyocyte and the noncardiomyocyte compartments of the heart[1]. Myocardial remodeling involves increased rates of cardiomyocyte cell death. Historically, there are three types of cell death: apoptosis, autophagy, and necrosis. Although the three types of cardiomyocyte death have been observed simultaneously in human hearts as a consequence of pressure overload or an ischemic insult, apoptosis is the most thoroughly characterized form of cell death in the failing and non-failing human myocardium[2].

In fact, cardiomyocyte apoptosis has been shown to be abnormally stimulated in the hypertrophied left ventricle of patients with arterial hypertension, no angiographic evidence of coronary artery disease, and normal cardiac function[3–5]. In addition, recent findings from our laboratory show that cardiomyocyte apoptosis is increased in patients with LVH and chronic heart failure (HF) compared with hypertensive patients with LVH and normal cardiac function[5–7]. Thus, it seems that cardiomyocyte apoptosis precedes the impairment in ventricular function and its exacerbation accompanies the development of HF in patients with HHD[8].

A number of observations suggest that besides changes in the composition of motor unit and cytoskeleton of cardiomyocytes, the transition from LVH to HF in arterial hypertension relates also to apoptosis. In fact, cardiomyocytes apoptosis may contribute to HF through several pathways[9,10]: (i) Reduction of contractile mass related to the loss of cardiomyocytes in which apoptotic cell deaths occurs; (ii) compromise of energy production due to the fact that apoptosis is associated with the loss of cytochrome *c* from the mitochondria and this may halt oxidative phosphorylation and production of ATP; (iii) impairment of contractility because of the activation of caspases that mediate the cleavage of myofibrillar proteins, resulting in an impaired force/calcium (Ca^{2+}) relationship and myofibrillar ATPase activity in viable cardiomyocytes; and (iv) facilitation of electrical disturbances since in the process of dying, a cardiomyocyte passes through phases of increased excitability or becomes automatic, at least until it is dead. In addition, from a random grouping of several such dead cells, the process of normal activation in that area of heart muscle must be deranged and redirected in a way that would provide a suitable anatomical substrate for re-entrant arrhythmias.

Role of the Renin–Angiotensin–Aldosterone System in Cardiomyocyte Apoptosis

Cardiomyocyte apoptosis may occur as a result of an imbalance between systemic and local factors which acting on the cardiomyocyte induce or prevent apoptosis (Table 14.1)[11]. Alternatively, it is possible that some abnormalities of the hypertensive myocardium facilitate the development of the apoptotic process (Table 14.1)[11]. Enhanced activity of the several components of the renin–angiotensin–aldosterone

Table 14.1 Mechanisms and factors that contribute to cardiomyocyte apoptosis in hypertensive heart disease

Enhanced activity of cardiomyocyte proapoptotic factors
Mechanical stretch
Ischemia
Angiotensin II
Aldosterone
p53
Bax
Loss of eficacy of cardiomyocyte survival factors
Insulin-like growth factor-1
Cardiotrophin-1
Bcl-2
WAF-1
Abnormalities that may facilitate the cardiomyocyte apoptotic response
Oxidative stress
Calcium overload
Mitochondrial defects
Alterations of caspases
Alterations of PPARα

system (RAAS), namely angiotensin II, appears to contribute critically to the aforementioned imbalance.

It has been shown that angiotensin II administration to normotensive rats results in increased cardiomyocyte apoptosis that is prevented by treatment with the AT_1 receptor blocker, valsartan[12]. Recent findings suggest that increased cardiomyocyte apoptosis may be related to exaggerated angiotensin-converting enzyme (ACE) activity in the hypertrophied left ventricle of adult spontaneously hypertensive rats (SHR)[13]. In addition, it has been shown that long-term treatment with an ACE inhibitor blocks apoptosis of cardiomyocytes in adult[13] and aged[14] SHR. Furthermore, it has been recently found that chronic blockade of AT_1 receptor with losartan prevents apoptosis in the hypertrophied left ventricle of SHR, independent of its hemodynamic effect[15]. These observations may be consistent with the possibility that angiotensin II may mediate directly cardiomyocyte apoptosis in this model of genetic hypertension.

In fact, angiotensin II has been shown to induce apoptosis of neonatal[16] and adult[17] rat ventricular cardiomyocytes in vitro. Angiotensin II binding to AT_1 receptors triggers apoptosis by a mechanism involving protein kinase C-mediated increases in cytosolic Ca^{2+} and the stimulation of Ca^{2+}-dependent DNase I, which results in internucleosomal DNA fragmentation[16,17] (Fig. 14.1). On the other hand, angiotensin II has been shown to downregulate the leukemia inhibitory factor receptor (LIFR) in rat cardiomyocytes via interaction with the AT_1 receptor[18] (Fig. 14.1). The importance of this observation is related to recent findings demonstrating that the gp130/LIFR signaling survival pathway protects cardiomyocytes against apoptotic stimuli[19]. Thus, besides a direct proapoptotic effect, angiotensin II reduces the resistance of the cardiomyocyte to apoptosis.

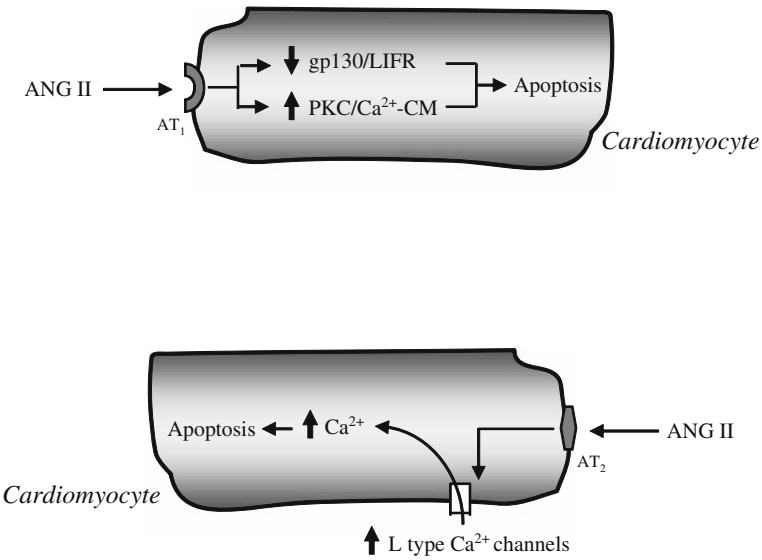

Fig. 14.1 Schematic representation of the different pathways through which angiotensin II (ANG II) may induce cardiomyocyte apoptosis acting on its type 1 (AT_1) (*upper panel*), and type 2 (AT_2) (*lower panel*) receptors located at the cardiomyocyte plasma membrane level

It is interesting to remark that stretching of cardiomyocytes in vitro leads to the autocrine formation of angiotensin II[20] and the increase in the number of AT_1 receptors[21]. Although unequivocal evidence remains to be obtained, it is tempting to speculate that the interaction of angiotensin II with its AT_1 receptor may be a significant component of mechanical load- and stretching-induced cardiomyocyte apoptosis[22].

Some of the cardiac effects of angiotensin II seem to be independent of the AT_1 receptor but may be the result of the ability of the peptide either to stimulate the AT_2 receptor or to operate in the intracellular space. It has been shown that angiotensin II-induced apoptosis in vitro in rat cardiomyocytes is blunted in the presence of the AT_2 receptor blocker PD123319[23]. The AT_2-mediated apoptotic mechanism is not related to the immediate angiotensin II-induced Ca^{2+} rise from intracellular stores, as it does the AT_1-mediated apoptotic mechanism, but it requires Ca^{2+} influx through L-type channel activity[23] (Fig. 14.1). Diep et al. have reported that the in vivo blockade of AT_1 receptors with losartan is accompanied by normalization of cardiomyocyte apoptosis in rats with angiotensin II-induced hypertension that exhibit increased expression of AT_2 receptors in the heart[24]. Therefore, it is still controversial whether the AT_2 receptor is a strong signal to induce cardiomyocyte apoptosis in vivo.

Recently, it has been reported that experimentally induced diabetes in the rat activates the cardiac intracellular renin–angiotensin system, which increases cardiomyocyte apoptosis[25]. Renin inhibition with aliskiren had a more pronounced

beneficial effect than AT_1 receptor blockade and ACE inhibition on this diabetic complication, suggesting a pro-apoptotic role for intracellular renin. This possibility is supported by in vitro findings showing that cytosolic renin, but not secretory renin, is targeted to mitochondria and induces apoptosis in rat cardiomyoblasts[26].

In this regard, Westermann et al.[27] have shown recently that subpressor pharmacological inhibition of renin with aciskiren is associated with decreased apoptosis and attenuation of cardiac dysfunction after myocardial infarction in mice. These effects were independent of blood pressure lowering and suggest a direct role for systemic and/or cardiac extracellular renin in cardiomyocyte apoptosis in this model.

Finally, it has been shown in vivo that the administration of exogenous aldosterone is accompanied by increased cardiac apoptosis in rats, this effect being abrogated by the simultaneous administration of either eplerenone[28] or spironolactone[12]. In vitro experiments have shown that aldosterone nongenomically induces cardiomyocyte apoptosis, probably through stimulation of NADPH oxidase-[29] and/or calcineurin-dependent[30] mechanisms.

Potential Clinical Implications

From the experimental data previously reviewed it is intriguing to speculate that pharmacological interference of the RAAS can also prevent cardiomyocyte apoptosis in patients with HHD and that this effect will not only depend on blood pressure lowering. However, there is just one clinical study showing that despite an identical antihypertensive efficacy, the AT_1 receptor antagonist losartan, but not the calcium channel blocker amlodipine, reduced cardiomyocyte apoptosis in patients with essential hypertension and LVH after 1 year of treatment (Fig. 14.2). Clearly, clinical trials are needed to evaluate the effect on cardiac apoptosis of drugs interfering with the RAAS and to prove that prevention of apoptosis might be suitable for preventing HF in patients with HHD.

In this conceptual framework, a noninvasive method to detect cardiomyocyte apoptosis would be ideal to assess its role in disease and to measure treatment outcomes in patients with HHD[31]. Recent clinical findings support the notion that annexin A5 (AnxA5) may be useful as a biochemical marker of apoptosis-related cardiomyocyte dysfunction in hypertensive patients. AnxA5 is a 32–35 kDa Ca^{2+}-binding protein that becomes upregulated in response to different apoptotic stimuli acting on cardiomyocytes[32]. It has been reported that whereas plasma concentration of AnxA5 was normal in hypertensive patients without LVH, it was abnormally high in patients with LVH[5]. In addition, plasma AnxA5 concentration was higher in patients with HF than in patients with LVH[5]. Interestingly, plasma AnxA5 was directly correlated with left ventricular end diastolic diameter and inversely correlated with ejection fraction in the whole group of patients[5].

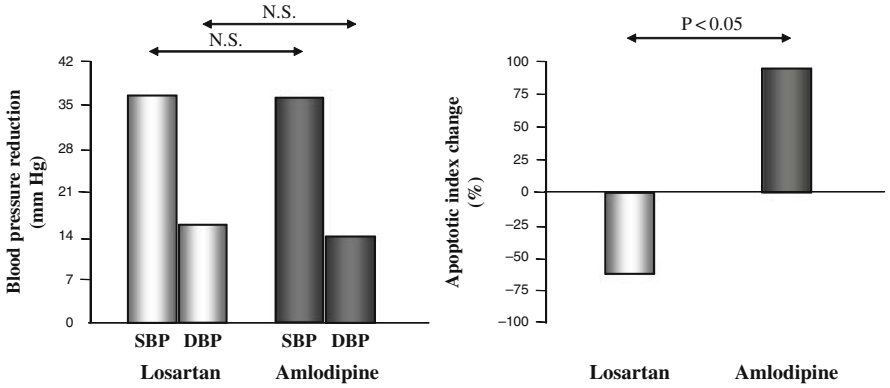

Fig. 14.2 Effects of treatment with losartan or amlodipine on systolic blood pressure (SBP) and diastolic blood pressure (DBP) (*left panel*) and cardiomyocyte apoptosis (*right panel*) in patients with hypertensive heart disease. (Adapted from reference[4])

Perspectives

Numerous hypotheses have been considered to explain the fundamental mechanism(s) for the development of HF in patients with HHD. Cardiomyocyte loss and dysfunction due to apoptosis is now being considered as one of the determinants of the maladaptive processes implicated in the transition from LVH to HF. Much work is being carried out regarding the mechanisms and the impact of cardiomyocyte apoptosis on the hypertensive myocardium but several methodological and conceptual issues still remain unsolved. Clarification of these is extremely urgent if one considers that the development of noninvasive tools for the monitoring of cardiac apoptosis and pharmacological strategies aimed to inhibit and/or counteract the apoptotic process could be of particular relevance to protect the cardiomyocyte and prevent the progression to HF in patients with HHD.

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Chapter 15
Upregulation of Angiotensin II Type 2 Receptor (agtr2) Attenuates Atherosclerotic Lesion Formation and Enhances Apoptosis in the LDL Receptor Knockout Mice Fed High Cholesterol Diet

Jawahar L. Mehta, Magomed Khaidakov, Changping Hu, Giusto Spagnolli, and Dayuan Li

Abstract Angiotensin II type 1 receptor (AT1R) exerts growth-promoting and anti-apoptotic effects, which contribute to atherogenesis. In contrast, type 2 receptor (AT2R) activation exerts anti-growth and pro-apoptotic effects. We tested the hypothesis that over-expression of AT2R will attenuate formation of atherosclerotic lesions. Low-density lipoprotein receptor knockout (LDLR KO) mice were injected via tail vein with recombinant AAV carrying AT2R (agtr2) cDNA (AAV/AT2R), AAV/Neo, or saline, and then put on a high cholesterol diet. At 18 weeks, all animals were sacrificed, and the aortas were harvested for AT2R expression and determination of atherosclerotic lesion formation. AT2R was highly expressed in mice given AAV/AT2R, but not in other groups. Atherosclerotic lesion formation and thickness of intima were significantly reduced in the LDRL KO mice with AT2R over-expression compared to other LDLR KO mice. Concurrently, there was suppression of oxidative stress (NADPH oxidase $p67^{phox}$ and the transcription factor NF-κB) and increase in the free radical scavenger superoxide dismutase activity. Importantly, there was a marked increase in apoptosis in the atheromatous tissues in the LDLR KO mice with over-expression of AT2R. Thus, upregulation of AT2R by gene delivery in the LDLR KO mice reduces oxidative stress and increases apoptosis resulting in a reduction in atherosclerotic lesion formation.

Keywords Angiotensin · Apoptosis · Atherosclerosis · Oxidative stress · Gene therapy

Renin–angiotensin system (RAS) plays a critical role in the pathophysiology of atherosclerosis. Activation of RAS results in the formation of angiotensin II

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(Ang II), which causes vasoconstriction, injures endothelium, induces oxidative stress and inflammation, and enhances smooth muscle cell (SMC) proliferation all of which are critical features in atherogenesis. The pathological effects of Ang II are thought to be mainly mediated by activation of its type 1 (AT1) receptor, a member of the superfamily of heptahelical G-protein-coupled receptors.

In recent years, there has been increasing interest in Ang II type 2 receptor (AT2R) since it has been suggested that the activation of AT2R has effects opposite of AT1R. The fetal tissues express high level of AT2R; the AT2R expression decreases with age and adult tissues have only low level of AT2R expression[1,2]. An in vitro study[3] showed that upregulation of AT2R in cultured adult SMCs antagonizes the growth-promoting effects of platelet-derived growth factor. Other studies have shown AT2R-mediated apoptosis in SMCs in vitro and in vivo[4–6]. In an in vivo study[3], AT2R expression by gene transfer attenuated neointimal proliferation in injured carotid arteries. Upregulation of AT2R expression has also been shown to mediate Ang II-induced vasodilatation in spontaneously hypertensive rats[7]. Studies from the Dzau's laboratory showed that Ang II caused a more pronounced hypertensive effect in the AT2R-null[8]. In these mice, the cardiac hypertrophy and fibrosis were more pronounced following the stress of aortic banding[9]. A recent study from this group[10] demonstrated that the loss of AT2R during atherogenesis in the ApoE KO mice enhanced the accumulation of macrophages, SMCs, and collagen in the atherosclerotic lesions, and decreased apoptosis of cellular components.

We have recently examined the hypothesis that upregulation of AT2R gene delivered via recombinant type adeno-associated virus (AAV) in the LDLR KO mice will reduce atherogenesis and modulate the apoptotic process.

Methods

Animal Protocol

Wild-type C57BL/6 mice and homozygous LDLR KO mice (on C57BL/6 background) were obtained from Jackson Laboratories. They were bred by brother–sister mating and housed in a room lit from 6:00 AM to 6:00 PM and kept at 21°C. Group 1: C57BL/6 mice were fed high cholesterol diet (4% cholesterol/10% cocoa butter) for the entire study period. This served as the negative control group. LDLR KO mice were divided into three groups, which were kept on high cholesterol for 18 weeks. Group 2: LDLR KO mice were injected with 100 μL of saline via the tail vein (positive control group). Group 3: LDLR KO mice were injected with 100 μL of AAV/AT2R virus (10^{11} e.g.) (experimental group). Group 4: LDLR KO mice were injected with 100 μL of AAV/Neo virus (10^{11} e.g.) (AAV control group). All experimental procedures were performed in accordance with the protocols approved by the Institutional Animal Care and Usage Committee. AAV/AT2R and AAV/Neo were provided by our gene therapy program.

Generation of AAV/AT2R

The method for insertion of cDNA in rAAV type 2 for various genes including AT2R has been recently published[11–13]. Essentially, total liver mRNA was isolated from SD rats using Trizol reagent (Invitrogen) and treated with 5U/μg of RNase-free DNase I (Promega) at 37°C for 1 h. Next, mRNA was separated using the Oligotex mRNA Mini Kit (QIAGEN). First-strand cDNA synthesis was performed using oligo(dT)15 primers. PCR amplification for the rat AT2R (agtr2) cDNA was performed using the following primer pair: 5′-CTCTCCGAAGTTCCGTGG-3′ and 5′-TCAGCTGCACTTG CAGGAC-3′ to amplify the sequence from nucleotides 380–1567. Finally, the AT2R cDNA was verified by sequencing. The map of AAV containing AT2R cDNA is show in Fig. 15.1.

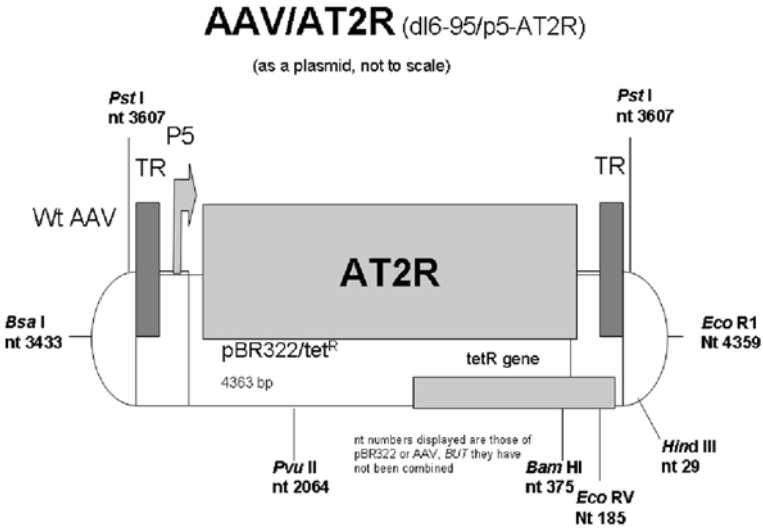

Fig. 15.1 Map of AAV/AT2R

Analysis of Transgene Vector DNA

The heart and aorta specimens were derived from the AAV or saline-injected LDLR KO mice. The total DNA was isolated from the frozen tissue specimens according to routine method[10,11]. PCR amplification was performed using the total DNA as the templates. The PCR upstream primer was complementary to AAV type 2 p5 promoter sequence (5′-GGAGGTCCT GTATTAGAG-3′). The downstream primer was designed for the *dl6-95* vector and complementary to AAV type 2 sequence (5′-CGCCATGCTACTTATCTAC-3′).

Analysis of Transgene mRNA Expression

Aortas from all animal groups were harvested at 18 weeks. The mRNA isolation and RT-PCR amplification analysis of AT1R and AT2R were carried out as described previously[12].

Analysis of Protein by Immunohistochemistry

Tissue specimens were obtained from aortic arch, fixed in formalin, and paraffin-embedded according to standard procedures. The 5 μm thickness sections from each representative block were cut, mounted on glass slides, deparaffinized, and rehydrated. Sections were incubated with blocking serum for 30 min and then incubated overnight at 4°C with primary antibody. AT1R and AT2R (Santa Cruz Biotechnology, Santa Cruz, CA) were analyzed using rabbit polyclonal antibody. Slides from all animal groups were then simultaneously processed for immunohistochemistry using double staining system (Bio-Rad Technology Inc) according to manufacturer's instructions and counterstained with hematoxylin. Negative controls were performed by omitting the primary antibody. Stained slides were observed under light microscopy[10,11,13].

Analysis of Atherogenesis

After sacrificing the animals, fatty deposits along the entire length of aorta were quantitated by the following method: 4–5 mice from each group were euthanized and the aortas were separated from surrounding tissues. After removal of the adventitial fat tissue, the aortas were opened longitudinally from the aortic arch to the iliac bifurcation and fixed in 10% formalin for 24 h. Then the aortas were rinsed in 70% alcohol briefly, stained with Sudan IV solution for 15 min, differentiated in 80% alcohol for 20 min, and washed in running water for 1 h. The aortas were mounted and photographed with a camera connected to a dissection microscope. The images were analyzed by software (Image Pro Plus, Media Cybernetics). In complementary method, 5-μm serial cross sections of the aortic root, thoracic aorta, and abdominal aorta were prepared and stained with hematoxylin-phloxine-saffron. To determine the average cross-sectional lesion area, four sections (interval 30 μm) of each specimen were analyzed blindly using the software (Image Pro Plus, Media Cybernetics)[11–13]. Data from several animals in each group were averaged.

Western Blot Assay

SDS-PAGE was performed on 10% separation gels with a 6% stacking gel. Proteins were transferred to nitrocellulose membrane (Bio-Rad). Blots were incubated

with primary antibody with 1:1000 dilution at 4°C for overnight. Blots were incubated with horseradish peroxidase–conjugated secondary antibody and signal was detected with enhanced chemiluminescence (Amersham Life Science)[12].

TUNEL and Caspase-3 Staining

Terminal dUTP nick end-labeling (TUNEL) staining was performed as described earlier with PI nuclear counterstaining. Negative control was performed without terminal transferase.

Successful Systemic Delivery of Transgene into LDLR KO Mice

Eighteen weeks after tail vein injection of saline, AAV/Neo or AAV/AT2R and feeding of high cholesterol diet, the animals were analyzed for vector DNA, RNA, and protein expression. The presence of AAV/AT2R vector DNA analyzed by RT-PCR is shown in Fig. 15.2a. The vector sequences with AT2R were PCR amplified

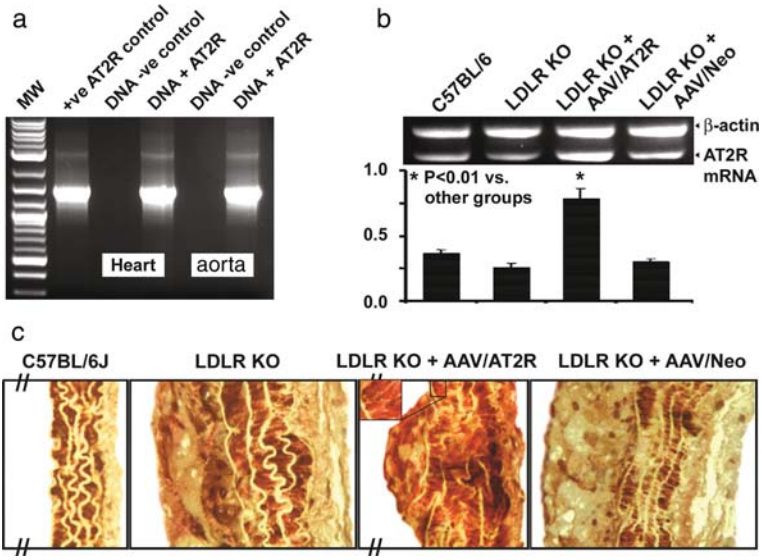

Fig. 15.2 Determination of vector DNA and AT2R mRNA and protein. **Panel a** shows that AT2R cDNA vector was highly expressed in the heart and aorta. Negative control (the same sample without RT) did not show the band. In **Panel b**, a single tail vein injection of AAV/AT2R markedly increased AT2R mRNA expression compared to the other groups. AT2R mRNA expression was increased in LDLR KO mice given AAV/AT2R. In panel C, double immunostaining showed that the AT2R protein was highly expressed in the LDLR KO mice given AAV/AT2R. AT2R protein (*red color*) was colocalized with smooth muscle cells (*brown*). This result is consistent with AT2R mRNA expression

using primers homologous to the ends of AAV vector. We found that with AT2R vector cDNA was highly expressed in various tissues. Figure 15.2b shows the RT-PCR data; again there was a marked increase in AT2R mRNA expression in mice given AAV/AT2R compared to the other groups. Figure 15.2c shows the results of immunostaining; the AT2R protein expression was highly expressed in the mice given AAV/AT2R and was found to be colocalized with SMCs. These data are consistent with the AT2R mRNA data. Note that the AT2R expression in LDLR KO mice given saline or AAV/Neo had the same level of AT2R as the wild-type mice.

AT1R mRNA and Protein in Atherosclerotic Lesions

In keeping with our previous observations in the hypercholesterolemic rabbits[14], AT1R mRNA (Fig. 15.3, right) and protein (Fig. 15.3, left) were markedly increased in the atherosclerotic mice aortas. The AAV/AT2R or AAV/Neo did not affect AT1R expression.

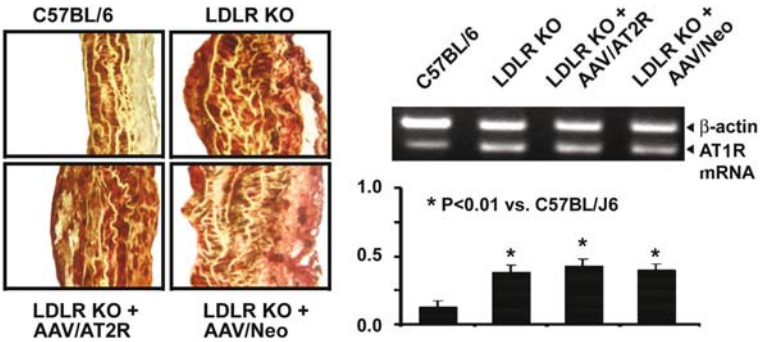

Fig. 15.3 AT1R mRNA and protein in the aortas of different groups of mice. The AT1R protein (*left panel*) and mRNA (*right panel*) were highly expressed in all LDLR KO mice groups. The AAV/AT2R or AAV/Neo did not affect the enhanced AT1R expression

AT2R Upregulation and Atherosclerotic Lesion Formation

To determine if AT2R over-expression results in an effect on atherosclerotic lesion formation, aortas were removed and analyzed for fatty deposits and plaque formation (index of atherosclerosis) at 18 weeks of high cholesterol diet. Representative aortas from each group and average of the extent of aortic lesions are shown in Fig. 15.4, top. Note that the AAV/AT2R-treated LDLR KO mice displayed dramatically smaller areas of sudanophilia than the LDLR KO mice treated with saline or AAV/Neo. AAV/Neo-treated animal showed no reduction in the areas of sudanophilia, indicating that the effect of AAV/AT2R was not a result of injection of AAV.

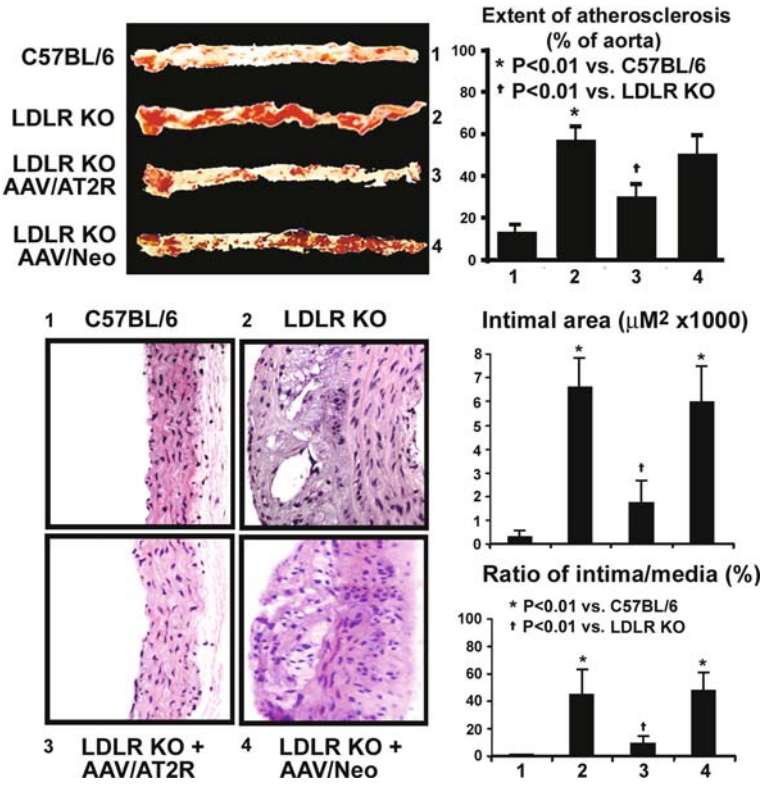

Fig. 15.4 AT2R and atherosclerotic lesion formation. Representative aortas are shown in the *top left panel*. Note that the AAV/AT2R-treated LDLR KO mice displayed dramatically smaller areas of sudanophilia than the LDLR KO mice treated with saline. AAV/Neo-treated animal showed no reduction in the areas of sudanophilia. The *top right panel* shows quantitation of the areas of Sudan IV staining (mean ± SEM). Data from 4 to 6 animals in each group. The bottom panel shows intimal area and the intima/media ratio in different groups. The intimal area and the ratio of intima/media were significantly increased in LDLR KO mice given saline compared to the wild-type mice. Over-expression of AT2R in the LDLR KO mice given AAV/AT2R resulted in a significant decrease in the intimal area and intima/media ratio. AAV/Neo did not affect these indices in LDLR KO mice. Data from four to six animals in each group in mean ± SEM

It is noteworthy that the wild-type mice also had 15% of aorta covered with atherosclerosis, perhaps an indication of feeding a very high cholesterol diet (4% cholesterol and 10% cocoa butter).

The data on sudanophilic areas were complemented by the data on cross sections of various parts of aorta wherein intimal thickness was quantitated. The intimal area and the intima/media ratio were calculated in each group of mice. The analysis revealed that intimal thickness as well as intima to media ratio were significantly increased in LDLR KO mice compared to the wild-type mice given identical high cholesterol diet. The over-expression of AT2R in LDLR KO mice markedly reduced the intimal thickness as well as intima to media ratio. AAV/Neo did not affect these

indices in the LDLR KO mice, indicating that the effect of AAV/AT2R was not due to AAV (Fig. 15.4, lower panel).

AT2R Upregulation and Oxidative Stress

To determine some of the potential mechanisms of AT2R over-expression in atherogenesis, expression of NADPH oxidase $P67^{phox}$ and the redox-sensitive transcription factor NF-κB were examined in this study. We found that NADPH oxidase $P67^{phox}$ was significantly increased in LDLR KO mice compared to that in the wild-type mice. The upregulation of NADPH oxidase was reversed by the over-expression of AT2R in LDLR KO mice. AAV/Neo itself did not affect expression of NADPH oxidase (Fig. 15.5).

Next we studied the expression of redox-sensitive transcription factor NF-κB p65 subunit, a signal downstream of NADPH oxidase. NF-κB p65 was significantly

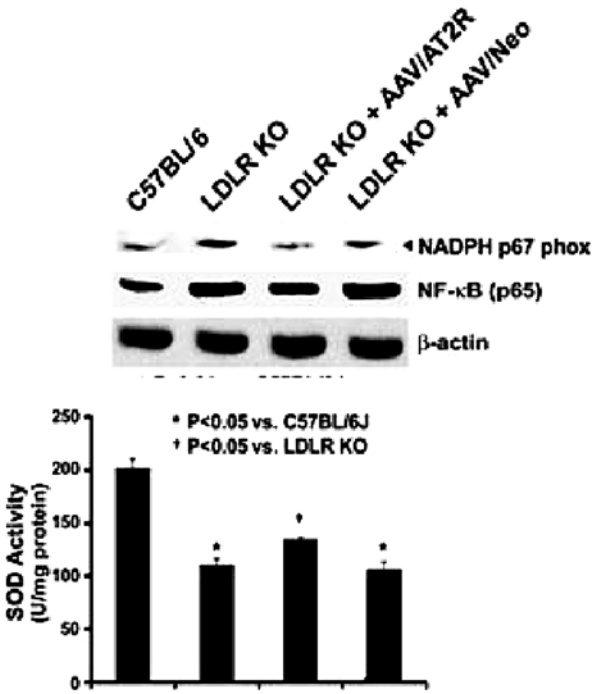

Fig. 15.5 Expression of NADPH oxidase $P67^{phox}$ and NF-κB p65 subunit was increased in the LDLR KO mice given saline (vs. wild-type mice). This was reversed by upregulation of AT2R in the LDLR KO mice. AAV/Neo per se did not affect the expression of NADPH oxidase or NF-κB. As anti-oxidant, SOD activity was measured and found to be significantly decreased in the saline-treated or AAV/Neo-treated LDLR KO mice (vs. wild-type mice). Upregulation of AT2R in the LDLR KO mice significantly enhanced the SOD activity. Data from four to six animals in each group in mean ± SEM

increased in the LDLR KO mice given saline or AAV/neo (vs. the wild-type mice). The increased expression of NF-κB was significantly inhibited by over-expression of AT2R in LDLR KO mice (Fig. 15.5).

As an anti-oxidant, superoxide dismutase (SOD) plays a critical role in scavenging free radicals and protecting SMCs from proliferation. We found that SOD activity was markedly decreased in LDLR KO mice given saline or AAV/neo (vs. the wild-type mice). The SOD activity was significantly preserved by upregulation of AT2R in LDLR mice (Fig. 15.5).

AT2R and Apoptosis in Atherosclerotic Lesion

There were very rare apoptotic cells in the atherosclerotic regions of the LDLR KO mice given saline or AAV/Neo. In contrast, apoptotic cells were often present in the atherosclerotic plaque in LDLR KO given AAV/AT2R (Fig. 15.6). The data on TUNEL staining was corroborated with the caspase-3 data (Fig. 15.6).

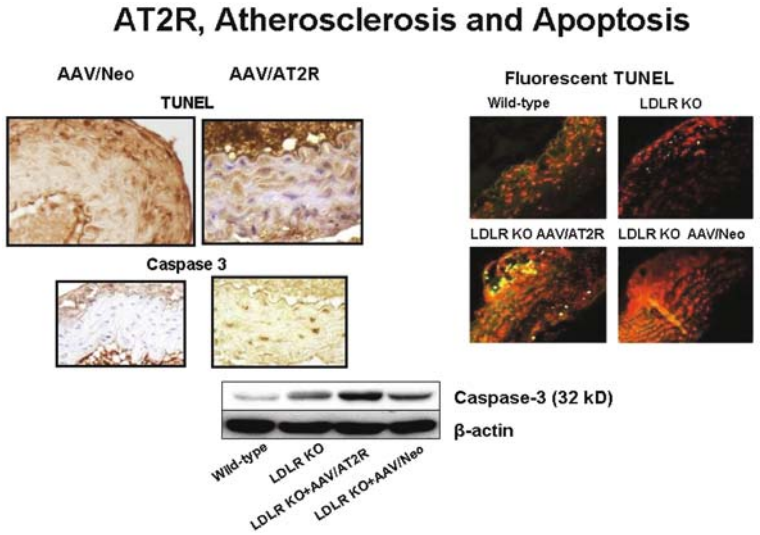

Fig. 15.6 AT2R and apoptosis in the atherosclerotic lesions. Very few apoptotic cells were seen in the atherosclerotic plaque in the saline-treated or AAV/Neo-treated LDLR KO mice. In contrast, the number of apoptotic cells were much greater in the atherosclerotic plaques of LDLR KO mice given AAV/AT2R. The data were confirmed by immunohistochemistry, fluorescent TUNEL staining, and caspase-3 measurements (immunostaining and Western blot). Data from four to six animals in each group in mean ± SEM

Comments

AAV is a very desirable vector to use when long-term expression is needed. Our recent studies have demonstrated that AAV can successfully deliver interleukin-10[11] and TGFβ1[10] genes in the mice. We document again that the

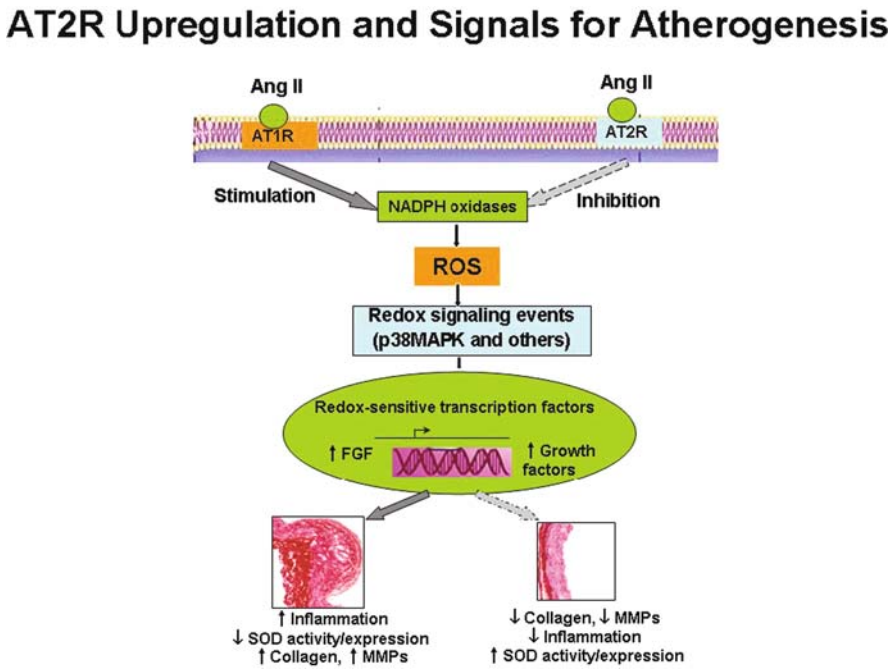

Fig. 15.7 A diagrammatic illustration of the effects of AT1R and AT2R on atherosclerosis. The concepts shown here are based on the observations made in this study

administration of AT2R with AAV by a single tail vein injection provides adequate and sustained high levels of transgene in a variety of tissues, including the heart, and aorta. This approach of intravenous administration has important clinical implications.

It is well known that AT1R is the predominant Ang II receptor in adult tissues and is highly expressed in the atherosclerotic lesions. It is believed that Ang II through AT1R activation stimulates intracellular signaling pathways such as NADPH oxidase[15], protein kinases, and redox-sensitive transcription factor NF-κB[14]. In a previous study[16], we found that AT1R expression is markedly greater than that of AT2R in arterial endothelial cells. Ang II through AT1R causes injury to arterial endothelial cells. In keeping with previous studies, we documented that AT1R is highly expressed in all LDLR KO mice fed high cholesterol diet. Importantly, we show that the over-expression of AT2R does not affect AT1R mRNA expression in atherosclerotic lesions; however, the AT1/AT2R balance shifts in favor of AT2R.

One study using immunostaining with cell type-specific antibodies[17] showed that AT2R is localized in macrophages and SMCs within the atherosclerotic lesions. In the present study, we found that AT2R, when its expression is unregulated by gene transfer technology, is mainly colocalized with SMCs and appears in abundance in the intimal region of the atherosclerotic plaque.

A number of investigators have shown that AT2R exerts anti-growth, pro-apoptotic, and anti-fibrotic effects[3–6,9]. However, AT2R signaling pathways remain incompletely understood. Some investigators[17] have suggested that stimulation of AT2R produces a kinin-dependent stimulation of nitric oxide (NO) generation and release. Actions of locally released NO may include a decrease in oxidative stress and subsequent decrease in the accumulation of monocytes, attenuation of collagen synthesis, a decrease in cell proliferation, and an increase in apoptosis. We also found that there was highly significantly increased expression of NADPH oxidase and the redox-sensitive transcription factor NF-κB in the atherosclerotic tissues. We also show that AT2R upregulation in the LDLR KO mice results in a marked reduction of the enhanced expression of NADPH oxidase and NF-κB.

Some investigators have suggested that besides an increase in the generation of oxidant species, atherosclerotic tissues exhibit reduced expression and activity of anti-oxidant species[18,19] and overexpression of catalase or both Cu/Zn-superoxide dismutase and catalase can retard atherogenesis[19]. We found that the activity of SOD was decreased in the LDLR KO mice given saline. It is of note that the over-expression of AT2R by gene therapy in the LDLR KO mice enhanced SOD activity concurrent with reduction in oxidant signaling and atherogenesis. Importantly, these alterations were not related to the AT2R vector AAV since the AAV/Neo group did not show the changes that were seen in the mice with AT2R over-expression.

AT2R Upregulation and Apoptosis

It is also possible that the pattern of apoptosis relates to the stage of atherosclerosis and plays different role in this process. Atherosclerosis is clearly characterized by increase in SMC proliferation and recruitment of monocytes/macrophages, all of which contribute to the growth of atherosclerosis. Apoptosis of SMCs may reduce bulk lesion in a mild-to-moderate atherosclerotic lesion. In a recent study, Steffens et al.[20] showed that a short-term treatment with anti CD3 antibody reduced atherosclerotic lesion formation in concert with enhanced apoptosis in mice. It is also logical that apoptosis of macrophages would combat inflammation, reduce local cytokine and protease release, and thus reduce plaque formation. At least two studies[5,21] have shown that upregulation of AT2R induces apoptosis of cultured vascular SMCs. Sales and his colleagues[10] found that apoE KO mice had a dramatic decrease in accumulation of macrophages, SMCs, lipids, and collagen. This phenomenon was accompanied by increased TUNEL staining. In the present study, we found that AT2R upregulation significantly enhanced the expression of caspase-3 and the number of apoptotic cells determined by TUNEL staining in the atherosclerotic plaque. Apoptosis of SMCs and/or macrophages may have contributed to the reduction of atherosclerotic lesion since AT2R was upregulated prior to the development of atherosclerotic lesion.

AT2R and Formation of Atherosclerotic Lesion

Genetic manipulation is a very desirable approach to change expression and component of genes. Recent studies from our laboratory[11,12] have demonstrated that an increase in the expression of IL-10 gene and active form of $TGF\beta_1$ dramatically attenuated formation of atherosclerotic lesion. It has been well known that Ang II through AT1R causes endothelial injury, inflammatory cell recruitment and oxidative damage to normal cells, cell proliferation, collagen synthesis, and lipid deposition. There is significant evidence that AT2R has effects. Since AT1R expression is much greater than that of AT2R in the adult tissues, manipulating AT1R/AT2R ratio is an ideal way to alter the AT1/AT2R balance.

In fact, the findings from the present study fully support our hypothesis. We demonstrated that a single injection of AT2R gene with AAV results in continuous expression of AT2R and markedly reduces lipid deposition and intimal thickening in the high cholesterol diet fed LDLR KO mice. AT2R acts constitutively to suppress the changes in vessel wall architecture via reduction of oxidative stress and induction of apoptosis in mice. A recent study[9] found that the deletion of AT2R strikingly increased SMC proliferation and collagen, thus providing clue to the changes in cellular composition of atherosclerotic tissues. Other studies have shown that AT2R activation reduces collagen deposition in the cardiovascular system. For example, Wu et al.[22] showed that AT1R blockade reduced cardiac remodeling in the AT2R KO mice. Akishita et al.[9] demonstrated an inhibitory effect of AT2R activation on coronary arterial remodeling after aortic banding in mice. Taken together, these observations support the hypothesis that the over-expression of AT2R can alter the response of the vessel wall during state of intense hyperlipidemia, and thereby limit initiation and progression of atherosclerotic lesions (Fig. 15.7).

Summary

This study demonstrates that systemic delivery of AT2R via AAV as vector is able to inhibit lipid deposition and formation of atherosclerotic lesions, possibly via antioxidant mechanisms and by inducing apoptosis in the constituents of the atherosclerotic region. These findings provide a novel view that alterations to AT2R/AT1R ratio can modulate the process of atherosclerosis.

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Chapter 16
Salt and Heart: RAAS Involvement

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Abstract The role of high dietary salt intake was previously thought to be restricted only to the effects on arterial pressure. However, a large body of evidence has accumulated over the last two decades pointing to the potent adverse effects of salt excess on cardiac structure and function. The role of the renin–angiotensin–aldosterone system as a key player in mediating these effects has been increasingly appreciated in recent years. Moreover, identification of angiotensin-(1-7), its receptor mas, and enzymes involved in its metabolism challenges our concept that only increased angiotensin II activity is responsible for cardiac injury in salt-sensitive hypertension.

Introduction

For many years the relationship between blood pressure and salt intake has attracted public and scientific attention. The high prevalence of hypertension in industrial societies has been linked to a high sodium intake[54]. The same is also true for age-related increase in arterial pressure[14]. Although some concern was raised from studies reporting greater risk of myocardial infarction[2] or higher sympathetic activity in subjects with lower urinary sodium excretion[27], wide-spread consensus for moderate reduction in sodium intake resulted in the current American Heart Association and US Agriculture recommendation for sodium intake equal or lower than 2,400 mg per day. This is of particular interest for individuals with increased risk for cardiovascular morbidity and mortality including patients with hypertension, diabetes, and hyperlipidemia. Hypertensive left ventricular hypertrophy, characterized by increased ventricular mass, pronounced interstitial and perivascular fibrosis,

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and increased medial thickening and endothelial dysfunction of coronary vessels, imposes a great cardiovascular risk that is independent of the level of arterial pressure[23,24]. Importantly, a large body of evidence derived from epidemiological and experimental studies suggests a potent trophic effect of dietary salt excess on the left ventricle that may be blood pressure-independent[10–12,22,41].

Left Ventricular Hypertrophy and Dietary Salt

The clinical importance of dietary salt intake in the development of left ventricular hypertrophy is underscored in many epidemiological, interventional, and experimental studies. High sodium intake (estimated by 24-h urinary sodium excretion) has been linked to increased left ventricular mass and albuminuria in hypertensive patients through blood pressure-independent mechanisms[11,12,52]. Few clinical studies, however, addressed the significance of salt intake in the development of cardiac dysfunction. Diastolic filling abnormalities were noted in hypertensive subjects in response to increased salt intake[44,51] even without a strong relationship between salt intake and left ventricular structure[51]. On the other hand, considerable evidence from animal experiments support the concept that salt excess adversely affects cardiac function, in part, through its pro-fibrotic effect that in some studies was shown to be independent from blood pressure[1,10,61,62,68]. In our recent studies, we demonstrated that high dietary salt intake in spontaneously hypertensive rats (SHR), a proven experimental model for human essential hypertension, impaired left ventricular function associated with increased arterial pressure and left ventricular mass[61]. Salt excess was also related to an exaggerated collagen deposition within the left ventricle and impaired coronary hemodynamics. Although exacerbated hypertension may in part explain left ventricular structural and functional abnormalities, it cannot account for similar changes observed in the right ventricle, as this side of the heart is not exposed to increased systemic arterial pressure. Thus, our observations of right ventricular relaxation impairment in SHR but not their normotensive counterparts Wistar-Kyoto rats (WKY) must be accounted for mechanisms other than increased afterload. In keeping with this interpretation, we showed that abnormal relaxation was also associated with an increased fibrosis (increased hydroxyproline concentration and collagen volume fraction) in the right ventricle of the hypertensive strain. Similar to the left ventricle, impaired coronary vasodilatory response to dipyridamole (i.e. minimal coronary vascular resistance) was observed in the right ventricle of SHR, a finding that in part might contribute to deterioration of ventricular function. Salt excess altered neither left nor right ventricular coronary hemodynamics in WKY. Taken together, these effects of salt loading on both ventricles in SHR, but not WKY rats, robustly suggest that non-hemodynamic mechanisms in hypertensive disease participate pathophysiologically with salt-loading hypertension.

Angiotensin II and Aldosterone in Salt-Related Cardiovascular Changes

What mechanisms may be involved in salt-induced cardiac injury and dysfunction? Besides the apparent role of increased arterial pressure, various non-hemodynamic mechanisms may contribute as well[16–18,29,42,43]. Among them, the renin–angiotensin–aldostereone system (RAAS) has been suggested to account for the detrimental effects of dietary salt excess[37,39,40,47,62,69]. This may seem paradoxical since high blood pressure in concert with higher sodium intake should suppress the activity of the RAAS[47,55,68]; however, inadequate suppression of the system may not occur since one study showed an inappropriately high level of plasma aldosterone and left ventricular diastolic filling abnormalities in response to an increase in salt intake of 5 g/day in young hypertensive subjects[51]. This is one of the few clinical studies that clearly related higher circulating RAAS activity for a given salt intake[51,53]. Moreover, in rats fed a high salt diet for four weeks, circulating angiotensin II was not suppressed in spite of decreased plasma renin activity[32].

Thus, we designed a study to examine whether angiotensin II via angiotensin II type 1 (AT_1) receptors participates in salt-induced changes in cardiovascular structure and function in SHR[62]. To this end, we treated salt-loaded SHR concomitantly with the AT_1 receptor antagonist candesartan. In this study we showed that the AT_1 receptor blockade ameliorated salt-induced target organ structural and functional damage in SHR at doses that had no effect on arterial pressure. Furthermore, candesartan prevented the salt-induced deterioration in coronary and renal hemodynamics and in that way reduced cardiac and renal functional impairment. Therefore, this study provides strong support to the concept that angiotensin II contributes to the detrimental cardiovascular effects of salt excess in a blood pressure-independent manner. Importantly, without decreasing blood pressure, the AT_1 receptor antagonism failed to prevent arterial stiffening and hypertrophy as reflected by the increased pulse wave velocity and aortic mass index suggesting that different mechanisms may be involved in mediating the adverse effects of salt excess on conduit vs. resistance vessels.

On the other hand, besides inadequately suppressed or even paradoxically activated circulating RAAS[65], compelling evidence points to the higher tissue RAAS activity in the face of increased dietary salt. Thus, increased angiotensin converting enzyme (ACE) protein and activity[39] as well as increased AT_1 receptor expression were found in heart, aorta, brain, and kidneys of rats fed a high salt diet[46,47,64,70]. In addition, in humans subjected to a high sodium intake increased de novo formation of angiotensin II in cardiac tissue was reported[45]. Dietary salt excess also stimulated aldosterone synthesis in rodent heart[55,56]; in some studies cardiac mineralocorticoid receptors were elevated and aldosterone blockade reversed cardiac hypertrophy and fibrosis in models of salt-sensitive hypertension[5,15,40,49].

Altered Salt Intake and Angiotensin-(1-7) Axis

As discussed above, a large body of evidence has accumulated in recent years underlying the inadequate response of angiotensin II to high salt intake and its contribution to the adverse cardiovascular events of salt excess. Characterization of the role of the heptapetide angiotensin-(1-7), its forming enzyme, a homologue of ACE, ACE2, and receptor, mas, during the last two decades by Ferrario and colleagues[8,19–21,36,50,58,59], suggests their possible involvement in explaining the unopposed effects of angiotensin II in cardiac response to excess salt intake. Angiotensin-(1-7) counterbalances the action of angiotensin II in many tissues[63] and we have suggested that hypertensive processes may in part depend upon suppression of the Ang-(1-7)/ACE2/mas receptor axis[34,36]. Genetic ablation of ACE2 or the mas receptor results in severe cardiac dysfunction and exaggerated hypertensive response to angiotensin II infusion[6,8,30,48]. Inactivation of the ACE2 gene also led to accumulation of angiotensin II in the heart[8,67], underlying the preferential role of ACE2 in metabolizing angiotensin II into angiotensin-(1-7). Both ACE2 overexpression[9,33] and supplemental angiotensin-(1-7) infusion[28] ameliorated cardiac remodeling due to myocardial infarction or angiotensin II excess. In addition, Trask et al.[60] recently demonstrated an exclusive dependence of angiotensin-(1-7) production on ACE2 in the hypertensive heart of transgenic mRen2 rats.

Considering the opposing actions of angiotensin II and angiotensin-(1-7) on cardiac hypertrophy and fibrosis, one can hypothesize that salt-induced cardiac remodeling may be, at least in part, the result of not only the undesirable action of Ang II but also a consequence of the diminished protective effects of the ACE2/angiotensin-(1-7)/mas-R axis (Fig. 16.1). Indeed, high dietary salt intake increased cardiac angiotensinogen and reduced cardiac ACE2 mRNA in the hypertrophied and fibrotic heart of Dahl salt-sensitive rats[57]. AT_1, but not mineralocorticoid receptor

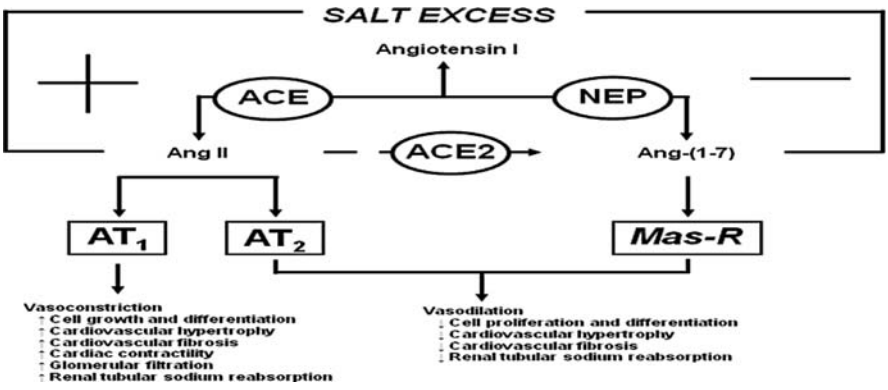

Fig. 16.1 Salt excess may induce cardiac remodeling and dysfunction by both facilitating the cardiac action of angiotensin II and reducing the counterbalancing angiotensin-(1-7) activity. ACE = angiotensin converting enzyme; NEP = neprilysin; Ang = angiotensin; AT_1 = angiotensin II type 1 receptor; AT_2 = angiotensin II type 2 receptor; Mas-R = Mas receptor

blockade, reversed the effects of salt excess on ACE2 gene expression ameliorating the salt-induced cardiac remodeling. This is in agreement with data from our laboratory showing that angiotensin II via AT_1 receptor negatively regulates ACE2 mRNA and activity both in vitro and in vivo[19,20,25,26,34,36]. Thus, in addition to reduced angiotensinogen and ACE mRNA in the heart of Dahl salt-sensitive rats, increased ACE2 gene expression after AT_1 receptor blockade may have resulted in accelerated angiotensin II conversion into angiotensin-(1-7) that in turn may contribute to the protective effects of an angiotensin II blockade in a salt-sensitive hypertensive heart. Therefore, it seems that high dietary sodium intake may facilitate the cardiac actions of angiotensin II by reducing the counterbalancing angiotensin-(1-7) activity. Our most recent studies found a reduced cardiac content of angiotensin-(1-7) in salt-loaded SHR underlying its critical role in the development of salt-induced cardiac remodeling (unpublished data).

On the other hand, low sodium intake did not affect ACE2 but reduced ACE mRNA and activity in normotensive Wistar rats[31]. Furthermore, the addition of a low-sodium diet to lisinopril did not affect the reduced renal ACE activity but the combined treatment was coupled with a profound increase in the angiotensin-(1-7)/angiotensin II ratio in plasma. The data are in agreement with clinical studies showing that beneficial effects of a RAS blockade may be more pronounced if followed by dietary sodium restriction[38]. The data also reflect the importance of ACE, not only in angiotensin II synthesis but also in angiotensin-(1-7) metabolism. Breaking of the Ile^5-His^6 bond by ACE results in the formation of inactive metabolite angiotensin-(1-5)[3,7] and ACE inhibitors increase the half-life of angiotensin-(1-7) in the circulation[66]. Furthermore, in salt-sensitive hypertensive patients, omapatrilat, a dual ACE and NEP inhibitor, increased urinary excretion of angiotensin-(1-7)[21] pointing that angiotensin-(1-7) of renal origin may contribute to the hypotensive effect of omapatrilat in the patients whose blood pressure is sensitive to sodium intake.

It is also necessary to pose a question as to the role of angiotensin-(1-7) activity in the development of salt-sensitive hypertension. In this context, Ferrario et al. were the first to report the counterbalancing hemodynamic action of angiotensin-(1-7) in rats with activated endogenous angiotensin II due to the chronic low sodium intake[35]. This tonic depressor action of angiotensin-(1-7) opposing increasing angiotensin II concentration was not a result from increased peptide formation since the plasma and renal heptapeptide levels did not change after salt depletion; an enhanced vascular sensitivity to angiotensin-(1-7) was proposed. Interestingly, acute vasodilation by angiotensin-(1-7) was augmented in rats fed a high sodium versus low sodium diet due to an increase in vasodilatory and a decrease in vasoconstrictor prostanoids[4]. It is intriguing to speculate that altered sodium status changes the vascular angiotensin-(1-7) receptor and/or angiotensin-(1-7) receptor-signaling interaction even more in response to dietary salt excess to oppose enhanced tissue RAS. Importantly, chronic angiotensin-(1-7) supplementation in Dahl salt-sensitive rats fed a high salt diet reduced the increase in blood pressure and improved aortic and renal blood flow[13]. Altogether, it seems that angiotensin-(1-7) posits as a

critically important factor in the development of salt-sensitive hypertension and target organ damage.

Conclusion

For many years, high dietary salt intake has been linked only to an increase in blood pressure. However, a large body of evidence now testifies to the potential of salt excess to adversely affect cardiac structure and function, as well. The important role of RAAS in mediating these effects has been increasingly appreciated in recent years. Moreover, identification of angiotensin-(1-7), its receptor mas, and enzymes involved in its metabolism challenges our concept that only increased angiotensin II activity is responsible for cardiac injury in salt-sensitive hypertension. Additional studies are warranted to further investigate the decisive role of angiotensin-(1-7) in the development of salt-sensitive hypertension and target organ damage.

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Chapter 17
Cardiac Effects of Aldosterone, the Bad, but Is There Also a Good?

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Abstract Several trials have demonstrated the important benefit of aldosterone, aldo, antagonists in patients with heart failure, HF, and the commonly accepted conclusion is that it is important to block aldo in every disease conditions. High doses of aldo generate myocardial fibrosis in the rat through pericoronary inflammatory reaction and necrosis. Low doses of aldo can be targeted in the myocardium by using a transgenic model of mice overexpressing aldo synthase, TGAS, in the cardiac tissue. In this model the normal cardiac content in aldo is enhanced by a factor of 1.7. TGAS mice have a normal cardiac function and no significant fibrosis.

A gender-specific coronary dysfunction was observed in males. The vasorelaxing response under acetylcholine + LNNA, a NOS inhibitor, was indeed reduced. The expression of BKCa-alpha and BKCa-beta1 subunits, which constitute the main channel responsible for relaxation in isolated coronary arteries, was reduced in TGAS. Iberiotoxin inhibits BKCa and induces a marked diminution in the coronary relaxation in both groups of coronary arteries. In contrast, the coronary function in female is normal. The reason for this is that estrogens themselves activate BKCa expression and by so doing compensate for the effects of aldo.

While aldo blockade was beneficial in diabetic patients with heart failure it was suggested that in diabetic patients without HF, spironolactone may not be so beneficial and, in particular, was unable to restore a normal endothelial function. Based on this observation, we generate TGAS and wild-type mice and treat them with streptozotocin to induce diabetes. In wild-type diabetic animals the fraction of shortening, capillary density, and VEGFa expression were diminished. Surprisingly, these parameters were normal in diabetic TGAS. Aldosterone blockade abolishes the beneficial effects of the TG induction of aldo.

Then, it was concluded that aldo has both detrimental and beneficial effects on the heart. In mice, aldo is cardioprotective in the presence of diabetes.

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Introduction

Both RALES 1 and EPHESUS 2 clinical studies[13,14] have demonstrated the important benefit of MR antagonists in patients with heart failure or left ventricular dysfunction after myocardial infarction, and the commonly accepted conclusion is that aldo plays a generally evil role and that it is important to block it in every disease conditions. The biological reality is not so simple, and to better understand its mechanisms and better anticipate the effects of treatment, several points should be highlighted. Aldosterone regulates electrolyte and fluid balance through translation of specific proteins into the adrenal gland and subsequent blood pressure homeostasis. However, the hormone has also unexpected rapid non-genomic effects, a wide-range of actions at many levels, including at that of non-epithelial tissues, and, finally, can be synthesized in a number of extra-adrenal tissues[5,17]. Probably, one of the main sources of such a pleiotropic activity can be found in the story of the ligand-receptor functional duo which did not evolve in parallel[3].

Aldosterone receptors are nuclear receptors and transcriptional factors as opposed to epinephrine or Angio II receptors which are transmembrane receptors regulating gene expression indirectly through a secondary messenger (Camp. . .). In addition, aldo receptors are unique examples during evolution of receptors, appearing nearly 40 million years before their ligand. The specific functional interaction between aldo and its partner, the mineralocorticoid receptor, is one of the first demonstrations that the assembly of complex systems evolved by a stepwise Darwinian process. Using gene resurrection it was indeed shown that, long before the hormone appeared, the receptor's affinity for aldo was present as a structural byproduct of its partnership with chemically similar but more ancient ligands. The vertebrate ancestor (~470 Myr) did not synthesize aldo, while it produces several corticosteroids and has a single receptor with affinity for both classes of ligands. A gene duplication of the receptor ancestor had occurred at ~450 Myr, which resulted into two separate receptors, the glucocorticoid receptor and the mineralocorticoid-receptor-to-be. The synthesis of aldo emerged 30–40 Myr later due to a modification of cytochrome P-450 1-betahydroxylase, and aldo, which is tetrapod-specific, is absent from teleosts and more ancient families[3,21].

The Bad

Aldosterone-Induced Fibrosis

Both clinical and experimental studies have convincingly shown a deleterious effect of aldosterone on both the myocardium and arterial wall. Christian Brilla and Karl Weber were the first to demonstrate, using an experimental aldo-salt model of hypertension, that aldo was able to induce myocardial fibrosis in both the left overloaded and the right non-overloaded ventricles[4]. This was further confirmed in our group[16] and in many other laboratories. Nevertheless, the experimental setting that showed the fibrogenic effects of aldo utilized very high hormonal concentrations

and an enormous dietary salt supplement. On another hand, clinical trials also provided direct arguments in favour of a cardiotoxicity of the hormone. F. Zannad et al.[24], for example, in a sub-trial analysis of RALES, convincingly showed that antialdosterone therapy reduces plasma markers of myocardial fibrosis (PIIP). In RALES, the pronounced protection against sudden death was often attributed to protection against a fibrosis-induced re-entry phenomena and severe arrhythmias. Nevertheless, the mechanisms of aldosterone-induced myocardial fibrosis in clinical conditions still remain unclear and it was suggested that in clinical conditions the augmentation of the plasma levels of aldo was probably too low to account for the fibrogenic process.

As initially shown in our group the myocardium itself is an endocrine which is capable of synthesizing the hormone[17]. The tissue aldo concentrations which have been measured in normal rat heart were approximately 15 times higher than the plasma concentration of the hormone and rose up to 50 times after a myocardial infarction[17]. Experimental settings have confirmed clinical and pharmacological data. In humans, the activation of several steroidogenic genes including the aldosterone-synthase has been detected in the failing heart, and the myocardial aldo production, as measured by the arteriovenous differences in the hormone, was significantly enhanced in the failing heart[10,22,23].

A pericoronary inflammatory reaction with macrophages, inflammatory markers, and necrosis is a very early step of cardiac damage in the aldosterone-salt model[19], and it had been suggested that at least in this model the coronary arteries could be a target for aldo in the heart and that vascular damages were likely to be one of the primary events in aldosterone-induced fibrogenesis. Then, it became more or less evident that the vessels are the main target of the aldo-induced myocardial damages[19].

Gender-Specific Coronary Dysfunction

To better define the specific cardiac effects of aldosterone and a possible compartmentalization of the hormone, a mouse model has been generated in which a genetic construct including the aldosterone-synthase gene was specifically targeted to cardiomyocytes using the myosin alpha heavy chain promoter[7]. In these transgenic mice, the aldo concentration was specifically, while moderately ($\times$ 1.7), enhanced within the heart, while the plasma level of aldo remained unchanged. In this model, contractile function remains normal, just like the electrical currents, and there were no more fibrosis.

In Males

The major findings that we observed were a coronary vascular dysfunction, which was, to our great surprise, gender-specific and only present in male transgenic mice. In males, the deficit in vascular function was not located, as usual, at the level of the endothelial cells, but at that of the vascular smooth muscle cell, VSMC, and

the impaired response to acetylcholine still persisted after the addition of a NOS inhibitor, LNNA (10^{-4} M)[1]. For example, the coronary relaxation decreased from 74 ± 6% in the wild-type to 53 ± 6% in the transgenic mice ($p < 0.01$) at 10^{-6} M acetylcholine. This coronary dysfunction was prevented by a three-week treatment with spironolactone. The relaxing responses to sodium nitroprusside were similar in the two groups of animals.

The altered acetylcholine-induced relaxation was unaffected by a COX inhibitor diclofenac and unrelated to prostaglandins. Nevertheless, the KCa blockers, apamin and charybdotoxin (in the presence of the NOS and COX inhibitors) almost completely abolished the relaxing responses in both experimental groups suggesting that such a deficit was almost entirely mediated by the KCa channels opening.

The coronary relaxation was assessed in the presence of the inhibitor of BKCa channels, iberiotoxin, a well-documented BKCa channel inhibitor. Iberiotoxin induced a marked diminution in the coronary relaxation in both groups of coronary arteries. Remarkably, the inhibition, which was induced by iberiotoxin, was much smaller in the transgenic animals than in the wild-types, and finally the curves obtained in the presence of the inhibitor were the same in the two groups. BKCa-beta1 are specifically expressed in the VSMC, the beta 1 isoform is smooth muscle cell specific ([8,12], our data). It is noteworthy that since BKCa-beta1 is expressed at a very low level, especially in MAS mice, immunolabeling failed to evidence quantitative changes. The expression of BKCa-alpha and BKCa-beta1 subunits, as measured by RT-qPCR in isolated coronary arteries, was reduced by 80% ($p < 0.01$) and 60% ($p < 0.05$) respectively in the transgenic animals. Identical results were observed at the protein level by Western blot analysis both on membrane-enriched fractions and total protein extracts. These results and the role of aldo were further confirmed on cultured rat aortic smooth muscle cells. Aldosterone indeed induced a major decrease in both BKCa-alpha and BKCa-beta1 mRNA in a concentration-dependent manner, and pre-treatment with spironolactone prevents this inhibition.

In Females

In the same conditions in female transgenic mice, the coronary relaxation in the presence of LNNA remains normal (unpublished data from our group). This was accompanied by a striking increased expression of BKCa-beta1 subunit, both at the transcript and protein levels. The beta1 subunit of the BKCa is in fact also a well-documented target of 17 beta-estradiol in arteries and estradiol induced a pronounced elevation of the level of both the messenger and the protein[11]. This effect was also observed in our hands. In isolated SMC, estrogen activates BKCa expression and this effect counteracts the inhibitory effects of aldosterone.

The Good

The EPHESUS study enrolled patients after an acute myocardial infarction and a subsequent heart failure, except if the patient were diabetic in which case only

systolic left ventricle dysfunction was required. All-cause mortality was reduced by 15% in every patient with heart failure treated with eplerenone including the patients with diabetes[13]. However, while aldo blockade was beneficial in diabetic patients with HF, it was shown that in diabetic patients without HF spironolactone had detrimental effects and in particular was unable to restore a normal endothelial function[6,20].

These data suggest that aldo may play a specific role in diabetic patients. To induce diabetes, transgenic male mice overexpressing aldosterone-synthase and their wild-type littermates were IP injected with streptozotocin (40 mg/kg/day) and streptozotocin-treated mice with blood glucose concentration >15 mmol/L were considered diabetic. After 8 weeks of diabetes the fraction of shortening, capillary density, and VEGFa expression were all significantly diminished in wild-type diabetic animals. Nevertheless, and surprisingly, these three parameters together with several biomarkers of the oxidative stress were normal in the transgenic diabetic mice. Aldosterone blockade using eplerenone totally abolishes the beneficial effects of the transgenic induction of aldo into the myocardium. Eplerenone has no effect in the wild-type animals. Then, it was concluded that aldo has a receptor-dependent cardioprotective effect in the presence of diabetes.

Diabetes is associated with three different cardiovascular abnormalities: atherosclerosis, which accounts for more than 50% of cardiovascular complications linked to diabetes, diabetic cardiomyopathy, and a pronounced microangiopathy. These three abnormalities were usually associated, which explains the well-documented severity of heart failure in diabetes. In our experimental settings the first two cardiovascular abnormalities were unlikely to be involved: (i) mice is an animal species known to be free from atherosclerosis under physiological conditions; (ii) diabetic cardiomyopathy is a multifactorial clinical entity characterized by fibrosis with diastolic dysfunction and the absence of coronary atherosclerosis, hypertension, and microangiopathy[2,15]. The last explanation which involves microvasculature is probably the best. Aldosterone indeed increases neovascularization in an in vivo model of ischemia secondary to right femoral artery ligature in mice[9], and we could speculate that the beneficial effect of aldo in diabetes may be related to protection against microangiopathy. As already suggested[20] the prescription of spironolactone in diabetes should be reevaluated.

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