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Cellular and Molecular Toxicology and In Vitro Toxicology

Edited by
Daniel Acosta, Jr.

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Cellular and Molecular Toxicology and *In Vitro* Toxicology

Editor

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PREFACE

This new volume on *Cellular and Molecular Toxicology and* In Vitro*Toxicology* reflects the importance of understanding the toxicity of xenobiotics at levels beyond the whole animal. An objective of this book is to provide a better understanding of the mechanisms by which xenobiotics are toxic to mammalian tissues and cells. The mechanism(s) by which toxic compounds injure or disrupt normal functioning of the whole organism is more clearly delineated by correlating changes at the whole animal level with discrete changes at the cellular and subcellular levels. The other aspect of the volume, as reflected in the title, concerns the use of *in vitro* systems as experimental tools to provide valuable insight into the biochemical mechanisms that underlie the toxicity of a xenobiotic. Thus, the purpose of the book is to focus on the cellular and molecular effects of toxicants on living systems and to highlight the use of *in vitro* experimental systems to assess toxic manifestations of xenobiotics.

This book concentrates on the cellular and molecular toxicity of selected well-known drugs or chemicals on the cardiovascular system. The chapters will highlight the most recent findings on the cellular and molecular mechanisms of toxicity of several important cardiotoxic agents: doxorubicin, ethanol, cocaine, and the catecholamines. In addition, an overview of vascular toxins and their biochemical effects and a summary on *in vitro* cardiovascular techniques for assessing toxicity of xenobiotics will be presented. We look forward to comments on this first volume, as well as to suggestions on topics for future volumes.

Daniel Acosta, Jr.

EDITOR

Daniel Acosta, Jr. was born in El Paso, Texas, on March 25, 1945. He attended the University of Texas at El Paso for his pre-pharmacy training from 1963 to 1965. He then transfered to the University of Texas at Austin College of Pharmacy in 1965 and received a B.S. in Pharmacy in 1968. He graduated first in his class with Highest Honors. In 1970, he was awarded a National Science Foundation Traineeship for his graduate work at the University of Kansas School of Pharmacy in the Department of Pharmacology and Toxicology. He received a Ph.D. in Pharmacology and Toxicology in 1974 under the supervision of Dr. Duane G. Wenzel.

In the fall of 1974 Dr. Acosta joined the faculty of the University of Texas College of Pharmacy as an assistant professor of pharmacology and toxicology. Before he was promoted to associate professor in 1979, he received a Ford Foundation Postdoctoral Fellowship from the National Chicano Council on Higher Education for the year 1978–1979. After his promotion to associate professor, he continued to develop his teaching and research program. His current research interests include cellular toxicology, cellular models of target organ toxicity, and cellular models of drug metabolism and toxicity. This research has been supported by grants from the National Heart, Lung, and Blood Institute, National Institute of Environmental Health Sciences, Environmental Protection Agency, American Heart Association, Pharmaceutical Manufacturers Association, Johns Hopkins Center for Alternatives to Animal Testing, Johnson & Johnson, and several pharmaceutical companies. Dr. Acosta has directed the research of 20 graduate and postdoctoral students. He has over 80 publications in such journals as *Toxicology and Applied Pharmacology, Biochemical Pharmacology, In Vitro,* and *Toxicology.*

In 1983 he was appointed to full professor and was the initial holder of the Eli Lilly and C.R. Sublett Centennial Fellowship and Alcon Professorship in Pharmacy. He is presently the initial holder of the Johnson & Johnson Centennial Professorship in Pharmacy. At the national level, he is a member of the Tissue Culture Association, Society of Toxicology, American Society for Pharmacology and Experimental Therapeutics, American Heart Association, Basic Sciences Council, Phi Kappa Phi, Rho Chi, and Phi Delta Chi. In addition to membership in these organizations, he has served as a consultant to the Minority Biomedical Support Program of the National Institutes of Health; member of the NIDA's Drug Abuse Biomedical Research Review Committee (Pharmacology II Subcommittee); member of the Board of Directors of the American Heart Association, Texas Affiliate; member of the Central Research Review Committee of the American Heart Association, Texas Affiliate; member of the National Research Council's Panel on Biomedical Sciences for the National Science Foundation Graduate Fellowship Program; member of the editorial boards of *In Vitro, Fundamental and Applied Toxicology, In Vitro Toxicology,* and *Toxicology In Vitro;* Formal Liaison between the Society of Toxicology and the Tissue

Culture Association; member of the Program Committee of the Society of Toxicology; chairman, Membership Committee of the Society of Toxicology; and President, Gulf Coast Regional Chapter of the Society of Toxicology. Most recently he was selected as the 1986 recipient of the Burroughs Wellcome Toxicology Scholar Program, a five-year program to develop a toxicology program at the University of Texas.

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Chapter 1

THE VASCULAR TOXICITY OF XENOBIOTICS

Paul J. Boor

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I. INTRODUCTION

Cardiac toxins have been studied for many years, and much is known about how chemicals injure the heart. This is not the case with vascular toxins, however. Perhaps this lack of investigative work in vascular toxicology is due to the diffuse anatomic and structural nature of the vasculature, which consists of many different types and sizes of vessel from large elastic arteries such as aorta to the smallest capillary network. Also, the endpoints of toxic damage to the heart (i.e., arrhythmia or myocyte necrosis) may be more dramatic and more easily measured than the more subtle alterations in blood vessels. Whatever the reason, relatively few specific vascular toxins are known and only recently has *in vivo* and *in vitro* experimental work begun to examine toxin-induced vascular lesions and the potential for vascular metabolism of xenobiotics.

This relative lack of interest in vascular toxic phenomena is surprising in view of the fact that the most significant modern disease of the Western population — atherosclerotic vascular disease — may well be induced by underlying toxic mechanisms. One of the earliest proposals to address the concept of environmentally induced vascular injury was the Benditts' theory of the "monoclonal origin of atherosclerotic plaques".[1,3] A basic tenet of this theory is that environmental toxins are capable of inducing vascular smooth muscle cell proliferation, resulting in the atherosclerotic plaque. Many environmental toxins have been associated with human coronary vascular disease, including carbon disulfide[4,5] and carbon monoxide (CO).[6,7] However, the most obvious and prominent adverse environmental factor causing cardiovascular disease in this country is cigarette smoking. Although the exact nature of the many toxins released by burning tobacco is not completely known, cigarette smoking clearly takes a massive toll in human morbidity and mortality,[8] largely due to well-demonstrated adverse effects on blood vessels.[9]

The first goal of this chapter is to review briefly the interesting epidemiologic and basic scientific work which suggests the involvement of toxic mechanisms in human atherosclerosis. A complete review of the many experimental models which attempt to mimic the atherosclerotic lesion, or of present theories of the origin of these lesions, is beyond the scope of this short work. Instead, the second goal is to focus in more detail on several xenobiotic compounds which have clear-cut toxic effects on relatively specific structural components of blood vessels.

II. ATHEROSCLEROSIS

Atherosclerosis is a degenerative, generalized disease of large- to middle-sized arteries which is characterized by intimal proliferative lesions and lipid storage within arteries resulting in obliteration of the vascular lumen and destruction of vascular wall. This disease, which is extremely common in modernized or Western societies, takes a massive toll, causing severe human

suffering and death related to heart attack, stroke, kidney failure, and other vascular complications such as peripheral vascular disease.

Cigarette smoking has been firmly established as a risk factor for the development of atherosclerotic disease, including most of the manifestations mentioned above.[10,11] The mechanisms involved in this increased susceptibility among smokers may include alterations in blood lipid dynamics, blood pressure, or the clotting system with resulting changes in hemostasis. The specific toxins in cigarette smoke which are responsible for its deleterious effects on larger blood vessels are largely unknown. Much speculation and a small amount of experimental work have suggested that CO, which combines with hemoglobin in high concentration in the blood of smokers, may have a direct effect on the heart[12] and an atherogenic effect on blood vessels.[6,7,13] Carbon disulfide, a common air pollutant in industrialized areas, has also been implicated in causing atherosclerotic cardiovascular disease.[4,5]

An interesting protective effect against atherosclerosis has been hypothesized by epidemiologists to be related to the degree of water hardness (or content of calcium carbonate) in differing areas in the U.S.[14] A few epidemiologic studies have suggested that it is the specific lithium content of water that affords protection against atherosclerotic disease.[15,16] A plethora of other trace elements have been suggested and studied for potential effects on the incidence of atherosclerotic vascular disease.[17,18] The contributory effects of hardness or softness of water supply, or of trace elements, to arterial injury are still under great debate, however, and no conclusive mechanisms through which such factors could exert an influence on blood vessels have been shown.

III. MEDIAL TOXINS

Atherosclerosis has been considered to be a process affecting the intima, endothelium, or subendothelium of larger blood vessels. A great deal of evidence exists, however, that the major proliferating cell in the atherosclerotic plaque is the smooth muscle cell, presumably derived from the vascular media.[19,20] Degenerative diseases of the media which are distinct from the atherosclerotic process occur in man and animals;[21] however, such medial degeneration results in vascular tears, aneurysm, and rupture rather than intimal lesions. It has been known for many years that several xenobiotic toxins, when administered to immature experimental animals, can induce these same medial degenerative changes known as angiolathyrism. Two unsaturated aliphatic amines (β-aminopropionitrile and allylamine) which act as potent medial or vascular smooth muscle toxins are the focus of this discussion.

A. β-Aminopropionitrile

In the 1950s, experimental work with the toxic sweetpea, *Lathyrus odoratus,* reproduced vascular medial injury consisting of smooth muscle cell necrosis, vascular dissection, aneurysm, and rupture in several animal species, especially

young weanling rats and turkeys. After the toxic substance of the sweetpea was identified as β-aminopropionitrile the extraordinary angiotoxicity of this chemical was extensively studied.[22] Although the underlying mechanism through which β-aminopropionitrile causes medial injury is still not completely understood, much valuable inferential information has been gained about its effects on the metabolism of the vascular wall. In addition to vascular effects, this compound has pronounced systemic effects on bone and connective tissue metabolism. Although several other classes of compounds, including hydrazine derivatives and hydrazides,[23] have been found to have similar lathyrogenic effects, the vascular toxicity of β-aminopropionitrile is best understood.

The unique vascular properties of great tensile strength and elasticity present in large vessels such as aorta (as well as medium-sized arteries) are largely due to abundant collagen and elastin in the vascular wall architecture. Each of these two structural proteins are extensively cross-linked by covalent bonds. Figure 1 illustrates normal vascular structure. In 1968, Pinnell and Martin[24] demonstrated that cross-linking of both proteins is initiated by oxidation of peptidyllysine residues to peptidyl-α-aminoadipic δ-semialdehyde, or allysine, which serves as a reactive precursor for the spontaneous production of cross-linkage between allysyl residues with other lysyl or hydroxylysyl residues in the molecule. In addition, the elastin molecule undergoes more complex reactions resulting in formation of the desmosines.[25] The enzyme which catalyzes the formation of allysine from the lysyl residues is lysyl oxidase, an enzyme found in virtually all types of connective tissues as well as vascular tissue.[26] β-Aminopropionitrile is a strong and relatively specific inhibitor of this enzyme, and this action is thought to be the molecular basis of the vascular toxicity of the compound.

Vascular lysyl oxidase has been extensively purified and characterized from bovine aorta;[27] the enzyme exists as four apparently distinct monomeric species of approximately equal size and molecular weight of 32,000 to 33,000. These four species may form the subunits of potentially differing functional polymers under physiologic conditions, although this possible role of the monomers is, at present, conjectural.[27] The enzyme presumably functions extracellularly in the cross-linking of collagen and elastin, and evidence exists that this process occurs early in the formation of extracellular collagen fibrils.[28,29] In aorta, the enzyme is localized at the interface of elastin and the surrounding microfibrils, indicating that lysyl oxidase is intimately involved in elastogenesis through cross-linking of tropoelastin.[30]

As early as 1966, the vascular toxic effect of β-aminopropionitrile, which was known to cause lathyrism in turkey chicks, was hypothesized to be due to inhibition of the cross-linkage in elastin.[31] Subsequent to the description of lysyl oxidase as a key enzyme in the cross-linking process, the inhibitory effect of ß-aminopropionitrile on lysyl oxidase was demonstrated,[26] and this enzymic interaction was proposed as the toxic mechanism of β-aminopropionitrile. Since this enzyme is copper-dependent, lathyrism induced by a copper-deficient diet has also been linked to lysyl oxide inhibition.[32,33]

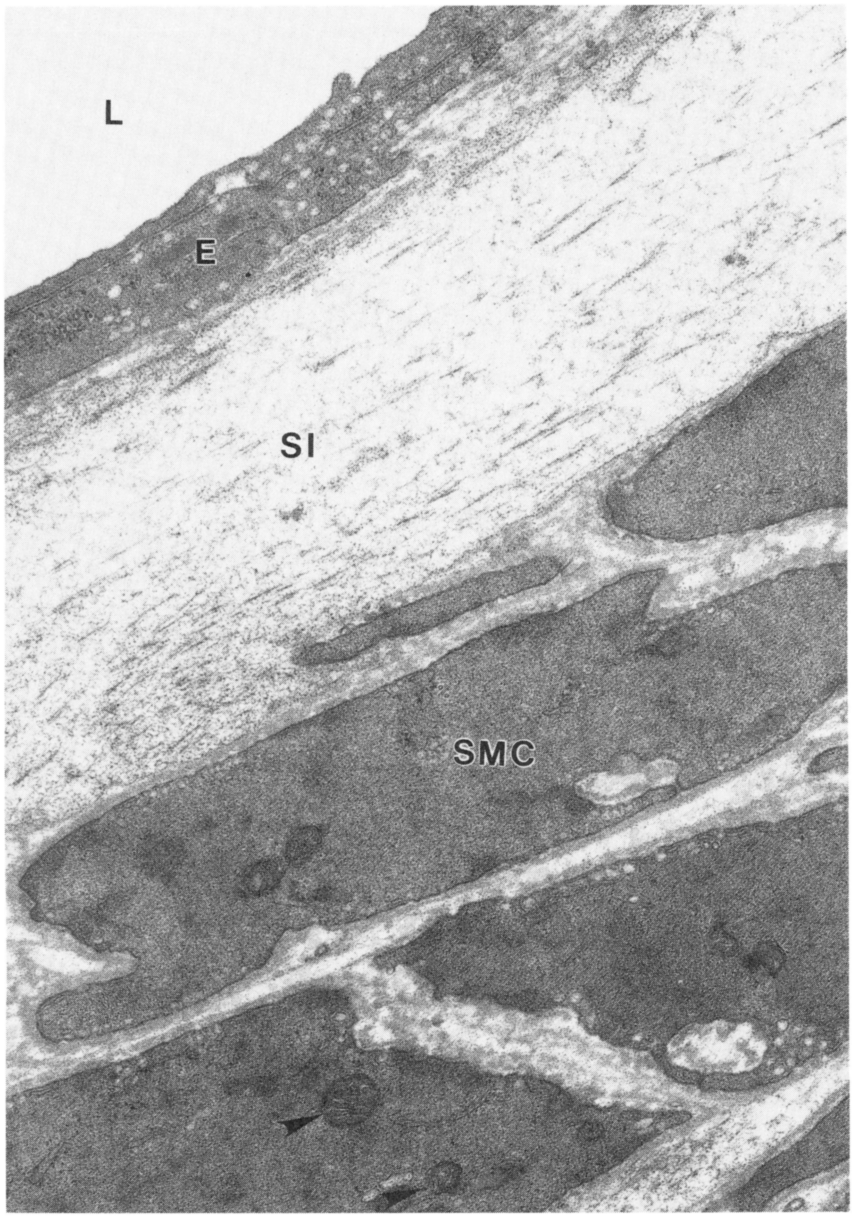

FIGURE 1. An electronmicrograph of rat coronary artery shows normal vascular structure. The lumen (L) is lined by the intima which consists of one or two layers of endothelial cells (E) with characteristic vesicles. Fibrillar extracellular material comprises the subintima (Si) and the media consists of vascular smooth muscle cells (SMC) with characteristic peripheral dense bodies, few mitochondria (arrowheads), and surrounding basement membrane material. The elastin and collagen interstitial matrix is not well seen in this micrograph. (Uranyl acetate and lead citrate; magnification × 98,000.)

Hence, β-aminopropionitrile is an example of one of the most potent angiotoxic substances, with an extraordinary toxic effect on the media of blood vessels of immature individuals of several species including turkeys, rats, mice, swine, and monkeys.[34] Its toxic mechanism, as outlined above, is believed to be linked to inhibition of the connective tissue enzyme, lysyl oxide, and to collagen and elastin metabolism. All details of this mechanism are not known, however. For example, although lysyl oxidase is believed to be of vascular smooth muscle cell origin because these cells express enzyme activity when grown in culture,[35] whether enzyme inactivation by β-aminopropionitrile occurs at the cellular level or extracellularly is not known. Indeed, the mechanisms by which chronic feeding of β-aminopropionitrile causes smooth muscle degeneration within the vascular media[36] and the reasons for the subsequent bizarre cartilaginous metaplasia[37] within vessels are not known.

Furthermore, although aberrations in lysyl oxidase activity have been linked to several human connective tissue disorders, including the Ehlers-Danlos,[38] Marfan's, an Menke's Syndromes,[39] as well as osteogenesis imperfecta, a clear-cut cause-and- effect relationship has not been proven, and many of the derangements seen in collagen metabolism in these disorders may be unrelated to lysyl oxidase alterations.[40]

It is interesting to note, however, that several therapeutic modalities used in human vascular diseases today were stimulated by early studies in β-aminopropionitrile-fed animals. Because these experimental lesions so closely resemble dissecting aneurysm in man, early investigators tested a variety of compounds as possible potentiators or inhibitors of the vascular medial damage induced by β-aminopropionitrile.[41-43] From such studies, two drugs — reserpine and propranolol — were identified as having beneficial effects in the treatment of spontaneous human aortic aneurysms. Thus, through experimental investigations of this medial toxin, therapeutic drugs have been discovered.

B. Allylamine

Allylamine, or 3-aminopropene, is another aliphatic amine which causes experimental vascular lesions as well as myocardial lesions which morphologically mimic myocardial ischemic disease in humans (see Figure 2). The chemical usage and general toxicity of this xenobiotic have been recently reviewed;[44] this discussion will address specifically the vascular toxicity and smooth muscle effects of this xenobiotic. The first study to focus on the vascular toxicity of allylamine was that of Mellon et al.[45] That 1935 study described a localized arteritis following intradermal injection of allylamine; the lesions were noted to occur at very low concentrations and at neutral pH. These authors illustrated a severe necrotizing vasculitis which they thought appeared similar to human polyarteritis nodosa. Interestingly, they compared these vascular lesions to those caused by histamine, which bears some chemical similarity to allylamine.

Vascular lesions have been induced in a variety of experimental animals by allylamine. The first extensive set of experiments to produce such lesions was

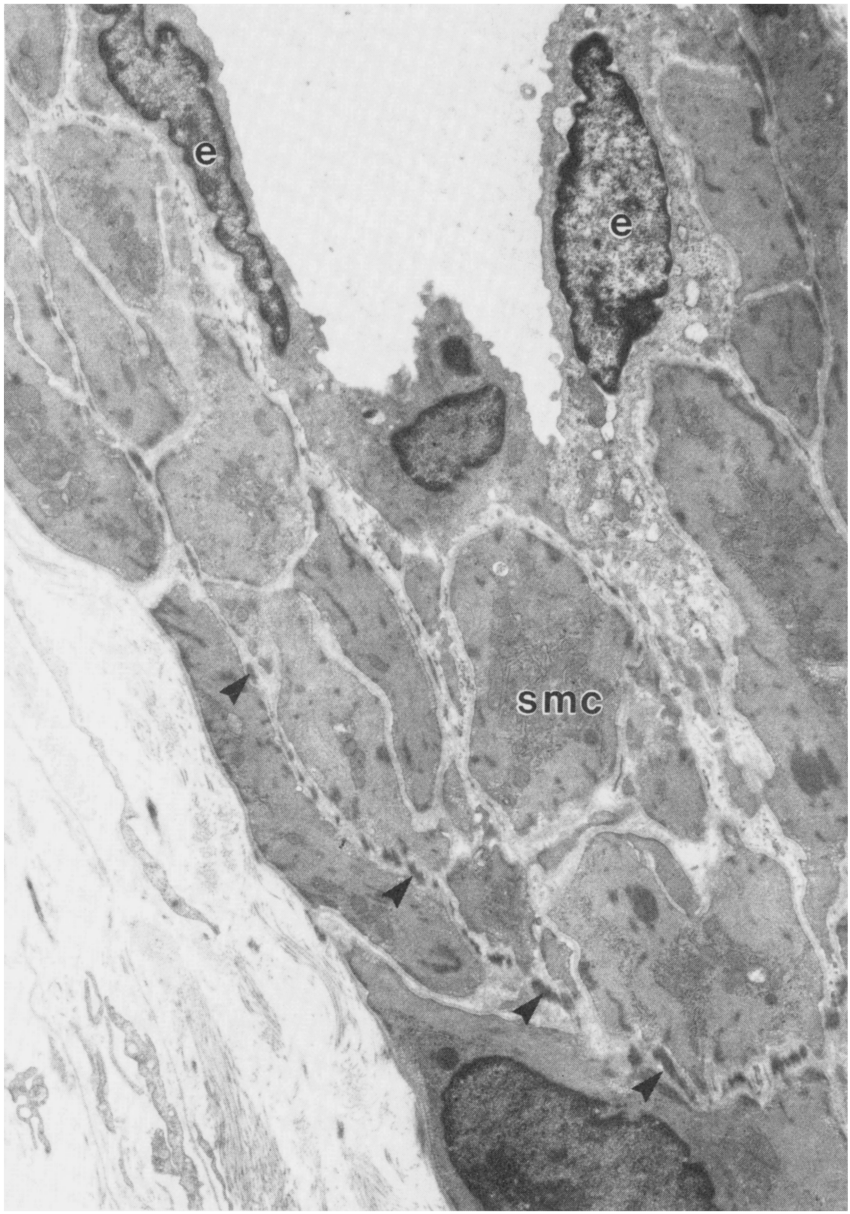

FIGURE 2. Electronmicrograph of an in vivo smooth muscle proliferative lesion induced by the xenobiotic allylamine. The lesion is in a smaller intramyocardial vessel; it consists of typical vascular smooth muscle cells (smc) proliferating in the subintimal space between the internal elastic lamina (arrowheads) and the endothelial cells (e). This lesion is similar to the hypothetical early atherosclerotic lesion of man. Rat given allylamine drinking water protocol[58,60] for 3 months. (Lead citrate and uranyl acetate; magnification × 100,000.) (From Boor, P. J. and Hysmith, R. M., *Toxicology*, 44, 129, 1987. With permission.)

that of Waters, [46-50] who administered allylamine to dogs primarily by i.v. injection. Waters and co-workers were studying arterial injury and the evolution of lesions which closely mimic the atherosclerotic process in man. They described medial edema and necrosis of coronary arteries following multiple injections of allylamine. Although they described the uncomplicated lesions as a "scarring" of the arterial wall, they induced secondary lipid storage in the lesions by a variety of manipulations — including cholesterol and lipid feeding[50] and i.v. injections of lipid[48] and lipid-rich human plasma components.[49] Thus, Waters and co-workers pioneered in the field of vascular injury and atherogenesis, utilizing allylamine to cause the initial vascular injury in their experimental model.

Since the early work of Waters, many investigators have similarly employed allylamine-induced vascular lesions in diverse studies of the vasculature. Donomae et al.[51] studied coronary vessels and myocardium in rabbits fed lanolin and given other manipulations — including i.v. allylamine. Their studies were the first to emphasize the extensive damage to the myocardium following allylamine administration. Bloor and Lowman[52] performed a histologic angiographic study of allylamine-induced lesions in the dog; their main interest was in coronary angiographic techniques which, at that time, were in a developmental stage. It is of interest that these authors noted the most extensive vascular lesions in medium-sized and small muscular arteries, rather than in the larger extramural vessels; this observation has subsequently been made by others in other species.[53,54] Bloor and Lowman also demonstrated the characteristic intimal proliferation lesions of these smaller vessels.

Another radiologic study which utilized allylamine is that of Lowman et al.,[55] in which allylamine was given by i.a. injections specifically to induce renal artery stenosis and secondary hypertension. Lowman et al. subsequently utilized allylamine to produce intracranial vascular lesions which could be visualized by angiographic techniques.[56]

The metabolism of allylamine has received extensive experimental attention during the past few years, and has been a major focus of our own laboratory. It appears that the vascular — as well as the cardiac — toxicity of allylamine is closely tied to its metabolism to highly reactive metabolites by specific vascular metabolic systems. Perhaps the first important data concerning the mechanism of allylamine vasculotoxicity were afforded by Lalich and Paik,[57] who in 1974 discovered that the hydrazine derivative, hydralazine, protected the myocardium from allylamine-induced injury. They suggested that this protective effect was due to hydralazine's proposed effects on arterial tone; however, we subsequently hypothesized that protection from allylamine's cardiotoxicity might be related to inhibition of some monoamine oxidase (MAO) with consequent alteration in metabolism of allylamine through a less toxic pathway.[58] We based this theory on the MAO-inhibitory action of many hydrazine derivatives.[59] *In vivo* studies in the rat, however, failed to show protection with some of the well-known MAO-inhibitors, whereas other types of amine oxidase inhibitors afforded remarkable, often complete, protection.[60]

Our subsequent demonstration of the metabolism of allylamine to the extremely reactive aldehyde, acrolein, by homogenates of rat tissues[61] afforded an opportunity to test the effects of a series of inhibitors of amine oxidase activity on the metabolic conversion of allylamine.[62] The results supported the concept that an amine oxidase was important in the metabolism of allylamine, but these specific inhibitor studies pointed to semicarbazide-sensitive amine oxidase (SSAO), not MAO, as the enzyme metabolizing allylamine. This enzyme has its highest activities in vascular tissue,[63'64] a tissue distribution which parallels closely the tissue distribution of the enzymic conversion of allylamine, i.e., with highest metabolic activities in aortic homogenates.[61,62] Furthermore, large elastic arteries such as aorta have been shown to have the highest SSAO activities of any organ examined in several species.[65] Indeed, SSAO appears to be of vascular smooth muscle cell origin[64] and we have recently shown that porcine aortic smooth muscle cells grown in culture produce the enzyme and secrete it into their media.[66] This is most interesting in light of our demonstration that aorta and other vascular tissues actually concentrate allylamine and/or its metabolites very rapidly after oral administration.[66,67] In fact, our recent study of the subcellular distribution of allylamine or its metabolites[67] in aorta suggests that the subcellular site of injury in vascular tissue may be at or near mitochondria where amine oxidase enzymes could hypothetically have access to the toxin as a substrate, and reactive metabolites of allylamine — including the highly reactive acrolein as well as H_2O_2 — could cause cellular damage.

Such a hypothetical scenario of toxic mechanism has been recently supported by *in vitro* work utilizing cardiac myocytes and fibroblasts (as well as an isolated mitochondrial system) to demonstrate that the toxic effect of allylamine is dependent upon its metabolism to acrolein *in vitro*,[68] and that allylamine may act as an inhibitor of heart mitochondrial electron transport[69] at or near Complex 2. Marked alterations in high-energy phosphate metabolism have also been noted *in vivo*.[70] Isolated aortic smooth muscle cells grown *in vitro* have been utilized to specifically address the vascular toxicity of allylamine, and similar observations about its metabolism to acrolein as an essential step of toxicity have been made.[70,71] The ability of allylamine to transform smooth muscle cells to a synthetic state from the contractile state which prevails soon after isolation has also recently been noted (see Figure 3).[73-75]

Investigations from our laboratory have pursued, through cell culture techniques, the apparent predilection which allylamine demonstrates for vascular tissue *in vivo*.[66] In a study of the comparative toxicity of allylamine to cultured aortic smooth muscle, endothelial, and fibroblastic cells,[76] smooth muscle cells appeared to be most sensitive to the cytolytic and cytotoxic effects of the toxin, whereas fibroblasts proved to be markedly resistant. Cell survival growth curves demonstrated that cultured vascular smooth muscle cells appear to be less able to recover from allylamine exposure, relative to the vascular endothelial or fibroblastic cells.

One hypothetical reason for this smooth muscle vulnerability may be a capacity to metabolize allylamine to a distal toxin, perhaps through the amine

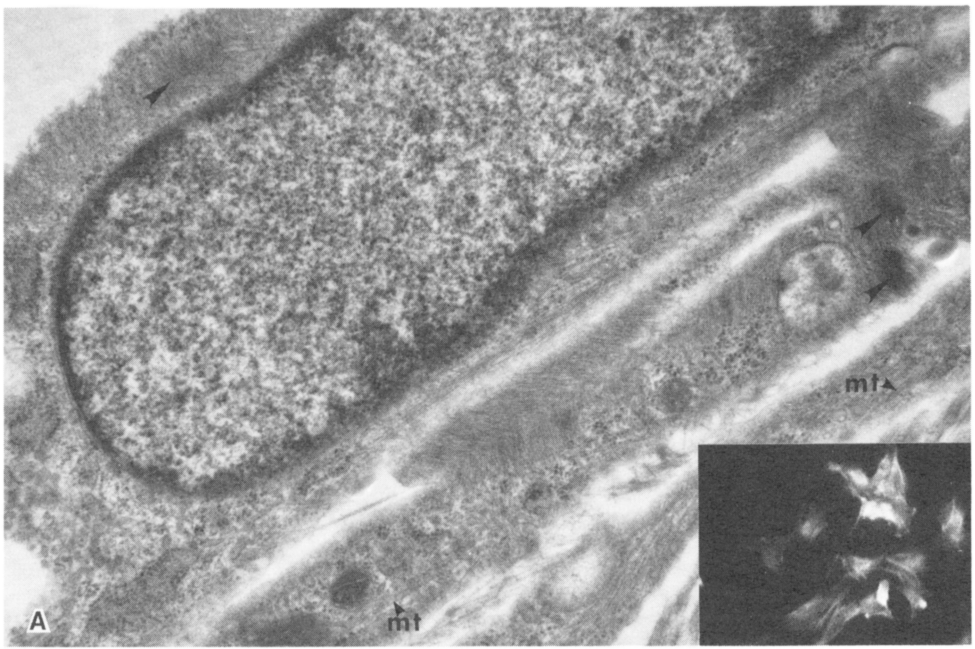

FIGURE 3. Electronmicrographs of cultured vascular smooth muscle cells derived from porcine aorta demonstrate the change in phenotype from contractile state (A) to synthetic state (B) which occurs during subculturing. Workers using such cells should be aware of marked differences in biologic response in cultured vascular smooth muscle cells.[19,20] Both electron micrographs are of cultured cells sectioned in a plane perpendicular to the plane of the culture flask; remnant of the flask is seen at bottom of cells in B. (A) Vascular smooth muscle cells in primary confluent cultures, and up to approximately the 8th to 12th population doubling, show diagnostic characteristics including

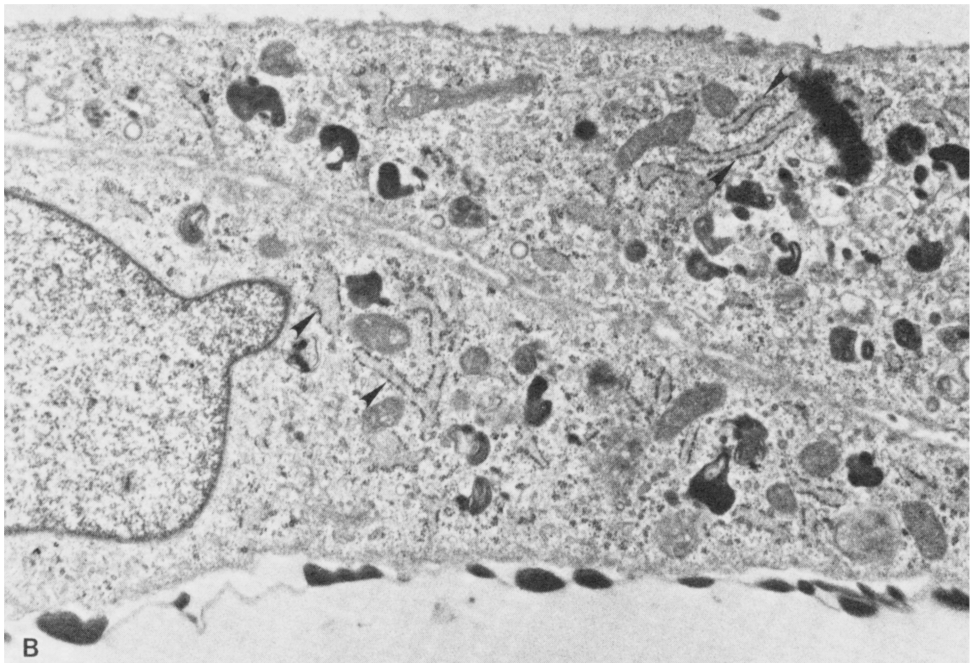

FIGURE 3 (continued).

abundant thin filaments, dense bodies (arrowheads), scattered ribosomes (but no rough endoplasmic reticulum), and mircotubules (mt). Such cells also stain intensely for intracytoplasmic myosin (inset). (Magnification × 41,000.) (B) Cells that have modulated to the synthetic state after 8—12 population doublings have decreased filaments and no dense bodies, but contain greatly increased ribosomes and rough endoplasmic reticulum (arrowheads). (Magnification × 25,000.) (From Hysmith, R. M. and Boor, P. J., *J. Cardiovasc. Pharmacol.*, 9, 670, 1987. With permission.)

oxidase enzyme discussed above, SSAO. *In vitro* data by Ramos et al.[72] and from our laboratory,[71] indicate that SSAO inhibitors capable of *in vivo* protection against allylamine-induced lesions also protect cultured smooth muscle cells from allylamine toxicity. This finding supports the concept that smooth muscle cell sensitivity is enzyme dependent. Continued studies of such cultured cell systems should help to elucidate the possible role of the vascular smooth muscle cell in allylamine cardiovascular toxicity.

IV. ENDOTHELIAL/CAPILLARY TOXINS

Since the two major cell types present in vascular tissue are the smooth muscle and the endothelial cell, a rational classification of vascular toxins should take into account toxic effects on both of these cell types. In reality, vascular injury will frequently involve both cell types. For instance, β-aminopropionitrile medial lesions eventually result in intimal or endothelial degeneration and thrombosis,[36] although the initial toxic effect is primarily on the media.

Nevertheless, there are several examples of toxic xenobiotics that have a relatively specific effect on endothelial cells, and, hence, cause aberrations concentrated in capillaries which are composed primarily of endothelial cells.

Capillaries serve as a target of several well-known vascular toxins, and generally manifest injury by leakage of intravascular contents including fluid (resulting in edema) and red blood cells (manifested as hemorrhage).

A. Snake Venoms

The venoms of a variety of snakes cause hemorrhagic complications, but the associated capillary injury has only recently been experimentally studied. A major hypothesis of how the venom of the Indian cobra, *Naja naja,* causes vascular injury in the capillaries of the lung and kidney is that the toxin causes acute inflammatory cells to lodge in capillary networks and activate their lysosomal systems which, in turn, generate highly reactive and destructive free radicals.[77] A more comprehensive discussion of the cardiovascular toxicity of snake venoms may be found in several reviews.[78,79]

B. Cyclophosphamide

Important therapeutic drugs also may injure the capillary. A vast variety of commonly used drugs may rarely cause vasculitis (inflammation of small vessels including capillaries) as an unusual drug reaction in rare sensitive individuals.[80] Because of the rarity of such reactions, however, little is known about the causes of this type of vascular injury.

Other drugs demonstrate more consistent capillary toxic damage. One example is the commonly used anticancer agent, cyclophosphamide. This drug, given in high doses to patients with cancer, can cause hemorrhagic complications which are especially pronounced in the heart and urinary bladder. Currently, patients may develop severe myocardial damage as well as dose-limiting

hemorrhage into the urinary bladder.[81,82] Experimental studies in several species have suggested that bladder toxicity of cyclophosphamide is caused by a toxic by-product of its metabolism, acrolein,[83,84] and several drug therapies have been devised to counteract this metabolite.[85] Although the mechanism of cyclophosphamide-induced myocardial injury is believed to be related to capillary injury, further experimental work is necessary to show this more conclusively.

C. 1,3-Butadiene

Tumors and proliferative lesions of vascular cells are extremely rare; hence, the recent report[86] of chemical induction of primary cardiac tumors of endothelial cell origin (hemangiosarcomas) in mice clearly demonstrates that vascular cells may undergo a tumorigenic response to toxic injury. 1,3-Butadiene is a colorless, flammable gas utilized extensively in the production of synthetic rubber and resins. Following long term inhalation exposure in mice, a high incidence of endothelial proliferative lesions and biologically aggressive hemangiosarcomas occur in the heart and frequently involve the liver and lungs.[51] The microscopic and ultrastructural studies performed by Solleveld et al.[86] demonstrate typical endothelial cell morphology of the proliferative, malignant cells. Although this demonstration of a xenobiotic-induced vascular tumor appears at this time to be unique for 1,3-butadiene, this study clearly raises the possibility of other tumorigenic responses by vascular cells.

V. CONCLUSIONS: EXPERIMENTAL SYSTEMS IN VASCULAR TOXICITY

This chapter has reviewed a few selected vascular xenobiotic toxins in order to focus on the way that *in vitro* experimental systems have added to our understanding of injurious vascular phenomena. As emphasized earlier, however, the application of such experimental approaches to the vascular system as a specific site of toxic manifestations has just begun, and doesn't presently approximate the extent to which *in vitro* methodology has been applied to myocardial and other cell systems.[87-89]

The first and foremost conclusion to be drawn from this review, therefore, is that a vast amount could be learned by continued applications of the many available *in vitro* experimental methodologies to vascular cells. Much has already been learned about *in vitro* culture of vascular cells; so much in fact that it is surprising to note that it was only about 16 years ago that investigators definitely cultured a pure endothelial cell line.[90] Now that a sizable body of literature on endothelial, smooth muscle, and fibroblastic cells of vascular origin is available, the vascular toxicologist should readily utilize such methods in his/her work.

Besides *in vitro* cultured cell systems, there are other potential *in vitro* systems (such as isolated perfused vessels) which could be adapted to toxic studies; these systems have been extensively studied for some years by physiolo-

gists[91] but have been applied rarely in the study of toxic phenomena. It is hoped that these and other techniques will see greater use in the future.

Thus, the continued and increased employment of methodologies such as cell culture, organ culture, and perfused organ systems in the study of vascular responses to toxic injury should help us understand toxic vascular lesions and — eventually — human disease. It is clear from this brief review, however, that work with *in vitro* models must also continue vigorously; this conclusion is emphasized by the recent study discussed above which demonstrates a remarkable tumorigenic effect of a xenobiotic in mice.[86] Such *in vivo* studies serve as the cornerstones of further mechanistic studies, and give relevance to *in vitro* work. A combination of careful animal studies, classic toxicologic studies, and basic cellular studies *in vitro* should result in a better understanding of human vascular lesions.

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Chapter 2

THE MOLECULAR AND CELLULAR TOXICOLOGY OF ETHANOL ON THE HEART

Philip I. Polimeni and Philip Posner

TABLE OF CONTENTS

I. INTRODUCTION

The belief in the beneficial effects of alcoholic beverages can be traced to the dawn of written history. Biblical admonitions against excessive ingestion of alcohol appear to have been concerned mainly with its negative socio-religious effects rather than any self-inflicted physical injury. Except for some concern for liver disease, the perception of excessive alcohol imbibition as an unhealthy activity seems not to have been widely appreciated until modern times. The recognition of alcohol as a potential cardiotoxicant and even a comparatively common cause of heart disease came about only in the last century. The existence of "alcoholic cardiomyopathy", i.e., a cardiomyopathy specifically associated with excessive and prolonged ethanol intake, was first recognized in the early 1960s and appears now to be generally accepted.[1,2]

Approximately one fourth to one third of the population in the U.S. abstains from alcohol entirely,[3] but 10% of the adult population accounts for half of all alcohol consumed.[4] A conservative estimate of the number of chronic alcoholics in the U.S. alone is ten to fifteen million individuals.[5] The frequent but unrecognized incidence of cardiac dysfunction among chronic alcoholics has led to the suggestion that alcohol abuse is a major cause of cardiovascular disease in the Western world.[6] Slightly more than 3% of all fatalities in the U.S. during 1980 in which cardiovascular disease was believed to be the main cause of death were attributable to alcoholic cardiomyopathy.[7]

II. CLINICAL OBSERVATIONS

After a long period of regular alcohol abuse, usually at least ten years, some heavy drinkers manifest a gradual onset of disturbing symptoms over a period of one or two years. Atrial arrhythmias or fibrillation may be among the earliest signs of alcohol-induced heart disease.[8] Later there may be complaints of breathlessness, palpitations, tachycardia, orthopnea, and paroxysmal nocturnal dyspnea. Vague chest pain may be present, but chest X-rays generally show little more than cardiac enlargement; pulse pressure may be shallow.[9,10] No pathognomonic criteria exist for alcohol-induced heart disease. The diagnosis is made by elimination of all other causes of cardiac dysfunction. The symptoms may be complicated and exacerbated by the beriberi syndrome in patients with inadequate intake of vitamin B_1, but the effects associated with this vitamin deficiency are reversed by the administration of thiamine. Cardiac malfunction is quite common among patients with chronic alcoholism.[6]

Alcohol by itself can result in myocardial dysfunction. However, its deleterious effects can be potentiated by other factors, including other diseases arising independently or secondary to alcoholism. Among the more common factors that aggravate the adverse effects of alcohol are smoking, malnutrition, hypertension, cirrhosis, diabetes, and obesity. Some of these factors lead to increased demand on the myocardial pump and potentially reduce coronary

nourishment. The effect of alcohol on coronaries is ambiguous, with different investigators reporting different effects of ethanol on coronary blood flow. Bing and Tillmanns[9] have suggested that the response of the coronary circulation to alcohol is mainly dependent on the concentration of blood ethanol. There is relatively little information on the synergistic effects of the many confounding factors involved, including dose and concentration of ethanol, duration of intake, animal species, and experimental conditions.[11]

The association between alcohol and coronary heart disease (CHD) is also uncertain, for while various studies suggest a positive correlation,[12] others report a negative correlation[13] or no association.[14] Although sometimes disputed,[15] substantial evidence suggests an inverse relation between chronic alcohol abuse and coronary artery disease.[8] Some studies indicate that even small or moderate intake of alcohol results in significantly lower scores for coronary artery occlusion compared to teetotalers.[16,17] An inverse relationship between moderate drinking and CHD also has been reported repeatedly.[16,18,19] Although many of the studies suggesting a lower incidence of CHD among moderate drinkers are open to various methodological criticisms, the suggestion is bolstered by the consistency of epidemiological data in many countries.[20,21] According to the Framingham Study, a prospective and systematic study of over 5000 adults, it appears that CHD and intermittent claudication are inversely related to the amount of alcohol consumed.[13] The relationships are weak, but statistically significant. Nonetheless, with respect to mortality from all causes, the risks for alcoholics and problem drinkers are certainly greater than for abstainers and moderate drinkers.[8,22,23]

Perhaps of pertinence is the evidence that ethanol consumption tends to elevate the concentration of high-density lipoprotein (HDL) and lower that of low-density lipoprotein (LDL).[24] These changes are generally believed to inhibit atherogenesis. LDL, which appears to be inversely related to alcohol intake, is positively correlated with cardiovascular disease.[25] In studies on large populations of men and women, the levels of HDL cholesterol were found to be higher among drinkers than nondrinkers.[24,26] In the absence of severe liver disease, alcoholics had much higher HDL levels than nonalcoholic control groups.[27,28] After two weeks of abstinence the difference in HDL levels between alcoholics and nonalcoholics disappeared.[24] Data from several studies suggest that the HDL levels of moderate drinkers, irrespective of the type of beverage favored, exceed those of teetotalers.[29] The mean HDL cholesterol level in men admitted to an alcoholic detoxification hospital was nearly twice that in the general population.[28] The recent study by Stampfer et al.[30] suggests that moderate consumption of alcohol decreases the risks of coronary heart disease in middle-aged women.

There is considerable epidemiological evidence that chronic alcohol abuse is associated with hypertension[31-34] and the hypertension is reversible upon abstention from alcohol.[33,35] The positive association between alcohol consumption and high blood pressure persists when precautions are taken to control for many of the confounding factors.[12,19,32,36] The relationship between alcohol and blood

pressure is more complex where moderate imbibition is concerned. In their recent review of the literature, Flegal and Cauley[29] conclude that most studies[12,32,37,38] indicate that ingestion of one to five drinks per day results in an approximately linear dose-response relationship between alcohol dose and systolic pressure of 1 to 3 mmHg per drink per day. Blood pressures generally were found to be slightly lower in teetotalers, or not significantly different, when compared with light drinkers.[31,32,37,39] Systolic but not diastolic pressure was sometimes found to be slightly higher in male nondrinkers.[12,19] There appears to be a consensus that women nondrinkers have slightly higher blood pressures.[31,32,37] In his recent literature review on the effects of alcohol on the cardiovascular system, McCall[40] concludes that whereas "two or fewer drinks per day may be safe, the regular use of three or more per day is associated with a significant risk of hypertension, which increases progressively as the daily consumption rises."

Although individuals who have been heavy drinkers for a long period often show no clinical evidence of heart disease, they frequently have abnormal myocardial function. Thus left ventricular depression, evidenced by an increase in the pre-ejection period (PEP) and the ratio of this period and left ventricular ejection time (LVET), PEP/LVET, correlated with the level of blood ethanol.[41] This subtle dysfunction, demonstrable by physiologic studies and indirect noninvasive tests without necessarily the challenge of alcohol, is thought to represent preclinical heart muscle disease.[8] Using PEP/LVET measurements as a criterion of preclinical cardiomyopathy, Wu et al.[42] concluded that females might be less susceptible to the toxic effects of alcohol, and this might account for the rarer occurrence of alcoholic cardiomyopathy among women even after adjustment for their lower incidence of alcoholism. Abnormal systolic time intervals can be detected in most chronic alcoholics, and these abnormalities are exaggerated in patients with alcoholic cardiomyopathy.[43] Because systemic vascular resistance is commonly low in alcoholics, a decline of left ventricular contractility may be concealed. Hemodynamic abnormalities, which often are difficult to detect in the resting state, may become apparent during various types of stress tests.[44] Lang et al.[45] showed that a slight depression of cardiac function could be detected in healthy drinkers with moderate consumption of ethanol even when the indices of contractility were relatively load-independent. With respect to the normal heart it is difficult to draw any firm conclusions from the literature, because studies have shown salutary, depressant, or no effects on ventricular contractility.[40] Presumably some of the apparent discrepancies are due to variations in dose, rate of ingestion, type of beverage, and other conditions. A mild dilation of the heart can be demonstrated in some asymptomatic alcoholics,[46] but the findings are nonspecific and differ little from those of patients with idiopathic dilated cardiomyopathy. Dancy et al.[10,47] have suggested that increase in end-diastolic dimension is perhaps the earliest occurrence that is detectable. According to Wodak and Richardson,[48] approximately 60% of patients whose hearts are dilated and function poorly have alcoholic myocardial disease.

Preclinical cardiomyopathy is detectable among many who abuse alcohol,[43,46,47] but the preclinical abnormalities of ventricular function are completely reversible by abstention from alcohol.[49] Whereas asymptomatic alcoholic cardiomyopathy is a common disorder which understandably goes unrecognized,[5] symptomatic alcoholic cardiomyopathy occurs in only 1 to 2% of chronic alcoholics.[50] These patients, individuals who generally drink over 80 g of alcohol per day for over 10 years, are likely to have other forms of tissue damage.[48] In the later stages of the disease, cardiac output is generally normal or low and left atrial and ventricular end-diastolic pressures tend to be elevated. Rhythm disturbances persist and there is a high incidence of embolic complications. Their breathlessness and night cough become exacerbated as the disease progresses, and anorexia, weakness, and fatigue on even mild exertion ensues. The latter generally reflects left ventricular failure and weeks or months later the abdominal swelling of ascites may appear. Frank right and left congestive heart failure are now apparent on physical examination, with the supporting signs of tachypnea, venous distention, pulmonary rales, cardiomegaly, and mitral regurgitation.[5,40,51] Except for the abuse of alcohol, the clinical findings are not distinguishable from chronic congestive cardiomyopathy due to any cause.[8]

There is sufficient evidence that alcohol injures the myocardium, probably by a direct effect, to justify designation of the general diagnostic term for primary myocardial disease, "cardiomyopathy", in conjunction with alcoholism. A considerable amount of evidence suggests a direct myocardial toxic effect probably by alcohol itself or less likely one of its metabolites (see below). As indicated above, since there are no specific symptoms, signs, tests, or pathologic findings in even advanced alcoholic cardiomyopathy, the diagnosis is always presumptive.[8]

It is widely known that the mainstay of treatment for alcoholic cardiomyopathy is abstinence from alcohol.[8,10] Even in nonalcoholic patients with heart disease, the evidence suggests that moderate amounts of alcohol are deleterious to ventricular function.[40] Alcoholic heart disease is completely reversible if recognized early and alcohol intake is eliminated. Both cardiomyopathy and the myopathy of skeletal muscles, characterized by progressive wasting and weakness of proximal muscle groups, tend to recover after extended abstention from alcohol.[8,52] After the development of frank heart failure, however, the prognosis is poor.[53] Based on an epidemiologic survey, it has been suggested[12] that six drinks of an alcoholic beverage per day over a period of years increases cardiovascular mortality. The ethanol consumption of alcoholic patients with congestive cardiomyopathy usually is considerably greater. Death usually occurs as a result of congestive heart failure, ventricular arrhythmia, or complete heart block.

III. EXPERIMENTAL ASPECTS

Although alcohol has been associated with cardiac failure since the middle of

the 19th century, it has been difficult to ascertain a direct link in clinical studies. The link between alcohol and cardiac myopathy had been thought to be secondary to nutritional deficits, toxic substances in the drink, volume overload, or even viral infection. Recent clinical studies controlling for nutritional state and liver damage have confirmed the direct cardiomyopathic effect of ethanol in humans.[54-56] Thus it appears that although nutritional, toxic, and infectious processes may modulate the onset of cardiomyopathy, ethanol *per se* is a prime risk factor. The problems in using a human model to study the mechanism of acute or chronic ethanol toxicity on the heart are many. The subjects must be controlled for genetic variability, sex, age, and nutritional state. In addition, previous exposure to ethanol and other cardiac risk factors (genetics, smoking, cholesterol, body weight) are important, as well as are emotional factors. If these factors are considered adequately in the data analysis, prospective and retrospective human studies can be quite valuable.

Because of the frequent unreliability of patient history obtained from alcoholic patients, and the ethical issues involved in human experimentation, the development of animal models for the study of ethanol toxicity has been important. The following animals have been used to study the acute and/or chronic effects of ethanol on the heart: cats,[57] dogs,[58] frogs,[59] guinea-pigs,[60] hamsters,[61] mice,[61] non-human primates,[62] rats,[63,64] and turkeys.[65,66] However, it is important to realize that different animal species may differ in sensitivity to ethanol in cardiac metabolism, electrophysiology, and contractile function. These differences must be considered in comparing data between species and extrapolating to humans. It is also important to be careful in extrapolating data from acute studies to chronic studies.[2] Few of the "chronic alcoholism" animal models approach the duration of heavy alcohol ingestion that is common among human alcoholics before the manifestation of serious cardiovascular problems. With all of these caveats observed, chronic animal studies have been quite useful. Ethanol can be effectively given up to 40% of total caloric intake using various modifications of the DeCarli-Lieber diet[67-69] for weeks, months, or years. Once the animal is ingesting ethanol, controlled studies can be devised using available physiological, pharmacological, biochemical, and anatomical analyses. These range from *in vivo* telemetry of cardiovascular function to analysis of function in isolated cell membranes.

In a review of rodent models of alcohol tolerance and dependence, Pohorecky[70] proposed criteria for an animal analog of alcohol dependence that should (1) approximate pharmacological levels of blood ethanol, (2) result in metabolic and functional tolerance, (3) produce physical dependence, (4) sustain its levels of blood ethanol, (5) be maintained on a controlled nutritional diet adequate for a prolonged period of health, (6) not involve excessive experimental intrusions, and (7) be suitable for repeated addiction cycles. Since no model satisfies all the criteria of an animal analog of human alcoholism, the method of choice should be decided by the particular objectives of the experimenter.

Gross cardiac dilatation and congestive heart failure have not yet been

produced in animal models of alcoholic heart disease.[1,8] Although Noren et al.[65] have reported that the Nicholas turkey develops congestive cardiomyopathy in a relatively short period secondary to the ingestion of a moderate quantity of ethanol, according to Dancy and Maxwell[10] no adequate experimental model exists that reproduces the features of alcoholic cardiomyopathy.

IV. EFFECTS OF CHRONIC ALCOHOL INGESTION

The chronic ingestion of ethanol has been correlated with changes in ultrastructure, electrophysiology, contractile function, and biochemical processes.[2,40,71] Because only a limited number of alcoholics develop a full-blown cardiomyopathy, there has been debate over the effects of ethanol alone and confounding factors in human alcoholics, such as genetics, viral myocarditis, and various dietary deficiency states. Because of this, several animal models have been developed to study the effects of ethanol alone while confounding variables were carefully controlled. In addition, several relatively well-controlled studies have been carried out on humans. This section presents a composite picture of the effects of chronic ethanol ingestion in humans and animal models on myocardial structure and function.

A. Ultrastructure

The classic picture of ethanol-induced cardiomyopathy is a flabby, dilated heart with minimal hypertrophy. There are degenerative changes in the myofibrils leading to cell death and healing by fibrosis. The mitochondria tend to have fractured cristae, increase in number and size, and fuse into giant structures (megamitochondria).[5,71-73] Histochemical staining of several mitochondrial oxidative enzymes is less intense. The sarcoplasmic reticulum and T-tubules are distended to cystic proportions and there is a dilatation of the intercalated disks. There may be an accumulation of glycoprotein-like material in the ventricular interstitium and an accumulation of neutral lipid droplets and lipofuscin granules in the myofibrils. Since these changes occur in controlled studies with ethanol ingestion as the only variable and are reversible with ethanol withdrawal, it appears that these structural changes are specifically—although not exclusively—associated with chronic ethanol consumption.[55,72-77]

In studies on animals (rats, mice, and dogs) chronically ingesting alcohol, the structural changes were similar to those seen in human biopsy studies. The changes were not found in all animals, but appeared to depend on a sufficient exposure to alcohol with respect to both time and dose. As in the human studies, the changes were similar to those found in myopathies of several different etiologies.[2]

Even among very heavy drinkers who died without clinical evidence of heart disease, post-mortem studies indicate that in most cases there is some cardiac abnormality, including myofiber swelling, vacuolization, fatty droplet infiltration, and structural irregularities of mitochondria, sarcoplasmic reticulum,

myofibrils, and intercalated disks. Focal scarring, diffuse interstitial fibrosis, and inflammation are also commonly found.[8] To date none of the degenerative features observed is significantly distinct to permit an absolute diagnosis apart from a history of excessive drinking.

B. Pathophysiology

1. Electrophysiology

The chronic ingestion of ethanol has long been correlated with clinical abnormalities of cardiac electrophysiology. As with the morphological changes, it is important to differentiate the direct effects of the ethanol from those induced by nutritional abnormalities, infection, or nonspecific myopathic changes.

Numerous clinical reports have linked alcohol ingestion with electrocardiographic abnormalities and arrhythmias. These include prolonged PR, QT, QRS intervals, bundle branch block, hemiblock, ectopic beats, tachycardia, flutter, and fibrillation.[40,54,71,77-79] Ettinger et al.[80] demonstrated that the acute arrhythmogenic effects of ethanol depend upon a history of drinking alcohol.

They coined the term "holiday heart syndrome" to refer to arrhythmias induced by binge drinking in people with a history of alcoholism. Greenspon and Schaal[81] demonstrated prolonged H-V intervals on His bundle recordings, as well as a decreased threshold for the induction of arrhythmias, using extra stimulus techniques, in alcoholic patients following 3 oz of whiskey. Various abnormalities of repolarization have been described,[77,78] including tall spinous T-waves in the precordial leads, cloven T-waves in leads I and II, and dimpled T-waves in lead I. These changes were shown to revert to normal with abstinence from alcohol. In chronic alcoholics these changes may be related to cardiac muscle damage[55] or hypomagnesemia.[82] On the other hand, Ettinger et al.[80] studied "pre-myopathy" alcoholics and found that acute ethanol ingestion triggered arrhythmias. Several clinical reports[80,83,84] have shown prolonged PRc, QRS, and QTc intervals, as well as the absence of septal Q waves and slurred, low-amplitude QRS complexes consistent with primary myocardial disease. There were also arrhythmias following heart failure and withdrawal from ethanol. Abbaskoor and Beanlands[84] showed a 39% incidence of arrhythmias in their alcoholic group using Holter monitoring.

In an effort to elucidate the spectrum of arrhythmogenic effects caused by chronic alcohol, independent of nutritional factors and overt failure, several well-controlled studies have been carried out in dogs receiving up to 30% of daily calories as alcohol for periods up to four years.[58,80,85,86] These studies showed atrioventricular and His bundle conduction delay. They also showed a reduced ventricular fibrillation threshold and increased susceptibility to arrhythmia induction using pacing techniques. Patterson et al.[87] found a similar sensitivity to induced arrhythmias using a dog model where ethanol was infused directly into the coronary circulation for periods up to 207 days (2 to 8 h/d). Several other electrophysiological studies have been carried out on isolated tissues or cells to learn the effect of acute and/or chronic ethanol. In Patterson's study, the

ventricular tissue from the region receiving coronary ethanol showed depolarized cells with a reduced amplitude and rate of rise of phase 0. They found a reduced action potential duration and an increased refractory period. These data are consistent with those of others[57,60,61,88-91] who found similar acute and chronic effects of ethanol on atrial and ventricular action potentials in rats, dogs, cats and frogs. In addition, Posner et al.[64,92] showed a reduction in baseline and maximum sinoatrial rate in rats receiving chronic ethanol. However, moderate ingestion of alcohol in this species did not modify sinus node automaticity or the membrane potential of the atrial myocardium.[93] Gimeno et al.[90] showed a depressed overshoot in the atrial action potential and a reduced conduction velocity in rat atria. Fisher and Kavaler[57] had similar reductions in conduction velocity in cat and frog ventricle. Takeda et al.,[59] studying single bullfrog atrial cells in culture, showed that acute ethanol reduced inward sodium current (i_{Na}), calcium current (i_{si}), and net potassium current.

In summary, both chronic and acute ethanol can trigger arrhythmias. These include abnormalities of ectopy, rate, and conduction. These can occur before as well as after overt myopathy takes place. The basic substrates for arrhythmias include myocardial defects in conduction, automaticity, and refractoriness.[94] These have been demonstrated in human subjects and animal models. In the "prefailure" stage of alcoholism, there is a reduced automaticity of the sinus pacemaker as well as reduced conduction velocity secondary to reduced action potential amplitude and *dv/dt* of phase 0. There is also a prolongation of the refractory period. These set the stage for potential ectopic, re-entrant tachyarrhythmias. These may be triggered by any increase of plasma catecholamines due to decreased clearance,[95] the presence of plasma acetaldehyde,[96,97] or coronary spasm secondary to Mg deficiency.[98] Later as the myopathy evolves, conduction is further compromised by swelling of intercalated disks and loss of myocytes. The remaining myocytes become more susceptible to ischemia and hypoxia due to reduced mitochondrial reserve.

2. Contractility

The effect of ethanol on cardiac contractile performance has been difficult to analyze because of its many effects in the body. One must consider the direct effect of ethanol on the myocardium, reflex responses due to peripheral vasodilation, effects of acetaldehyde, release of catecholamines, and possible nutritional and ionic imbalances caused by chronic and acute ethanol ingestion. The role of the sympathetic nervous system in the heart's response to ethanol remains to be elucidated.[99] Several investigators have demonstrated the depressant effect of chronic or acute ethanol on isolated cardiac tissue from various animals. Ethanol has been shown to reduce baseline and maximum contractile function in atrial and ventricular tissue of the rat.[90,92,100-102] Similar findings have been made in hearts of dog,[101,103] cat,[57] and turkey.[65]

The myopathic effects of chronic ethanol in the rat have been shown to be reversible with abstinence,[104-106] but data on reversibility in humans with absti-

nence are still unclear.[1] Notwithstanding the experimental difficulties, nearly all experimental data suggest that either acutely or chronically administered ethanol results in depression of myocardial contractility.

3. Excitation-Contraction Coupling

The preceding sections have presented information on ethanol's chronotropic and inotropic effects on the heart. In order to present what is known about the mechanisms for these effects at the cellular level, this section examines the interaction between alcohol and electromechanical coupling. In normal cardiac muscle, contraction is preceded by an electrical event which triggers a rise in intracellular calcium ions. These ions interact with the contractile proteins to activate the contraction. The force of contraction is dependent on the concentration of intracellular Ca^{2+}. Depending upon the species and its maturity, intracellular Ca^{2+} may be modulated by binding to mitochondrial membranes and sarcoplasmic reticulum, as well as Mg^{2+} and MgATP concentrations, intracellular pH, and Na^{+}-Ca^{2+} exchange. In most mammalian hearts, the "trigger" calcium enters during the latter stage of the action potential upstroke and the beginning of the plateau. The maintenance calcium and replenishment of calcium stores occurs during the remainder of the plateau and possibly phase 3. The calcium enters the cell through voltage-gated membrane channels and through the Na^{+}-Ca^{2+} exchanger. Therefore, anything that would reduce trigger calcium, intracellular calcium stores, or calcium release would lead to a depressed contractile state.

Several studies presented earlier showed a reduction in action potential duration and amplitude of phase 0 in response to acute and chronic exposure of the myocardium to alcohol.[57,60,87-90] Takeda et al.[59] showed a reduction in i_{si} with acute ethanol in bullfrog atrial cells and Posner et al.[218] found a reduction in peak slow net inward current in guinea-pig ventricular myocytes exposed to acute ethanol. Even though these studies point to a reduction in transmembrane Ca^{2+} movement, Gimeno et al.[90] have calculated that the reduction in Ca^{2+} caused by shortening the action potential would not explain the entire reduction in contractility. Several laboratories have shown an ethanol-induced defect in sarcoplasmic reticulum binding of calcium.[104,107,108] This defect correlates with reduced contractile force and prolonged relaxation kinetics.[88] Thus, while it appears that alcohol adversely affects the electrical trigger for elevating and removing intracellular calcium, to the best of our knowledge the effect on release and replenishment of stores has not been studied.

C. Biochemistry

The effects of ethanol on electrophysiology, contraction, and excitation-contraction coupling having been discussed, this section addresses the effects of alcohol on myocardial cell biochemistry. Since the effects of ethanol on the cardiac cell membrane and its components will be discussed elsewhere, this section focuses on the metabolism of ethanol and mitochondrial function in the

heart. The systems which exist in the body for metabolizing ethanol are alcohol dehydrogenase, a microsomal ethanol oxidizing system, and catalase. Approximately 90 to 98% of all ethanol ingested is completely oxidized. However, no appreciable metabolism of alcohol takes place in muscle, including the heart.[109] These tissues lack alcohol dehydrogenase; thus most of the dehydrogenation is carried out in the liver.[110]

One of the metabolites of ethanol, acetaldehyde, has long been suspected of playing some role in the adverse effects of alcohol,[96] because aldehydes are known to be highly reactive compounds that bind to many categories of biological molecules. Some of the literature on acetaldehyde predating the early 1980s is now questionable, because it appears that the earlier assays of acetaldehyde in biological fluids overestimated its concentration.[111] Dancy and Maxwell[10] have suggested that since the heart muscle lacks alcohol dehydrogenase, alcohol *per se* and not one of its metabolites is likely to injure the heart, although an effect by a hepatic metabolite via plasma cannot be ruled out. Taraschi and Rubin[112] have argued that this compound cannot easily be incriminated in the pathogenesis of extrahepatic diseases. Recent and more reliable analytic techniques demonstrate that the circulating levels of acetaldehyde associated with chronic alcohol abuse are very low, although acetaldehyde concentrations in cardiac myocytes might be up to tenfold higher than the plasma concentration.[97] In an *in vitro* study Williams et al.[113] demonstrated that at concentrations present in plasma after alcohol consumption, ethanol but not acetaldehyde exerted a direct and reversible effect on the electrical activity of guinea-pig cardiac Purkinje fibers.

Recent investigations suggest another possible mechanism of alcohol-induced myocardial injury involving a nonoxidative metabolism of ethanol. In ventricular tissue the nonoxidative biochemical pathway produces fatty acid esters of palmitate, palmitoleate, stearate, oleate, linoleate, arachidonate.[114] These esters reportedly are present in high concentration in several organs, including heart, and they are synthesized at high rates in those organs most susceptible to injury by long-term alcohol abuse. There appeared to be a rough correlation between the production of the esters and susceptibility to alcohol damage among the several organs tested. Given the emerging importance of plasma membrane dysfunction as a root cause of cellular damage, and the possibility that some of the esters produced by the nonoxidative pathway might become incorporated in the membrane, one might speculate that such incorporation might in the long run alter the membrane's fluidity and permeability characteristics (see below).

The ingestion of even small amounts of alcohol by Orientals with genetically defective acetaldehyde oxidation results in an intense enhancement of left ventricular function and facial flushing.[115] In a study of healthy volunteers (nine Japanese and ten Finnish), five of the Japanese subjects had high blood acetaldehyde concentrations (ca. 40 μmol/l) one half hour after ingestion of a small amount of alcohol, whereas no acetaldehyde was detectable in the remaining subjects. Kupari et al.[115] attributed the hemodynamic effects in the

responsive subjects to an indirect sympathetic cardiac stimulation secondary to acetaldehyde-induced catecholamine release. Although there seems to be an emerging consensus against the idea of a significantly deleterious effect of acetaldehyde on the heart, the issue still remains open.

Catalase is also abundant in the liver, where it functions primarily for protection against hydrogen peroxide formed by various oxidases. Fahimi et al.[116] found an increase in cardiac catalase activity in rats chronically fed ethanol. This increased activity was shown to have a protective effect on myocardial function.[117] When available, fatty acids are the predominant source of myocardial energy. However, with chronic ethanol ingestion, the mitochondria show abnormal respiration and oxidative metabolism *in vitro*. There is a reduced oxidation of fatty acids by cardiac mitochondria[69] as well as a reduced ability to oxidize other substrates. These changes are not surprising considering the mitochondrial structural changes presented earlier. There is an inhibition of mitochondrial respiration and a depression of tricarboxylic acid enzyme activity with chronic ethanol that is, however, reversed with abstinence.[105] Based upon several studies using substrate substitution, the reduction in mitochondrial oxygen uptake and respiratory control index is found[105,108,118] to be due mainly to defects in respiratory states 3 and 4. The decrease in fatty acid oxidation leads to an increase in esterification of triglycerides.[69,76] Lange and Sobel[119,120] have shown that fatty acid ethyl esters which are metabolites of ethanol induce mitochondrial dysfunction. The mechanism of triglyceride accumulation in the cells is controversial.[5,72,74-77] This is due to the confounding metabolic influences of catecholamines, which are acutely released with ethanol ingestion,[121] and the possible effects of acetaldehyde.[76,118] In addition to the mitochondrial effects, acute and chronic exposure to alcohol leads to an inhibition of protein synthesis.[122,123] One of the oddities of this inhibition in the heart is that it apparently occurs concurrently with tissue hypertrophy.[124] The work of Segal et al.[100,104] points to a reduction in cardiac reserve, perhaps due to a defect in chemical to mechanical energy transfer.[108] This information, taken with the morphologic findings of mitochondrial swelling and lipid accumulation,[71,72,76,77] correlates well with the reduced cardiac function seen in chronic alcoholism.

The development of a fatty liver after heavy ethanol consumption has been considered to involve lipid peroxidation, partly because this development is inhibited by antioxidants.[125] This view has been controversial, mainly because some investigators have failed to find confirming evidence, and where found, very high acute doses of ethanol generally have been used. In a chronic study, however, Shaw et al.[126] found that chronic alcohol consumption increases various hepatic microsomal enzymes, including those of the ethanol oxidizing system, and acute ethanol administration at doses equivalent to commonly observed human intakes resulted in hepatic lipid peroxidation. This was potentiated by fasting. Free radicals generated by a microsomal ethanol-oxidizing system are believed to combine with fatty acids to form lipid peroxides,[127] although the specific radical species involved remain uncertain. The increased

production of free radicals can overwhelm the normal antioxidant defenses found in all cells, particularly after inadequate intake of nutrients participating in those defenses. Free radicals are known to bind to unsaturated membrane lipids, and subsequent peroxidation may result in a loss of membrane fluidity.[128] Redetzki et al.[129] have proposed lipid peroxidation as a possible mechanism of ethanol injury in the heart. These investigators found that pretreatment of mice with alpha-tocopherol partially prevented morphological and enzymic changes commonly associated with ethanol-induced myocardial damage, suggesting that some toxic effects of alcohol might be mediated via a free radical mechanism. Oei et al.[130,131] presented evidence of an ethanol-induced oxidative stress in liver and heart, suggesting that lipid peroxidation may be one of the mechanisms mediating alcoholic cardiac pathology. Reinke et al.[132] have recently found that rats on an ethanol and high-fat diet for two weeks or more generate free radicals in liver and heart. Significantly less free radical generation was observed when fat intake was low, and there was none in control animals on an ethanol-free isocaloric diet. In these experiments electron paramagnetic resonance spectroscopy suggests that the radical species are carbon-centered. Although there has been considerable activity in hepatic research involving ethanol-induced free radical production, cardiac studies have been relatively few and it remains to be seen how many of the findings in liver are applicable to the heart.

In a histochemical study of human ethanol-induced cardiomyopathy Ferrans et al.[72] demonstrated a decreased staining for several myocardial oxidative enzymes. These included succinic dehydrogenase, cytochrome oxidase, isocitric dehydrogenase, and lactate dehydrogenase. Gilmiyarova et al.[133] used a rabbit model to show that chronic ethanol (2 months) resulted in a reduction of the oxidized nicotinamide coenzymes as well as a reduction in the use of citric acid cycle substrates in the heart. This resulted in the accumulation of dihydroxyacetone phosphate, lactate, pyruvate, malate, oxaloacetate, and 2-oxoglutarate. In a comparison between dilated myopathies (ethanol and non-ethanol-induced), Richardson et al.[134] studied enzyme activity in 50 endocardial biopsies from two groups of patients. They found that creatine phosphokinase (CPK) activity correlated well with alcohol intake. However, CPK levels did not return to normal following 3 to 6 months of abstinence. They also found malic dehydrogenase (MDH), lactate dehydrogenase (LDH), and alpha-hydroxybutyric dehydrogenase (alpha-HBD) differed significantly between the two myopathy groups. Their data imply that the alpha-HBD/LDH and LDH/CPK enzyme activity ratios correlate well with ventricular ejection fraction. Based on earlier reports[118,135] of release of myocardial enzymes into the serum and reduced activity of isocitrate dehydrogenase (ICDH) and myocardial ATP content, Edes et al.[136] employed a rat model to look more closely at myocardial enzyme activity following 6 and 12 weeks of ethanol ingestion. They measured the activity of LDH, MDH, aldolase (ALD), ICDH, CPK, and glutamate-pyruvate transaminase (GPT). Whereas ICDH, ALD, and GPT activities did not change, those of MDH, LDH and CPK were reduced in the ethanol group. In addition, the distribution of LDH and MDH

isozymes coupled with MDH/LDH and MDH/ALD ratios points to a move from oxidative metabolism to glycolysis, secondary to a reduction in both pathways. The reduction in these enzyme pathways may be caused by a loss of enzyme from the cell[135] and an inhibition of protein synthesis.[122] The decrease in oxidation increases the availability of acyl-CoA and increases glycerol acyltransferase activity.[118] This may partially explain the triglyceride accumulation reported by several groups[71,73,76,77] when taken in concert with an increased uptake from the plasma fatty acid pool.[137]

In addition to its effects on metabolic enzyme systems, ethanol also modulates enzymes involved in contraction and ion movement. Sarcolemmal Na^+-K^+-activated ATPase and the sarcoplasmic reticulum calcium pump are both affected by ethanol.[138] Work by several investigators has shown that alcohol inhibits myofibrillar ATPase.[139,140] The effect on Ca movement appears to be modulated through phospholamban-Ca^{2+} pump ATPase interactions, whereas other effects which show tolerance with chronic exposure may be mediated through membrane fluidity changes.[138] The changes presented above are seen in states of chronic ethanol intake. However, some are also seen in several other types of cardiac myopathy. In addition, some of the changes in enzyme function do not correlate well with reduced contractile function.[105,139,140] This may, however, signal a final common pathway for myocardial malfunction and insufficiently sensitive tests for contractile function. Several workers have implicated changes in autonomic function as mediating the effects of ethanol on the heart.[2,55] Segel and co-workers et al.[2,141] have shown a reduced ventricular response to beta-adrenergic stimulation as have Posner et al.[102] Both groups have found that chronic ethanol (40 weeks) in rats does not change ventricular beta-adrenergic receptor density or binding. These data agree with those of Banerjee et al.[142] at 24 h after ethanol withdrawal. In addition, it was found that chronic ethanol ingestion did not affect alpha-adrenergic or muscarinic receptor density or binding in rat ventricles.[102] It would appear from these studies that the autonomic effects of ethanol are mediated beyond the receptor binding level, possibly at the level of the G proteins. To our knowledge, studies on this system have not yet been published.

V. ACUTE ALCOHOL INGESTION

Moderate drinking is known to slightly increase the heart rate, blood pressure, and cardiac output of healthy humans. In a study of 22 healthy volunteers, Kupari[143] concluded that modest doses of ethanol did not impair myocardial performance, but only altered extramyocardial influences on left ventricular function, *viz.*, heart rate, preload, and afterload. The effect of acute ingestion of alcohol on coronary blood flow appears to be variable,[8] with reports that the flow increases,[5] decreases, or remains unchanged. Ingestion of alcohol in amounts sufficient to cause severe intoxication, however, is associated with hypotension, arrhythmias, and, if extreme, cardiac standstill. This terminal event is thought to

be triggered by neurogenic reflexes.[8] In one study of 36 acutely intoxicated chronic alcoholics who were monitored electrocardiographically over a 12-h period, only 3 were completely free of arrhythmias.[84]

Gould et al.[144,145] looked at the acute effect of ethanol on cardiac output, heart rate, and stroke volume in groups of chronic alcoholics and nonalcoholics with and without cardiac disease. Theirs and other studies[41,146,147] showed mixed results in the healthy nonalcoholics. There appeared to be a direct depression of systolic function which was compensated by sympathetic reflexes triggered by vasodilation.[146] In nonalcoholics with cardiac disease, 2 to 3 oz. of whiskey caused a drop in stroke work and cardiac output.[144] Chronic alcoholics without signs of overt heart disease have had episodes of arrhythmias after bouts of binge drinking described by Ettinger et al.[80] as the "holiday heart syndrome", because the episodes usually occurred after heavy drinking on holidays and weekends. The prevalent arrhythmias observed in 24 patients were atrial fibrillation and flutter and isolated ventricular premature beats. Cardiac catheterization failed to demonstrate any significant anomalies at rest, but left ventricular response to angiotensin was abnormal. Alcoholics without cardiac disease showed a tolerance to the negative effects of acute ingestion of whiskey.[145,147] However, alcoholics with depressed baseline function were quite sensitive to acute alcohol intake. In this group, 2 oz. of whiskey caused a 20% reduction in cardiac index.

In an effort to describe the mechanisms seen clinically, numerous studies have been carried out in intact animals and in isolated hearts and myocardial tissue. Using the dog model of chronic ethanol ingestion,[86] a decrease in baseline cardiac function has been demonstrated[148] as well as a reduced cardiac reserve.[69,149] Impaired myocardial function was also observed in rats ingesting 36% of their total calories as ethanol over a 5-week period.[101] Stratton et al.[150] and Wong[151] showed an acetaldehyde-triggered release of catecholamines as well as the same compensatory reflexes seen in normal humans in response to vasodilation.[152] Studies using isolated perfused hearts,[100,104,149,153] atrial muscle,[90,102] and ventricular muscle[57,88,102,154] from either control animals or those chronically ingesting alcohol were carried out. A summary of the findings shows that acute ethanol reduces tension and contractility by an amount that is dose dependent. In the animals ingesting alcohol, there is an increase in baseline contractile function after short periods of consumption (3 to 5 weeks) followed by no change in baseline performance until 30 to 40 weeks, when it is depressed. The absolute times for these changes vary with species, age, and quantity of alcohol ingested. Once the cumulative level for depression is reached, the baseline contractility and reserve are reduced. Several investigators have shown this depression to be reversible following ethanol withdrawal.[105-107]

In summary, acute ethanol has a direct depressant effect on myocardial contractile function. In the whole organism, there is a compensation by cardiovascular reflexes, vasodilation, and catecholamine release. In weakened myocardial states, caused by ethanol or other means, the net effect of acute ethanol is cardiodepressant.

VI. INTERACTIONS

A. Nutrition

Although several physicians had recognized the association between excessive alcoholic drinking and heart disease before the turn of the century, interest in this association was sporadic until Aalsmeer and Wenckebach[155] first gave a comprehensive description of beriberi heart disease in 1929. The syndrome that they described was rapidly reversed by the administration of thiamine. However, thiamine deficiency might be expected to result in high-output failure, whereas alcoholic patients were subject to both high- and low-output failure. Keefer[156] reported that some alcoholic patients with low cardiac output also had beriberi, which suggested that both low- and high-output states were primarily a consequence of thiamine deficiency rather than alcohol *per se*. This view began to be altered several decades later when it became apparent that the low-output heart disease usually suffered by alcoholics was not ameliorated by thiamine, and such heart disease was observed among prosperous alcoholics who had a relatively low incidence of nutritional deficiency.[157] Numerous experimental studies on various animal species, where nutritional requirements were carefully maintained, clearly demonstrate that ethanol is deleterious to the heart by several criteria irrespective of nutritional status.[8,78] However, frank heart failure is rarely achieved in animal models of alcoholism, presumably because the duration of alcoholic intake is short relative to the period of abuse among human alcoholics.

In the 1960s, an unusual number of reports appeared in the medical literature relating fatal cardiomyopathies to excessive consumption of beer. The deaths were traced to specific brands of beer[158] and turned out to be caused by the myocardial toxicant cobalt, used as an antifulminant in beer. Another cardiac disease induced by beer falls under the rubric "Munich beer-drinkers' syndrome". This dilated, congestive failure is thought to be caused by the massive volume of fluid ingested by some beer drinkers.[71] There is a high incidence of myocardial ischemia in the alcoholic population[159] which may be caused by acute vascular spasm. The spasm may be secondary to the reduced magnesium levels found with chronic ethanol ingestion. The reduced magnesium levels may also lead to depressed sodium pump activity and consequent calcium overload of myocytes.

B. Aging

Alcoholism is a common problem in the older population and this group is more susceptible to cardiomyopathy.[160] Arrhythmias such as atrial fibrillation and tachycardia are common in the elderly who are heavy drinkers.[161]

Aging has been shown to produce a reduction in heart rate and rhythmicity in addition to a lower chronotropic and inotropic reserve.[106,162,163] In a study by Posner et al.,[92] the chronotropic and inotropic effects of chronic alcohol on rat atria were studied in senescent rats. They found that baseline and maximum rate were depressed after 20 weeks on ethanol in the old rats. The young rats did not

show depressed atrial function until 30 weeks on the alcohol diet. Similar findings were made with regard to baseline and maximum tension development in isolated atrial tissue. This same group demonstrated that the cardiac depression was reversible upon withdrawal of ethanol. Both groups (young, senescent) recovered over the same time course.[106] In a similar study using old rats, Morvai and Ungvary[164] found that old rats were more susceptible to the ethanol-induced changes in heart weight, blood pressure, and redistribution of blood flow.

It appears that senescent animals and man are more susceptible to the negative cardiovascular actions of ethanol than their younger counterparts. This susceptibility may be due to several factors, including a reduction in the size and metabolic function of the liver with aging. Ingestion of equivalent amounts of alcohol by the young and elderly results in higher levels of blood alcohol for longer durations in the aged.[165,166] Notwithstanding their greater susceptibility to ethanol, drinking tends to increase in older retired people.[167] The cardiovascular system adapts to the stress by intrinsic modulation of myocytes and peripheral resistance as well as extrinsic modulation by the autonomic nervous system. It is well known that the ability of the aging cardiovascular system to respond to stress is reduced, sometimes markedly. This functional decline may be due to direct cellular changes and a diminished capability of the aging autonomic system to adequately regulate cardiac output.[168] Thus the aging population, given its impaired metabolic function, reduced cardiac reserve, and altered drinking behavior, are at increased risk of ethanol-induced cardiac dysfunction.

C. Pharmaceuticals

As with the previous risk factors, the negative cardiac effects of alcohol may be modulated by pharmaceutical agents. The ones which are of current interest include nicotine, calcium channel blockers, and beta-blockers. Nicotine has been implicated in cardiac disease and sudden death.[169] In recent animal studies[170,171] an interaction between acute and chronic ethanol and nicotine on sinus node and atrial function has been demonstrated. Posner et al.[172,173] demonstrated that the chronotropic effect of verapamil and the inotropic effect of nifedipine are potentiated by alcohol. Wu et al.[174] found that verapamil prevented several metabolic and functional abnormalities in hearts of hamsters that had been on an alcohol regimen for six months, leading the authors to hypothesize that the protective effect of the Ca-blocker was exerted at the level of the plasma membrane. King and Hirst[175] have shown that beta-blockade in rats can block the cardiac hypertrophy induced by ethanol. Antiarrhythmic drugs such as quinidine, procaineamide, and propranolol, which are cardiodepressants, should be used with caution in patients whose cardiac reserve has been depressed by ethanol. The effects of cocaine may be potentiated by acute ethanol ingestion due to a sharp elevation of circulating catecholamines, and this potentiation is particularly hazardous in the chronic alcoholic whose cardiac reserve is already compromised.

VII. MEMBRANE EFFECTS

The mechanism by which alcohol causes cellular abnormalities and eventually irreversible injury remains unknown at present,[176] but the hypothesis that alcohol-induced derangements are due to direct or indirect effects of ethanol on myocardial plasma membranes[177,178] has been gaining adherents over the past decade.[179] Much of the evidence for this view has been obtained from a variety of organs, including myocardial tissue, where ethanol has been shown to alter the phospholipid composition of cell membranes.[180,181] The compositional changes tend to be small and no clear pattern has yet emerged. However, because membrane enzymes generally appear to require specific and vicinal "boundary" lipids for optimal activities, it is possible that major modifications in enzymic activities might follow barely detectable alterations in composition and content[182] and need not involve bulk lipid.[180]

The lipoproteins that constitute plasma membranes are highly ordered, particularly by the presence of the lipid moiety consisting mainly of various phospholipids and cholesterol. This order is an intrinsic determinant of the physical attributes of biomembranes, including their permeability properties which regulate transmembrane fluxes. The cascade of biochemical reactions that directional membrane fluxes induce or modify encompasses virtually all metabolism.

A. Electrolyte Transport and Distribution

It has long been known that ethanol alters the cellular-extracellular distributions of sodium and potassium in a variety of tissues, leading to the proposal that an inhibition of active transport by ethanol might be a ubiquitous phenomenon. This ionic redistribution appears also to occur in the heart. After administration of 12 oz. of ethanol there occurs a leakage of potassium and phosphate from the left ventricle, reflected by elevations of their coronary concentrations, and recovery follows after about 2 h.[44] Similar losses of myocardial potassium (and gains of sodium) were found in dogs receiving 36% of their calories as ethanol over a period of 18 months.[177] This reciprocal shift in sodium and potassium was also observed in rats.[63,183] The finding that ethanol causes a dose-dependent inhibition of Na^+,K^+-activated ATPase activity of plasma membranes prepared from the guinea-pig heart[184] suggests that the redistribution of the two cations is due to inhibition of active Na^+-K^+ exchange. However, the concentrations of alcohol required for Na^+,K^+ ATPase inhibition are high and this mechanism might not pertain to the *in vivo* condition. Another explanation for the redistribution of myocardial sodium and potassium is that the cations run down their respective electrochemical gradients following an ethanol-induced, nonspecific increase of sarcolemmal permeability.[63] The evidence supporting this hypothesis is that all the major cations (Na, K, Ca, Mg) are redistributed—all ions running down their respective electrochemical gradients—in the myocardium of the rat alcoholic model, and this redistribution is associated with the entry of an

extracellular tracer (radiosulfate) which is normally restricted to the outside of the cellular space.[63] The modifications of both cation cellular concentrations and sarcolemmal permeability were reversible soon after ethanol intake ceased. In a rat model of binge drinking, where the animals were rendered unconscious, the extracellular tracer did not penetrate into myocytes and there was a paradoxical redistribution of the monovalent cations, i.e., intracellular sodium concentration fell and potassium rose.[185] It was surmised that contrary to the inhibitory effects of ethanol on Na^+,K^+-activated ATPase activity found *in vitro,* where dosages are usually an order of magnitude greater than the highest concentrations reached *in vivo,* the *in vivo* effect is a *stimulation* of Na-K pump activity. It is known that plasma catecholamine concentration rises during binge drinking,[121] perhaps by stimulating sympathoadrenal activity[111] and/or inhibition of catecholamine clearance,[95] and there is evidence that catecholamines enhance Na-K pump activity.[186,187]

A clinical association of hypomagnesemia and hypermagnesuremia with chronic alcoholism is well established.[82] In several studies the hypomagnesemia was shown to be accompanied by hypocalcemia. Acute ethanol intoxication in humans results in relatively little change of plasma Mg and Ca concentrations, but their renal excretion is increased significantly.[188] It appears that the mechanism of divalent cation loss is specific for each ion at the level of the renal tubule rather than due to diuresis *per se*.[189] The loss of Mg is particularly marked during ethanol withdrawal even in well-nourished individuals and its significance is suggested by the fact that cardiac arrhythmias and tetany are relieved by Mg supplementation.

In contrast to the loss of plasma Mg commonly observed in human alcoholics, small increments are sometimes observed in acute animal studies.[185,189,190] This suggests that there may be an early stage of Mg efflux from the musculature into the plasma compartment which exceeds the rate of loss from the kidneys. Given the loss of enzymes and other large molecules from the skeletal musculature that is known to occur in binge drinking,[191,192] suggesting a partial loss of the sarcolemmal permeability barrier, the escape of Mg (mainly as MgATP) down its electrochemical gradient into the plasma compartment is not surprising. Isoenzymic studies suggest that the myocardial sarcolemma is more resistant than that of skeletal muscle to the alcohol-induced permeability breakdown.[50] Comparison of myocardial electrolyte distributions in acute[185] and chronic[63] alcohol rat models suggest that even at very high ethanol doses single bouts of binge drinking have little effect on cellular cation concentration.

The hypocalcemic effect of ethanol observed in alcoholic patients[193] has also been seen in both chronic[63] and acute[185,189,190,194,195] rat models. This effect cannot be explained by a putative increment of urinary Ca output, because direct measurement of the total output during the experiment revealed that it decreased and the effect occurred even after nephrectomy.[194] Peng and Gitelman[190] reported that the fall in plasma Ca was found in all fractions (total, ionic, ultrafilterable, and protein-bound) except complexed Ca. To eliminate the

possibility that the release of parathormone might be suppressed by a hypermagnesemic response to ethanol,[82] they demonstrated that high doses of parathormone do not prevent ethanol-induced hypocalcemia in thyroparathyroidectomized animals (this response might be offset by a hypomagnesemia shown to occur after thyroparathyroidectomy *per se*).[196] Parathormone does not reverse ethanol-induced hypocalcemia,[195] despite the fact that ethanol does not interfere with the release of the hormone in response to hypocalcemia. These results suggest that ethanol causes a movement of Ca from plasma into either the osseous reservoir of Ca or some cellular space. Some evidence exists against a transport of Ca into bone.[195] It was noted above that the cellular Ca concentration was high in ventricular tissue of the "chronic" alcoholic rat,[63] but no change was detected in acute experiments.[185] Given the effect of even single large doses of ethanol on the sarcolemmal permeability of skeletal muscle cells towards protein molecules (see below), the mass of this musculature, and the tremendous inward electrochemical gradient of Ca, it is reasonable to suspect that the movement of plasma Ca is into this tissue.

Although no change of cellular Ca content was detected in a rat model of acute alcoholic intoxication,[185] the content increased significantly in myocardial tissue obtained after *in vitro* perfusion of the guinea-pig heart for 30 min with a physiological saline solution containing 2% ethanol.[197] There was a concomitant functional depression of the heart which was reversible after removal of the alcohol. Verapamil protected the heart against the ethanol-induced depression, particularly against a marked elevation of end-diastolic pressure. However, the mechanism of protection is unlikely to be based simply on the Ca-blocking action of the drug, because diltiazem had little influence on the effects of ethanol.

B. Permeability

As noted above, there is a considerable amount of clinical and experimental evidence that alcoholic toxicity results in a loss of myoglobin, taurine, various enzymes, and other substances from cells and intracellular organelles of striated muscle.[191,198] Not surprisingly, there have been numerous speculations relating a putative disintegration of membrane molecular structure or order and the cardiac derangements—metabolic, electrical, and contractile—typical of alcoholic heart disease.[55,199-202] Wendt et al.[200] postulated that cell membrane integrity was impaired by ethanol, thereby changing membrane permeability.

Acute alcohol intoxication of chronic alcoholics frequently results in a leakage of large molecules from striated muscles.[191,198] Leakage of serum CPK and presumably other proteins also occurs in some acutely intoxicated but otherwise healthy individuals not given to excessive drinking on a regular basis.[192,203] Kettunen[198] failed to detect any CPK-MB isoenzyme, an enzyme of cardiac origin, in the serum of nonalcoholic volunteers or chronic alcoholics after acute ethanol intoxication. This suggests that the leakage of CPK from striated muscle is from skeletal and not myocardial derivation. This finding is in accord with that of Fink et al.,[50] who found increased CPK but not CPK-MB

activity in the serum of some chronic alcoholic patients. These negative findings suggest that either there is no breakdown of the sarcolemmal barrier in cardiac myocytes, in contrast to skeletal myocytes, or the change in permeability is relatively slight and protein leakage is too slow to be detected.

The ability of ethanol to render the sarcolemmal membrane of cardiac myocytes more permeable to small ions was tested *in vivo* in the rat using the extracellular tracer [^{35}S]sulfate.[63,185] This tracer is restricted to the extracellular compartment for long periods in the normal rat, but enters the cellular space under a variety of conditions associated with cell membrane injury. When rats drinking alcohol over a 3-month period were injected with the tracer, its distribution space increased throughout the 6-h experimental period. This space remained unchanged in control rats given water alone to drink. The fact that an apparent increase of the space of tracer distribution in alcoholic rats represented tracer entry into the cell, and not an actual expansion of the extracellular space, was confirmed by simultaneous morphometric measurement of the space. This effect was reversible when ethanol was eliminated from the drinking water. The fact that four cations each moved down their respective electrochemical gradients suggested that the change in permeability towards sulfate was not particular to this anion, but instead represented evidence for a reversible, nonspecific increase of sarcolemmal permeability in ventricular myocytes.[63] This permeability was not altered significantly when nonalcoholic rats were injected intraperitoneally with a single dose sufficient to render the animal comatose.[185]

C. Fluidity

Since the binding of alcohols to plasma membranes is nonsaturable, it is unlikely that the effects of ethanol are directly mediated by changes in enzymic activities or receptor responses. Optimal function of many enzymes, however, may require specific "boundary" lipids,[112] i.e., the lipid molecules immediately surrounding the enzyme molecule that exert much greater influence on enzymic activity than do the more distant bulk lipid molecules. Membrane molecular order is inversely related to the partitioning of lipophilic agents which is governed by the Meyer-Overton rule. Many lipophilic substances, including ethanol, readily enter the membrane phospholipid bilayer, disrupt its ordered molecular arrangement,[204-206] and alter the "fluidity" of the membrane. This disruption of membrane order, which can be detected by several physicochemical techniques, including electron paramagnetic resonance, fluorescence polarization, nuclear magnetic resonance, differential scanning calorimetry, and optical transmission, presumably effects the cellular membranes of all organs and it is concentration dependent. Whether the primary site of action is the lipid or protein moiety is uncertain,[207] but the demonstration that pure phospholipid vesicles are disordered by ethanol suggests that proteins are not required.

Cholesterol and increased saturation of phospholipid fatty acyl chains tend to enhance the order and stiffness of biomembranes.[207] In membrane vesicles synthesized with a mixture of phospholipids, the addition of cholesterol to the

vesicles not only increased membrane order, but it also blunted the disruptive effect of ethanol.[205] Membranes obtained from animals drinking alcohol chronically become more ordered[208] and frequently (but not always) contain more cholesterol[204] or increased saturation of fatty acids.[209] Membranes from such animals have been found to be resistant to the disordering effect of ethanol.[204,208-211] Although membranes of animals genetically resistant to alcohol intoxication also are resistant to molecular disordering,[212] not all changes in sensitivity to ethanol are reflected in alteration of membrane fluidity.[207] For example, synaptosomal membranes of old mice are more resistant to ethanol-induced disorder *in vitro* than those of young mice, but the older mice are more sensitive to the intoxicating effects of ethanol.[213] This relationship involving ethanol, cholesterol, membrane order, and resistance to ethanol-induced membrane disorder has led Goldstein[207] to suggest that alteration of the lipid content with prolonged alcohol intake might be regarded as an adaptive response tending to attenuate the deleterious effects of alcohol on the cell. Beauge et al.[214] and Wood et al.[215] recently demonstrated differences between ethanol-induced fluidization in erythrocytes of alcoholic patients and control subjects consistent with earlier animal studies.

Warren et al.[216] have presented evidence suggesting that the region immediately surrounding proteins embedded on the membrane lipid bilayer has a low cholesterol content. Thus any direct effects of ethanol on transport systems or enzymes might be accentuated by allosteric effects of vicinal phospholipids particularly sensitive to ethanol-induced disorder. This might account for an inhibition of Na-K-ATPase activity in cardiac plasma membranes of heavy drinkers.[184] Although the techniques for assessing membrane fluidity have not yet to our knowledge been applied to membranes obtained from the heart, the view that ethanol probably disorders myocardial sarcolemmal and organelle membranes as it does extracardiac membranes has been argued.[136,217] In our opinion the membranes of the heart probably are more resistant to molecular disorganization than those of some other organs, but the difference is likely to be one of degree rather than one of character. The eventual collapse of the permeability barrier might be viewed as the consequence of an insidious undermining of membrane order due to unrelenting exposure to a mild solvent. This ensuing nonspecific permeation of the cell barrier would result in a slow loss of vital cellular substances, with simultaneous influx of substances normally kept out, and this would be expected to result in a cascade of aberrant metabolic interactions ultimately leading to functional disintegration of the cell.

VIII. CONCLUSIONS

There can be little doubt that alcoholic cardiomyopathy is a real and distinct disease entity, even if its symptoms cannot be specifically attributed to this myopathy. Both human and animal studies have revealed extensive changes of ultrastructure, electrophysiology, contractility, and metabolism in the heart after

prolonged alcohol abuse. Although alcoholic cardiomyopathy is a relatively uncommon cause of death, cardiac dysfunction is commonplace among chronic alcoholics, and it may occur among binge drinkers. The deleterious effects of alcohol on the heart are exacerbated and complicated by a multitude of conditions and factors, some of which are frequently associated with alcohol abuse. Although most evidence presently suggests that drinking alcohol reduces the incidence of coronary heart disease, there can be little doubt that morbidity and mortality from all causes is increased among those who drink more than moderate amounts of alcohol. It is the impression of the authors that most epidemiological studies suggest that one or two alcoholic drinks per day is a safe intake in the absence of disease, and thus intakes exceeding these amounts on a sustained basis might be excessive. Since beer, wine, spirits, and other alcoholic beverages generally contain similar amounts of ethanol per drink, an intake limit of two drinks per day might generally be considered to be "moderate" for *healthy* individuals. For patients with heart disease, including those not given to alcohol abuse, even moderate intakes of ethanol may be detrimental. Patients with alcoholic cardiomyopathy tend to recover after an extended period of abstention, but after the development of frank heart failure the prognosis is unfavorable.

The precise mechanism by which ethanol causes cellular injury remains unknown. However, evidence is growing that injury to the plasma membrane is a ubiquitous effect of ethanol, which readily penetrates into the membrane structure and interrupts its lipoprotein organization. Since the protection of membrane structural organization is vital to the maintenance of permeability and transport processes that govern metabolism, disintegration of this organization is bound to result in a crescendo of defective activities at various levels of cellular function via both direct and secondary effects. Such a mechanism of cellular injury would be expected to demonstrate a spatial and temporal complexity resisting facile explanation despite extensive and prolonged study.

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Chapter 3

MECHANISMS OF ANTHRACYCLINE CARDIOTOXICITY: ARE METABOLITES INVOLVED?

Richard D. Olson and Phillip S. Mushlin

TABLE OF CONTENTS

I. INTRODUCTION

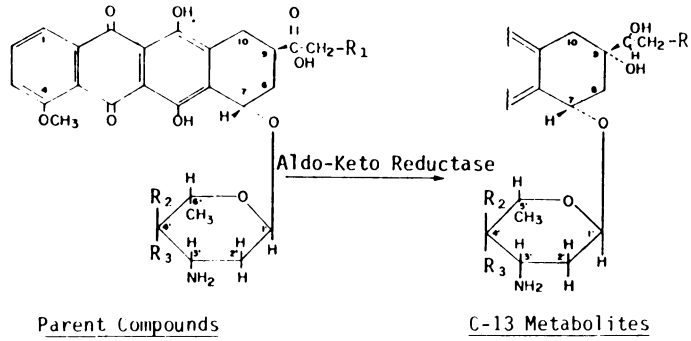
The anthracyclines, doxorubicin (Adriamycin) and daunorubicin (Daunomycin), are highly effective anticancer agents. These natural products of *Streptomyces peucetius* var. *caesius*[1] share similar chemical structures, differing only by an oxygen in the C-14 position (Figure 1). Despite similarities in structure, important differences exist in antineoplastic spectra. Daunorubicin is the single most effective agent against acute nonlymphoblastic leukemias in adults. Doxorubicin is also used to treat acute leukemias but has a much broader antitumor spectrum than daunorubicin, being effective against lymphosarcomas, soft tissue sarcomas, breast cancer, and small cell carcinoma of the lung.

II. CLINICAL SIGNIFICANCE OF CARDIOTOXICITY

As with most anticancer drugs, anthracyclines are highly toxic to host cells, producing bone marrow depression, stomatitis, and alopecia. Unlike other antineoplastic agents, however, anthracyclines produce a potentially fatal cardiotoxicity that relates to the total cumulative dose administered.[2-5] For example, vonHoff et al. reported that 10% of patients receiving a cumulative doxorubicin dose of 550 mg/m^2 developed a congestive cardiomyopathy; the cardiomyopathy was fatal in 60% of these patients.[4] Left ventricular dysfunction (via radionuclide angiography) was observed in more that 50% of asymptomatic patients receiving a cumulative doxorubicin dose of 430 to 600 mg/m^2.[6-8] Even relatively low cumulative doses of doxorubicin (i.e., less than 250 mg/m^2) often produced cardiac injury that was evident only from subendocardial biopsies or studies of diastolic function.[9-11] Thus, anthracycline doses in the therapeutic range usually produce some cardiotoxicity.

A. Risk Factors

Several clinical factors increase the risk for developing anthracycline cardiotoxicity. These factors include hypertension, liver dysfunction and cardiac



Parent Compounds	R_1	R_2	R_3
doxorubicin	OH	H	OH
daunorubicin	H	H	OH
epirubicin	OH	OH	H
C-13 Metabolites			
doxorubicinol	OH	H	OH
daunorubicinol	H	H	OH
epirubicinol	OH	OH	H

FIGURE 1. Structural formulas for three anthracyclines — doxorubicin, daunorubicin, and epirubicin — and their C-13 alcohol metabolites — doxorubicinol, daunorubicinol, and epirubicinol. The metabolites are formed by the reduction of the C-13 carbonyl group to an alcohol via anthracycline reductases (? aldo-keto reductase enzymes).

disease, previous treatment with mediastinal radiation, treatment with chemotherapeutic agents such as streptozotocin or cyclophosphamide,[2,3,12-14] and, most importantly, the age of the patient.[4,7,9,15] Patients below 10 or above 40 are very likely to develop cardiac dysfunction at therapeutic doses of anthracyclines.[7,15] At cumulative doses between 430 and 600 mg/m^2, 82% of patients above 40 years of age, but only 38% of patients between 10 and 39 years of age, developed cardiac dysfunction.[7] How clinical factors such as age predisposed to anthracycline cardiotoxicity is unclear.

B. Approaches to Limiting Clinical Cardiotoxicity

At present, the best way to prevent the cardiomyopathy is to limit the dose of anthracycline, accepting that this may compromise antineoplastic efficacy. The

current approach to minimize cardiotoxicity is to limit the total cumulative dose of doxorubicin or daunorubicin to 550 mg/m^2 or to use higher doses but slowly infuse the drug over hours to days.[16] This latter approach, which attempts to minimize the peak plasma level produced by any dose of anthracycline, is based on the assumption that the peak plasma level of anthracycline is the primary determinant of the development of the cardiomyopathy.

Another way to minimize anthracycline cardiotoxicity is to develop analogs that retain anticancer activity but produce less cardiac injury than currently available anthracyclines. Of the hundreds of analogs tested, only a few, which include epirubicin and idarubicin, have been suggested to have increased therapeutic potential.[17-20] Nevertheless, these new analogs are cardiotoxic. Identification of the structural features of anthracycline molecules responsible for cardiotoxicity or tumor cytotoxicity could provide a basis to synthesize safer, more effective anthracyclines. The development of such analogs should be possible if anthracyclines exert antitumor and cardiotoxic effects by different mechanisms.

III. MECHANISM OF ANTITUMOR ACTIVITY AND DISSOCIATION FROM CARDIOTOXIC ACTIVITY

Mechanisms of antitumor activity will be briefly reviewed before discussing cardiotoxicity. Antitumor activity probably involves an interaction between anthracyclines and DNA; anthracyclines intercalate with DNA and inhibit several functions of DNA, including DNA and RNA synthesis.[21,22] Brox et al. showed that doxorubicin caused DNA strand scission and cross-linking,[23] possibly by stabilizing complexes of DNA and topoisomerase II.[24,25] Damage to DNA may also occur via anthracycline-induced free radical generation.[26,27] In addition, anthracyclines may kill cancer cells by direct actions on the plasma membrane. For example, Tritton and Yee and Wingard et al.[28,29] observed that cancer cells were killed even when doxorubicin was cross-linked to microspheres of agarose or polyvinyl alcohol to prevent its entry into cells. Taken together, the above studies suggest that the mechanism of the antitumor effect is unclear.

Whatever the mechanism of antitumor effect, recent data indicate that it can be dissociated from the cardiotoxic effect. Speyer et al. demonstrated that ICRF-187 decreased the cardiotoxicity of doxorubicin without interfering with antitumor activity in women with advanced breast cancer.[30] In addition, ouabain did not prevent the cardiotoxicity of doxorubicin in isolated preparations[60] but did decrease cytotoxicity in human and hamster cancer cells (probably by preventing topoisomerase-induced DNA strand breakage).[31] These findings suggest that it may be possible to enhance the therapeutic potential of anthracyclines by developing interventions (e.g., anthracycline analogs) that decrease cardiotoxicity.

IV. ACUTE VS. CHRONIC CARDIOTOXICITY

A. Definitions of Acute and Chronic Cardiotoxicity

Acute toxicity refers to toxic effects of a single dose of doxorubicin whereas chronic toxicity refers to effects of the total (cumulative) dose administered. Acute toxicity, manifested by ECG abnormalities (nonspecific ST-T changes, decreases in voltage, widening of the QRS complex, and dysrhythmias), myocarditis, pericarditis, or reversible cardiac dysfunction, is rarely a clinical problem.[32-36] In contrast, chronic cardiotoxicity often culminates in a congestive cardiomyopathy that is refractory to conventional medical therapy.[2-4] It is not always clear when anthracycline therapy should be terminated because chronic cardiotoxicity may not become apparent for weeks to months after discontinuation of doxorubicin therapy.[37] Thus, chronic rather than acute cardiotoxicity limits the therapeutic potential of anthracyclines.

To design experiments that will yield meaningful insights into mechanisms of acute or chronic cardiac injury, one must know the relevant manifestations of doxorubicin cardiotoxicity. Are histopathologic changes more informative than cardiac mechanical abnormalities? What is the significance of ECG changes? Are changes in plasma creatine kinase (CK) or lactate dehydrogenase (LDH) activities sensitive indicators of doxorubicin-induced myocardial injury? How do the manifestations of doxorubicin cardiotoxicity relate to each other? Answers to such questions are not obvious, even after a thorough review of the literature.

B. What is an Appropriate Animal Model of Cardiotoxicity?

After defining the appropriate manifestations of cardiac injury, one must also decide how to study anthracycline cardiotoxicity.[20] What is the appropriate animal model? Should one administer multiple doses of anthracyclines to animals and thereafter perform *in vivo* or *in vitro* studies on cardiac function? Multiple dose studies are expensive and require a much longer time to complete than single dose or *in vitro* studies. Can valid inferences be made from studies in isolated cardiac preparations (e.g., atria or papillary muscles) incubated with varying concentrations of anthracyclines for various periods of time? Although this latter approach appears to provide an acceptable model of acute toxicity, its relevance to "chronic cumulative" toxicity is unclear. Nevertheless, recent data suggest that models of acute toxicity may provide provocative insights into mechanisms of chronic cardiotoxicity.[38]

C. Histopathology after Acute or Chronic Anthracycline Therapy

It seems plausible that acute cardiac injuries summate with each dose of doxorubicin to produce chronic cardiotoxicity. This concept is supported by similar patterns of histopathology after either a single dose or multiple doses of doxorubicin. In both acute and chronic models, sarcoplasmic reticulum and

mitochondria are the organelles most sensitive to anthracycline cardiotoxicity; myofibrillar degeneration occurs with high doses of anthracyclines.[39-48] In single-dose studies in mice, microscopic lesions in mitochondria and sarcoplasmic reticulum were detected 14 h after injecting doxorubicin. These lesions progressed, and, after 3 to 5 d, marked histopathologic changes were noted, including vacuolar degeneration of sarcoplasmic reticulum, swelling and disruption of mitochondria, and myofibrillar disorganization.[44,45] In isolated myocytes incubated with doxorubicin, histologic injury to sarcoplasmic reticulum (disruption, hypertrophy, and vacuolization) was more prominent than injury to other organelles. Nucleolar fragmentation and muscle fiber loss was also noted.[46,47]

Histopathologic changes in humans are similar to those in animals following acute doxorubicin administration.[48] Endomyocardial biopsy specimens revealed marked histologic abnormalities 4 and 24 h after patients received an initial dose of doxorubicin (30 to 60 mg/m^2). Nucleolar changes were occasionally observed after 4 h, and mitochondrial swelling was often apparent in 24 h, but the most consistent and dramatic abnormalities were swelling of the sarcoplasmic reticulum and T tubular system. All changes became less prominent 24 h after doxorubicin administration.[48]

Histopathologic lesions with chronic dosing protocols appear more severe than with acute dosing. Chronic dosing with doxorubicin causes perivascular and interstitial fibrosis with edema and myocytolysis. Large vacuoles, which appeared to result from distended sarcoplasmic reticulum, displace both contractile elements and mitochondria. Mitochondria exhibit electron-dense bodies that presumably represent inclusions of calcium.[39-43] Thus, chronic administration of doxorubicin may exacerbate injuries to mitochondria and sarcoplasmic reticulum caused by acute doxorubicin exposure.

D. Cardiac Dysfunction After Acute or Chronic Anthracycline Therapy

Does cardiac dysfunction with chronic doxorubicin exposure represent a progression of injuries following multiple acute exposures to doxorubicin? Ditchey et al. studied the acute cardiac effects of doxorubicin in dogs.[49] They reported that 20 min after injection of doxorubicin (1.5 mg/kg, i.v.) left ventricular peak systolic pressure had decreased by 36% and cardiac output had decreased by 42%.[49] This impairment of systolic performance (also reflected by rapid decreases in percentage segment shortening, normalized shortening rate, and maximum left ventricular dP/dt) occurred despite a reduction in afterload (a change that by itself would tend to improve systolic performance). A single dose of doxorubicin also acutely impaired cardiac relaxation, as indexed by a decrease in compliance and an increase in the time constant for the decline of isovolumic pressure.[49] The authors concluded that doxorubicin is a direct myocardial depressant because the impairment of systolic and diastolic function was independent of loading conditions and occurred at very low systemic concentrations of doxorubicin (i.e., doxorubicin was infused directly into the coronary arteries). Further support for this conclusion comes from rabbit studies by

Rabkin et al. wherein doxorubicin (1 mg/kg) acutely decreased cardiac output without decreasing myocardial blood flow.[50]

Acute models of cardiotoxicity and chronic dosing protocols produce generally similar impairment of systolic and diastolic function. In the chronic dog model, doxorubicin was injected at 1 mg/kg/week for 8 weeks, then at 1 mg/kg every other week for 8 weeks.[51] Doxorubicin caused a cumulative dose-dependent decrease in and a time-dependent reduction of systolic and diastolic function as assessed by echocardiography (left ventricular systolic diameter, pre-ejection period index, and ratio of pre-injection period to left ventricular ejection time); 18 weeks after beginning the doxorubicin treatments, cardiac index had decreased by 40%, maximum systolic dP/dt had decreased by 25%, and left ventricular diastolic pressure had increased by 250%. In the chronic rabbit model,[52] injections of doxorubicin (1 mg/kg i.v. twice weekly for 8 weeks) produced a 35% decrease in cardiac index. Thus, active (systolic) and passive (diastolic) cardiac dysfunction occur in both acute and chronic models of anthracycline cardiotoxicity. This provides a basis for studying the acute model since chronic dysfunction could possibly represent a summation of acute persistent injuries.[38]

E. Cardiac Dysfunction in Humans

The pattern of doxorubicin-induced cardiac dysfunction in humans appears similar to that observed in animals. Lee et al. reported that a relatively low cumulative dose of doxorubicin (193 mg/m^2) reduced diastolic function (decreased left ventricular filling velocity) but failed to diminish systolic function (via radionuclide angiography).[11] The dose of doxorubicin that impairs systolic function varies considerably,[6] but cumulative doses under 400 mg/m^2 do not usually cause clinically significant reductions of ejection fraction.[7,8] The presence of diastolic dysfunction in the absence of overt systolic dysfunction may partially relate to compensatory reflexes that maintain cardiac output and offset direct negative inotropic effects. Thus, diastolic dysfunction may be a sensitive indicator of an anthracycline-induced decrease in cardiac reserve.

V. ISOLATED CARDIAC PREPARATIONS

Isolated cardiac preparations are useful because they allow observations of direct rather than reflex cardiac effects, and they afford precise control of experimental conditions. In Langendorff rat and rabbit heart preparations, doxorubicin (perfusate concentrations between 10 and 200 μM) decreased contractility,[53-56] relaxation rate,[54] and cardiac compliance.[53,56] As will be discussed in detail later, the magnitude of these effects was highly dependent on duration of exposure to doxorubicin.[53]

A. Validity of *In Vitro* Models of Cardiotoxicity

Although observations in isolated cardiac preparations parallel *in vivo* observations, some investigators have questioned the validity of isolated preparations

as models of "chronic cumulative" cardiotoxicity. There are several reasons for this skepticism. First, the development of anthracycline cardiomyopathy in humans usually follows multiple anthracycline injections and relates to the cumulative dose of doxorubicin administered (as if the heart "remembers" prior exposure to anthracycline.) Second, overt cardiac dysfunction is often delayed, not occurring for weeks to months after patients complete a course of anthracycline therapy. Third, studies reporting anthracycline-induced cardiac dysfunction in isolated preparations often used concentrations of doxorubicin or daunorubicin (between 10 and 200 μM) that were 10 to 200 times higher than plasma anthracycline concentrations in patients receiving antharcycline therapy.[57,58] Finally, there is disagreement about the qualitative nature of toxicity of anthracyclines in isolated heart. For example, some investigators reported negative inotropic effects[53-56,59] whereas others reported positive inotropic effects in rabbit papillary muscles and atria,[60,61] guinea pig atria,[62] and rat Langendorff preparations.[63] Such disparate observations have raised questions about the validity of isolated preparations as models of anthracycline cardiotoxicity.

B. Cardiotoxicity: Dependence on Frequency of Contraction

Some confusion may arise from an incomplete understanding of anthracycline effects in isolated cardiac preparations. In cardiac tissue isolated from rat and rabbit, the degree of doxorubicin toxicity was highly dependent upon contraction frequency.[38,64] Doxorubicin (44 to 350 μM) enhanced contractile function in isolated rabbit atria beating once per second, but essentially obliterated active contraction in atria beating 3 times per second (Figure 2). A literature review indicates that doxorubicin lacked cardiac depressant effects in isolated preparations,[60-63] when studies utilized muscles contracting at rates below those required to cause cardiac dysfunction.

C. Cardiotoxicity: Dependence on Duration of Exposure

Another source of confusion about effects of anthracyclines on isolated cardiac preparations may stem from a failure to appreciate the importance of duration of exposure to anthracyclines.[53,60,62,65,66] Between 30 and 210 min after adding doxorubicin (175 μM) to isolated rabbit atria, systolic function (developed force) decreased from 100% to 35% of the predoxorubicin value at 2 beats/s and from 135% to 90% at 1 beat/s (Figure 3). Thus, duration of exposure to doxorubicin can influence conclusions about the potency of doxorubicin to impair cardiac function. In fact, many studies reporting that doxorubicin lacks toxicity have utilized muscle preparations exposed to doxorubicin for relatively brief periods of time.[60-63] Note that in intact animals, the heart may be exposed to doxorubicin for long periods of time.[44,67,68] Cardiac concentrations of doxorubicin may remain elevated for days to weeks after a single injection of doxorubicin.[44] Thus, evaluations of anthracycline effects at various contraction rates and after appropriate exposure times could possibly provide valuable insights into the mechanism of the "chronic" cardiotoxicity of anthracyclines.[38]

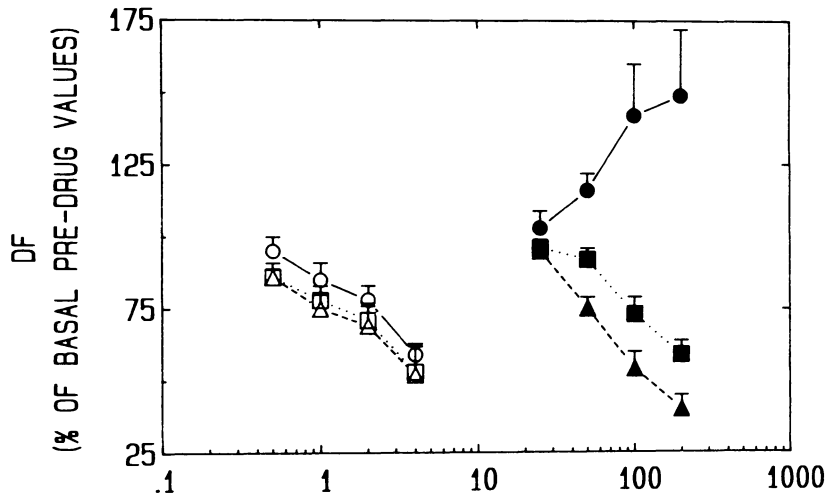


FIGURE 2. Effects of doxorubicin (solid symbols) and doxorubicinol (open symbols) on contractile strength of isolated rabbit atria contracting at 1 beat per second (circles), 2 beats per second (squares) and 3 beats per second (triangles). The x-axis shows concentration (μg/ml) of doxorubicin and doxorubicinol. The y-axis shows developed force (DF, an index of systolic function) expressed as percentages of pre-drug values. Data are mean ± SEM. (References 60 and 97 provide further information about methods.)

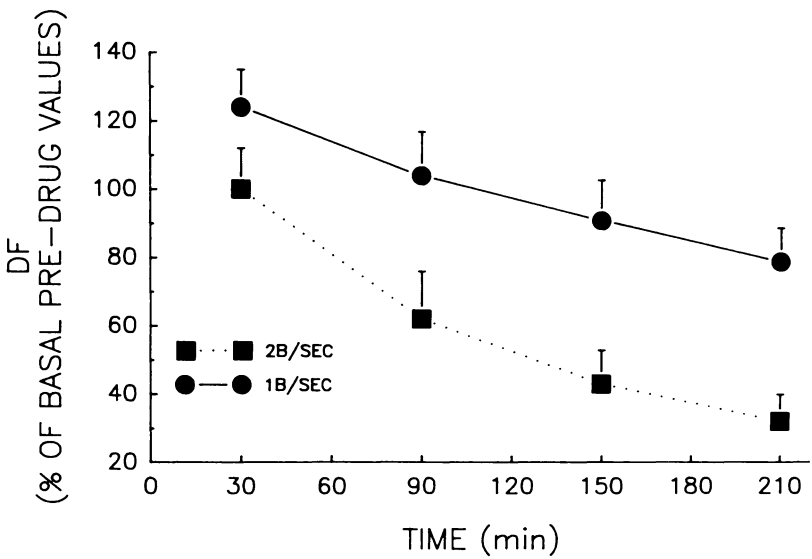


FIGURE 3. Effects of doxorubicin (175 μ*M*) on contractile strength at various times after adding doxorubicin to isolated rabbit atria contracting 1 beat per second (circles) or 2 beats per second (squares). The x-axis shows time, in minutes, after exposing the atrial preparations to doxorubicin. The y-axis shows developed force (DF, an index of systolic function) expressed as percentages of pre-doxorubicin values. Data are mean ± SEM. (References 60 and 97 provide further information about methods.)

VI. MECHANISMS OF ALTERED CALCIUM HOMEOSTASIS AND SUBCELLULAR DYSFUNCTION

A. cAMP-Dependent and Independent Mechanisms

Many investigators have suggested that doxorubicin causes cardiotoxicity by altering calcium homeostasis.[66,69,70] Azuma et al.[71] reported that doxorubicin (100 n*M*) stimulated calcium influx via the slow channel in isolated perfused chick hearts, and Combs et al.[72] showed that calcium uptake into rat myocytes was increased by doxorubicin (5×10^{-8} *M*). Verapamil, a blocker of the slow calcium channel, abolished the increase.[72] Doxorubicin elevated cAMP concentration in chick hearts,[71] inhibited cAMP phosphodiesterase activity of *in vitro* and *in vivo* mouse tissues (especially heart),[73] and stimulated adenylate cyclase activity of rat heart sarcolemmal preparations at very low concentrations (1 to 10 n*M*).[74] Thus, low concentrations of doxorubicin may enhance calcium channel influx via phosphorylation (by cAMP-dependent protein kinase) of the slow calcium channel. High concentrations of doxorubicin (greater that 1 μ*M*), however, probably increase calcium influx via cAMP-independent mechanisms since 1 μ*M* doxorubicin inhibited adenylate cyclase activity in rat hearts[74] and inhibited the ability of isoproterenol to enhance calcium influx via the slow channel in chick hearts.[71]

B. Sarcoplasmic Reticulum

Injury to the sarcoplasmic reticulum, which releases, sequesters, and stores the calcium that regulates the contractile state, may be an important component of anthracycline cardiotoxicity. Anthracyclines markedly alter the function of isolated sarcoplasmic reticulum and exert profound effects on the terminal cisternae.[79] Terminal cisternae and longitudinal tubules of sarcoplasmic reticulum are distinct sites of calcium metabolism. Sarcoplasmic calcium is taken up into longitudinal tubules[75] and transported to the terminal cisternae for storage and subsequent release to the myofibril apparatus.[76-78] Terminal cisternae, unlike longitudinal tubules, exhibit Ca-induced Ca-release suggesting the presence of Ca-gated release channels.[79] Sorazato et al. showed that doxorubicin (25 μ*M*) caused a rapid and near complete release of calcium from isolated terminal cisternae without affecting calcium uptake into isolated longitudinal tubules.[79] Doxorubicin activated specific calcium releasing channels, and this effect was antagonized by ruthenium red, a known inhibitor of calcium release from terminal cisternae.

The calcium pool depleted by doxorubicin appears to be the same pool that caffeine depletes.[79,80] Thus, one action of doxorubicin on sarcoplasmic reticulum is to release and thereby deplete calcium from terminal cisternae. The consequence of depleting releasable calcium stores from sarcoplasmic reticulum would be impaired systolic function. In addition, the ability of heart to relax could be compromised by a large "leak" in the terminal cisternae, especially if calcium sequestration into the longitudinal sarcoplasmic reticulum is impeded

(doxorubicinol, the metabolite of doxorubicin, is a potent inhibitor of calcium sequestration into longitudinal tubules; see Section IX). This would increase the calcium concentration in the sarcoplasm during rest (diastole), producing an increase of muscle stiffness.

C. Mitochondria

Mitochondria may also be important targets for anthracycline-induced cardiac injury. Mitochondria, which provide the energy to maintain calcium homeostasis and also act to buffer changes in intracellular calcium, are injured (i.e., histopathologic data) in both acute and chronic models of anthracycline cardiotoxicity. Doxorubicin inhibited the ability of isolated mitochondria to sequester and retain calcium[66,81-83] and compromised high-energy phosphate availability in a variety of preparations. In isolated perfused rat hearts, doxorubicin (17 μM) decreased cardiac ATP and creatine phosphate content by nearly 25% after 60 min.[56] Relatively high concentrations of doxorubicin (60 and 100 μM) decreased ATP and creatine phosphate content by nearly 70% after 60 min. These decreases were time dependent and correlated with decreases of cardiac function.[56] Doxorubicin also inhibited the function of mitochondria from rat heart, but concentrations required to do this *in vitro* were substantially higher (i.e., 300 to 800 μM) than *in vivo*.[84] At concentrations between 300 and 800 μM, doxorubicin changed the redox state of the carriers of respiration and produced a 50% inhibition of respiration (sites 2 and 3), oxidative phosphorylation, and mitochondrial ATPase activity.[84] Of interest, mitochondria of heart, liver, or tumor cells were equally sensitive to toxic effects of doxorubicin.[84] Thus, it is difficult to ascertain whether direct mitochondrial effects can account for the selective toxicity of anthracyclines to heart. It is also difficult to know whether studies using isolated mitochondria can provide relevant information about cardiotoxicity because of the very high concentrations of doxorubicin required to inhibit their function.

1. Free Radicals

Mechanisms of doxorubicin-induced mitochondrial dysfunction have been explored. The quinone moiety of anthracyclines may be involved with membrane dysfunction or impaired electron transport because a nonquinone anthracycline, 5-iminodaunorubicin, was much less potent than doxorubicin as an inhibitor of mitochondrial function.[85] The quinone moiety is readily reduced to a semiquinone in the presence of electron donors such as NADPH and cytochrome P-450 reductase, NADH and NADH dehydrogenase, or iron (Figure 4).[86-95] As the anthracycline semiquinone cycles back to a quinone, electrons are transferred to molecular oxygen to produce superoxide anion. Superoxide anion can either initiate lipid peroxidation or be dismutated (via superoxide dismutase) to hydrogen peroxide. Free radicals generated from hydrogen peroxide, lipid peroxides, or iron can damage membranes or macromolecules.[96,97] Doroshow reported that substantial amounts of superoxide anion were formed by adding doxorubicin and NADH to the mitochondrial fraction of rat hearts.[86] Reduced

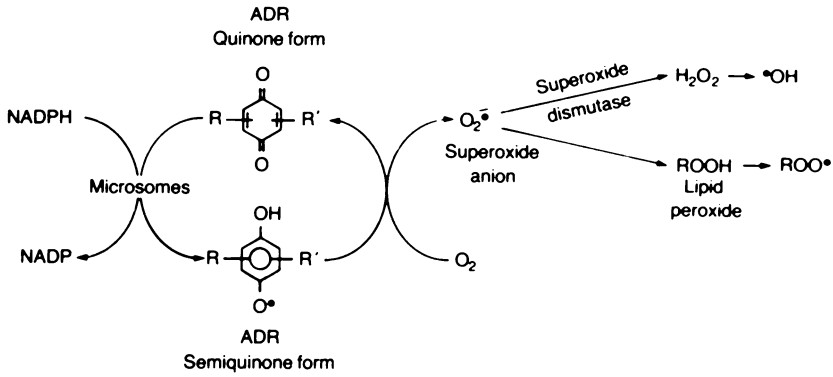


FIGURE 4. Mechanism of free radical generation by doxorubicin (ADR). Doxorubicin in the quinone form accepts an electron from an electron donor yielding the semiquinone free radical. Numerous reducing substances support this reaction, such as cytochrome P-450 reductase and NADPH from liver microsomes. The semiquinone cycles to the quinone and transfers its electron to molecular oxygen to form superoxide anion. Superoxide anion is dismutated to hydrogen peroxide which decomposes to hydroxyl free radicals. (This reaction is very rapid when metal cations such as iron or copper are present.) Alternatively, superoxide anion initiates the formation of lipid peroxides that decompose to peroxyl (ROO·, as shown) or alkoxy (RO·, not shown) free radicals.

glutathione (G-SH), which presumably protects against injury associated with peroxidation,[98] decreased the toxicity of doxorubicin to isolated rat hepatocytes; doxorubicin toxicity (lactate dehydrogenase leak[99]) was inversely related to the size of the reduced glutathione pool of mitochondria.

2. Cardiolipin and Coenzyme Q_{10}

Another possible mechanism of mitochondrial dysfunction relates to an interaction of anthracyclines and cardiolipin of the inner mitochondrial membrane.[100,101] Doxorubicin apparently inactivates cytochrome c oxidase (enzyme in final step of the respiratory chain) by altering its lipid environment rather than by directly inhibiting the enzyme.[100] A linear relationship has been reported between anthracycline concentration that inhibited cytochrome c oxidase by 50% (3 μM for doxorubicin) and dissociation constants of cardiolipin-anthracycline complexes.[100] Thus, anthracycline interactions with the cardiolipin environment of cytochrome c oxidase could cause mitochondrial dysfunction.

Another mechanism of mitochondrial dysfunction may involve an interaction of doxorubicin and coenzyme Q_{10} (i.e., ubiquinone; 2,3 dimethyl-5 methyl-6-decaprenyl benzoquinone), a redox carrier in the respiratory chain.[102] Exogenous CoQ prevented doxorubicin from producing dysfunction in mitochondria isolated from beef heart.[103] Exogenous CoQ_{10} protected against electrocardiographic changes and decreased the lethality of doxorubicin in rodents.[104,105] Crane et al. suggested that doxorubicin interacts with coenzyme Q_{10} and interferes with electron flow down the respiratory chain.[102] Doxorubicin may also inhibit CoQ enzymes directly and inhibit the biosynthesis of CoQ.[106,107]

TABLE 1
Proposed Mechanisms of Anthracycline-Induced Cardiac Injury

I. Injurious agents
   A. Free radicals[85-95, 99, 127-136, 137]
   B. Anthracycline metabolites[12,64,65,134,147-157,160-167, 170]
   C. Histamine[110-112]
   D. Calcium overload[41, 66, 69-72, 81-83]
II. Subcellular sites of injury
   A. Sarcoplasmic reticulum[39-48]
      1. Terminal cisternae[79]
      2. Longitudinal cisternae[160-167]
   B. Mitochondria[39-45,48,66,81-86,56,103]
      1. Cardiolipin[100,101]
      2. Electron transport[84, 103]
      3. Proton pump[160, 167]
   C. Sarcolemma
      1. Na-K ATPase[160, 167]
      2. Na/Ca exchange[163]
   D. Myofibril apparatus[39-47]

CoQ has antioxidant and membrane-stabilizing effects.[108] Tabora et al. recently showed that CoQ antagonists, which are themselves cardiotoxic, acted synergistically with doxorubicin to produce enhanced toxicity.[109] CoQ prevented antagonist toxicity, but neither vitamin E nor CoQ decreased the enhanced toxicity observed with doxorubicin plus CoQ antagonists.[109]The data are difficult to interpret, but these authors and others[56]concluded that indirect effects of CoQ, rather than effects on the respiratory chain, may be responsible for protective effects of CoQ in doxorubicin-induced toxicity.

VII. AGENTS THAT MODIFY ANTHRACYCLINE CARDIOTOXICITY

Although there is a dearth of compelling information about specific mediators or modifiers of anthracycline-induced cardiac injury, there is no shortage of ideas (see Table 1). Bristow et al. reported that doxorubicin released histamine from Langendorff-perfused rabbit hearts[110]and postulated that histamine mediates anthracycline cardiotoxicity. Indeed, histamine infusions produced histologic lesions similar to those observed with doxorubicin, and pretreatment with inhibitors of histamine release (e.g., sodium cromolyn, etc.) prevented doxorubicin-induced histologic lesions.[110-112]The clinical relevance of these observations is unclear. Taurine, an amino acid present in high concentrations in heart, may reduce cardiac injury caused by calcium overload.[113] Hamagiuichi et al. recently reported that exogenous taurine decreased the cardiotoxicity of doxorubicin in mice and concluded that doxorubicin produces cardiac injury via calcium overload.[114]

Amelioration of doxorubicin cardiotoxicity has been reported using amrinone, a bipyridine derivative that selectively inhibits phosphodiesterase isozyme III. Salutatory hemodynamic effects in refractory congestive heart failure result from vasodilation and an enhanced inotropic state.[115] Amrinone decreased ECG abnormalities and lethality in mice, and it prevented cardiac dysfunction, but not histological lesions, in isolated guinea pig atria treated with doxorubicin.[116,117] Inosine, an ATP metabolite that stimulated the synthesis of high-energy phosphates[118-120] and reduced ischemic myocardial injury,[121] also diminished doxorubicin cardiotoxicity as assessed by ECG and histological techniques.[122] Other protectants include carnitine[123] and 3,5,5-trimethyl-2-morpholinon-3yl radical dimer,[124] a drug that reacts directly with doxorubicin to form the doxorubicin aglycone metabolite (presumably a nontoxic species).

ICRF-187 ((+) -1,2,-bis(3,5 dioxopiperazinyl-1-yl) propane) lessened the acute and chronic cardiotoxicity of anthracyclines in animal studies.[125,126] This is the only agent documented to protect humans from the cumulative cardiotoxicity of doxorubicin.[30] ICRF-187 (1 g/m^2), administered to women with advanced breast cancer 30 min before each dose of doxorubicin, decreased the impairment in systolic function (ejection fraction) and protected against histological lesions without compromising the anticancer activity of doxorubicin. It has been suggested that ICRF-187 may exert its salutory effects by scavenging free radicals or chelating iron.[30]

VIII. FREE RADICAL HYPOTHESIS OF ANTHRACYCLINE CARDIOTOXICITY

A. Evidence For the Free Radical Hypothesis

Among the explanations for anthracycline cardiotoxicity, the free radical hypothesis has received the most attention. Anthracyclines that contain the quinone moiety can form superoxide anion, peroxides, and other free radicals *in vitro* (Figure 4).[86-95] Iron-containing species markedly enhance the ability of doxorubicin to form free radicals that induce oxidative injury.[87,89,93,127-130] Pretreatment of rodents and rabbits with free radical scavengers such as cysteamine, *N*-acetylcysteine and vitamin E[131-135] has been reported to decrease lethality and lessen the severity of cardiac histologic lesions produced by doxorubicin. Furthermore, it was argued that doxorubicin predisposed to free radical injury via depletion of reduced glutathione, which is believed to protect cells from oxidative stress and free radical injury (by reducing peroxides to alcohols).[98,131-136] Several investigators observed that doxorubicin lowered the concentration of reduced glutathione and elevated the concentration of oxidized glutathione in erythrocytes, cardiac myocytes, and hepatocytes.[133,134,136] Proponents of the free radical hypothesis argue that heart is highly susceptible to doxorubicin toxicity because anthracyclines accumulate in heart, and cardiac tissue is less able (i.e., contains less reduced glutathione, catalase, and superoxide dismutase) to detoxify free radicals than metabolic tissues such as liver and kidney (for review see Reference 137).

B. Evidence Against the Free Radical Hypothesis

Although the free radical hypothesis can theoretically account for the selective cardiotoxicity of anthracyclines, considerable data appear inconsistent with this hypothesis. For example, van Vleet et al. reported that the lipid-soluble free radical scavenger, vitamin E, did not attenuate the cardiotoxicity (histopathology) resulting from multiple injections of doxorubicin in dogs.[39] In addition, susceptibility to doxorubicin cardiotoxicity was not increased by making hearts deficient in vitamin E. Julicher et al.[55] reported similar negative inotropic effects of doxorubicin in vitamin E-deficient and non-vitamin E-deficient rat hearts. Furthermore, vitamin E deficiency failed to enhance the ability of doxorubicin (100 μM) to (1) decrease activities of superoxide dismutase and glutathione peroxidase, (2) decrease tissue content of glutathione or (3) increase degree of lipid peroxidation as assessed by tissue malondialdehyde content.[55] Moreover, the water-soluble free radical scavenger, *N*-acetylcysteine (20 mM), failed to prevent the decrease in cardiac contractility and relaxation rate in rabbit papillary muscles exposed to 100 or 200 μM doxorubicin.[138] Similarly, in dogs treated with multiple doses of doxorubicin, *N*-acetylcysteine (140 mg/kg i.v. 30 min before and twice a day for 5 d after each doxorubicin dose) failed to ameliorate systolic and diastolic dysfunction as assessed by echocardiography (e.g., left ventricular systolic diameter, ratio of pre-ejection period to left ventricular ejection time, pre-ejection period index) and cardiac catheterization (cardiac index, left ventricular dP/dt, and left ventricular end diastolic pressure).[51] *N*-acetylcysteine (5.5 g/m^2 p.o., 1 h before each doxorubicin injection) pretreatment also failed to attenuate cumulative dose-dependent decreases in ejection fraction associated with doxorubicin therapy in a randomized clinical trial.[139] Thus, free radical scavengers have often failed to decrease the toxicity of anthracyclines.

A number of studies have suggested that anthracyclines, at doses that produce cardiotoxicity, fail to generate significant quantities of free radicals. For example, a large single dose of doxorubicin (10 mg/kg i.v.) in rabbits[140] or rats (20, 45, or 65 mg/kg i.p.)[141] did not cause lipid peroxidation (assessed by malondialdehyde or ethane production), and multiple doses of doxorubicin (e.g., 1.1 mg/kg i.v. twice weekly for up to 10 weeks) did not substantially alter components of the glutathione pathway in rabbits.[140] Oxidized glutathione concentration and glutathione peroxidase activity were unchanged from controls, and concentrations of reduced glutathione actually increased 30% after 10 weeks of doxorubicin therapy.[140] Also, doxorubicin (25 mg/kg i.p.) failed to decrease the concentration of reduced glutathione or glutathione reductase activity of mouse heart for the duration of a 24-h experiment.[142] Similar results were reported after incubating neonatal rat myocytes with doxorubicin (100 μM).[143] In another study using neonatal rat myocytes, Damant and Wasserman concluded that doxorubicin (0.16, 1.6, and 16 μM) did not peroxidize membrane lipids as assessed by fatty acid analysis of total membrane phosphoglycerides.[144] Porta et al. reported that doxorubicin (15 mg/kg) increased plasma CK activity and produced mitochondrial swelling and myofilament fragmentation in rat hearts without decreasing

the activity of catalase or glutathione peroxidase or causing lipid peroxidation in the heart (assessed by diene conjugates or malondialdehyde).[145] Thus, there is often no evidence of oxidative stress or lipid peroxidation at doses of doxorubicin that cause cardiac injury.[146] Consequently, the role of free radicals in the mechanism of anthracycline cardiotoxicity is unresolved.

IX. ANTHRACYCLINE METABOLITES AND CARDIOTOXICITY

A. Pharmacokinetics

Recent studies with anthracycline metabolites offer an alternative to the free radical hypothesis — namely that C-13 hydroxy metabolites contribute to the cardiotoxicity of anthracycline therapy. Cytoplasmic anthracycline reductases catalyze the metabolism of doxorubicin and daunorubicin by reducing the C-13 carbonyl moiety to yield corresponding alcohol metabolites (doxorubicinol and daunorubicinol; Figure 1).[147-153] Pharmacokinetic studies have demonstrated substantial amounts of these metabolites in plasma, which are cleared from plasma at a rate similar to the parent compounds (e.g., $t_{1/2}$ approximately 24 h). Daunorubicinol plasma concentrations actually exceeded those of daunorubicin during the entire plasma elimination phase;[147] the area under the plasma concentration vs. time curve for doxorubicinol was approximately $^1/_3$ of that for doxorubicin.[12] Plasma concentrations, however, often fail to reflect tissue concentrations of the parent anthracycline or its C-13 alcohol metabolite.[149,154]

Pharmacokinetic profiles of anthracyclines are highly dependent on tissue type. Muscle accumulates C-13 alcohol metabolite more avidly than other tissues following single or multiple injections of doxorubicin. In rats receiving a series of 9 or 24 injections of doxorubicin (1 mg/kg i.p.), concentrations of doxorubicin and doxorubicinol in plasma and liver peaked 1 h after the final dose and fell rapidly within 24 h.[155] By contrast, concentrations of doxorubicin in cardiac and skeletal muscle slowly decreased, and doxorubicinol concentrations in heart muscle actually increased over the 24-h period.[155] After 24 h, heart contained more doxorubicin and doxorubicinol than other organs, with concentrations that were 2 to 10 times higher than in skeletal muscle or liver.[155]

Doroshow et al. showed that mouse hearts also accumulated doxorubicin 24 h after a single injection of doxorubicin (15 mg/kg); doxorubicin levels were similar in heart and liver, but doxorubicinol levels were 2 to 3 times higher in heart than in liver.[134] Del Tacca et al. reported that doxorubicinol concentration in rat heart, which correlated with widening of the QRS complex and ST segment changes, reached the highest value after the last of 3 weekly injections of doxorubicin (2 mg/kg i.v.).[156] In other words, the half-life of doxorubicinol in cardiac tissue exceeded the dosing interval, leading to doxorubicinol accumulation in heart. This accumulation was unrelated to plasma levels of doxorubicinol, which had declined 3 h after the doxorubicin injection without producing detectable amounts of doxorubicinol in heart.[156] In contrast, elevated plasma

levels of doxorubicin produced increased cardiac and pulmonary concentrations of doxorubicin, allowing computation of the cardiac (or pulmonary) to plasma ratio of doxorubicin. Multiple injections of doxorubicin failed to alter the cardiac (or pulmonary) to plasma ratio of doxorubicin; in both heart and lung, tissue levels were approximately 50-fold higher than plasma levels. Despite similar amounts of doxorubicin in heart and lung, doxorubicinol levels were 8 times higher in heart than lung (after the last of the 3 weekly injections of doxorubicin).

Heart has also been shown to selectively accumulate the C-13 alcohol metabolite of 5-iminodaunorubicin. Peters et al.[157] measured tissue concentrations 1, 2, 4, 8, and 24 h after injecting rats with 5-iminodaunorubicin (16 mg/kg i.v. or i.p.) In plasma, liver, lung, and brain, the concentration of the alcohol metabolite peaked in 2 to 4 h and then leveled off or decreased. In contrast, the metabolite concentration in heart steadily increased during the 24-h period following an i.p. injection. After the i.v. dose, area under the time-concentration curve (AUC) for metabolite was greatest in lung and heart (265 for heart, 299 for lung, 84 for liver, 5 for plasma, and 2 for brain nmol-h/g for 0 to 24 h). However, only in cardiac tissue was AUC greater for metabolite than for parent drug (AUC for metabolite was nearly twice that for parent drug after i.p. injection).

The cardiac concentration of metabolite may be a determinant of the degree of cardiac dysfunction caused by treatment with the parent anthracycline. Injections of doxorubicinol into rats (either a single dose of 9 mg/kg i.v. or 3 weekly doses of 3 mg/kg i.v.) caused ECG changes that were associated with very low levels of doxorubicinol (2.5 ng/g wet weight).[158] Rat atria contained 1.2 μg doxorubicinol per gram of wet weight 24 h after a single dose of doxorubicin (6 μg/kg),[159] and mouse heart contained 4.1 μg doxorubicinol per gram of wet weight 24 h after a single dose of doxorubicin (15 mg/kg).[134] Based on the above studies coupled with data from isolated heart, it would appear that the intracardiac doxorubicinol concentrations which result from *in vivo* treatment with doxorubicin are sufficiently high to exert cardiotoxic effects (Figure 2).

Heart does not appear to sequester doxorubicinol via transsarcolemmal influx since plasma concentrations rise and fall without producing measurable cardiac concentrations of doxorubicinol.[134,155,156,158] Cardiac doxorubicinol levels observed *in vivo* probably result from intracardiac metabolism of doxorubicin to doxorubicinol, as observed in rabbit heart slices incubated with doxorubicin (Figure 5).[160] Rabbit atria incubated with 175 μ*M* doxorubicin generated microgram amounts of doxorubicinol per gram wet weight of tissue in 180 min (Figure 5).[160] In another study, various tissues were incubated with 35 μ*M* daunorubicin,[161] and after 90 min of incubation, daunorubicinol appeared in brain, liver, lung, heart, small intestine, kidney, and skeletal muscle. Interestingly, kidney and heart, two organs that are highly susceptible to daunorubicin toxicity, produced more daunorubicinol than the other tissues. (Heart formed 4.94 μg daunorubicinol per gram of wet weight of tissue.) It appears that the heart accumulates daunorubicinol more rapidly than doxorubicinol (Figure 6),[160,161] probably because daunorubicin has a greater affinity than doxorubicin for anthracycline reductase

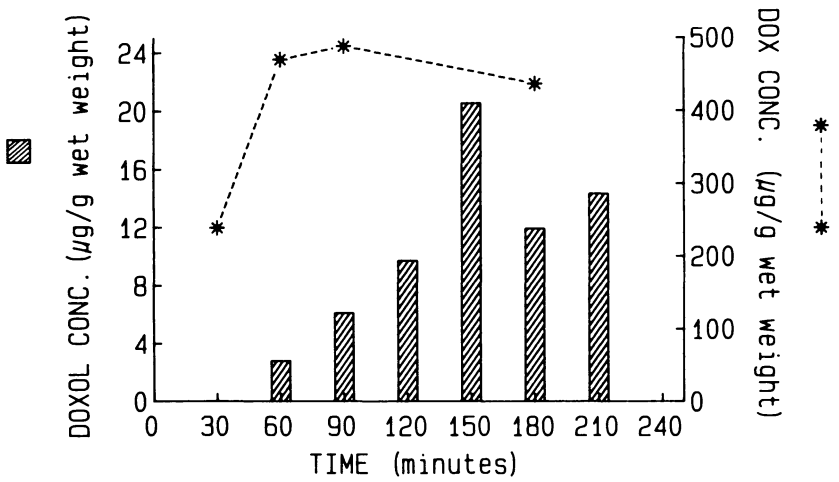


FIGURE 5. Concentrations of doxorubicin (stars connected by dashed line) and doxorubicinol (bars) in isolated rabbit atria incubated for various times with doxorubicin. The x-axis shows time of exposure (min) to 175 μ*M* doxorubicin. The y-axis shows tissue concentrations (μg per gram of wet weight) of doxorubicin (right) and doxorubicinol (left).

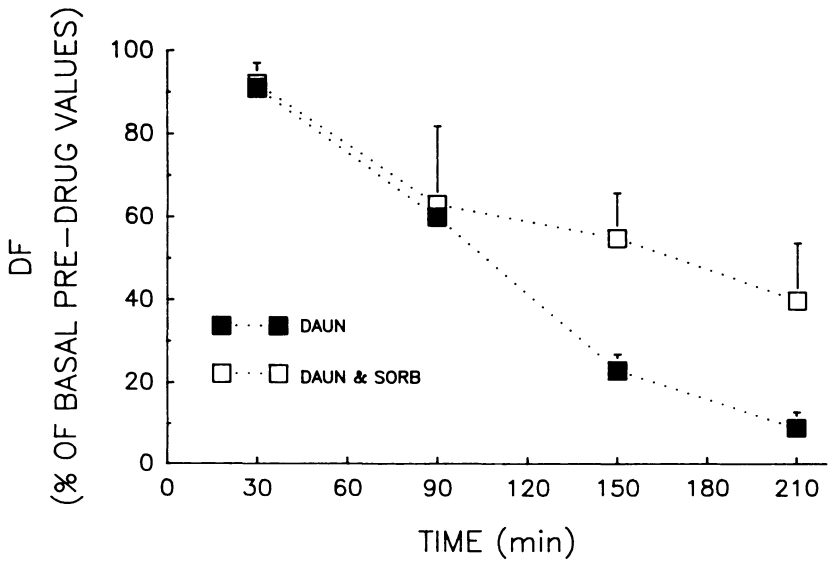


FIGURE 6. Effects of sorbinil (200 μ*M*), an aldo-keto reductase inhibitor, on the time-dependent decay of contractile strength caused by daunorubicin (175 μ*M*) in isolated rabbit atria contracting 2 beats per second. The x-axis shows time of exposure to daunorubicin. The y-axis shows developed force (DF, an index of systolic function) expressed as percentages of pre-daunorubicin values. Solid squares show data from atria incubated with daunorubicin, and open squares show data from atria treated with sorbinil 30 min prior to addition of daunorubicin. Data are mean ± SEM.

enzymes, as suggested by experiments in hepatocytes isolated from rats and rabbits.[162]

B. Metabolite Effects on Cardiac Function

What are the effects of anthracycline metabolites on cardiac function? The C-13 metabolites are 30 to 500 times more potent than the parent compounds to decrease systolic function (Figure 2).[64,65,160,163-166] In isolated rabbit atria contracting twice per second, 6 μM doxorubicinol inhibited systolic function by 50% whereas similar decreases required 298 μM doxorubicin (Figure 2). In addition to these quantitative differences in toxicity, there appear to be major qualitative differences between the cardiac effects of doxorubicin and doxorubicinol. For example, doxorubicin produced a frequency-related inhibition of contractile function whereas doxorubicinol depressed contraction function in a manner that was relatively independent of contraction frequency (Figure 2). Thus, comparisons of potencies of doxorubicin and doxorubicinol as cardiotoxic agents can be confounded by the differing effects of these two agents on the force-frequency relationship.

At slow rates of contraction (e.g., 1 beat per second), potencies to depress cardiac function could not be compared because doxorubicin, at concentrations up to 350 μM (the highest concentration studied), actually increased systolic function (Figure 2). On the other hand, doxorubicinol at 9μM decreased systolic function by 50% in atria contracting once per second (Figure 2). Another variable that confounds attempts to compare potencies of doxorubicin and doxorubicinol as cardiodepressent agents is duration of exposure to doxorubicin or doxorubicinol (Figure 3). We have observed that contractile strength at 1 or 2 beats per second decreased with time in the presence of doxorubicin but not in the presence of doxorubicinol. Doxorubicinol can also impair cardiac relaxation, although the degree of impairment for a given reduction of contractile strength is greater with doxorubicin than with doxorubicinol (data not shown). Studies using ventricular muscle preparations show results similar to the above-mentioned studies in atrial muscle.[160,167]

Intracardiac formation of doxorubicinol offers one explanation of the time-related cardiodepressant effects of doxorubicin. Preliminary data suggest that inhibitors of aldo-keto reductase may be able to modify anthracycline cardiotoxicity. One such inhibitor, sorbinil, used at 100 times the Ki for inhibition of aldo-keto reductase,[168,169] appeared to decrease the time-related depression of cardiac function by daunorubicin (but not the direct effects of daunorubicin) in isolated rabbit atria, conceivably by inhibiting formation of intracardiac daunorubicinol (Figure 6). Furthermore, rabbit atrial tissue exposed to 175 μM doxorubicin for 210 min (Figure 5) produced tissue concentrations of doxorubicinol that were associated with a significant impairment of systolic function.[160] Thus, it seems possible that doxorubicinol mediates the acute, time-related depression of cardiac function by doxorubicin. Whether doxorubicinol contributes to *in vivo* cardiotoxicity remains to be determined, but the above-discussed *in vitro* results

are compelling because the concentrations of alcohol metabolites observed in cardiac tissue *in vivo* cause profound cardiac dysfunction *in vitro*.

C. Metabolite Effects on Subcellular Function

How might doxorubicinol impair cardiac function? Doxorubicinol is a potent inhibitor of the specific ion dependent pumps of sarcoplasmic reticulum, mitochondria, and sarcolemma.[160,167] Doxorubicinol (approximately 4 μM) produced a 50% inhibition of calcium loading of sarcoplasmic reticulum, Ca-Mg ATPase activity of sarcoplasmic reticulum, and F_0F_1 proton pump activity of mitochondria (Mg ATPase).[167] At 10 μM, doxorubicinol inhibited the Na-K ATPase activity of sarcolemma by 50%.[167] This metabolite was also a potent inhibitor of Na/Ca exchange of sarcolemma.[163] By contrast, doxorubicin lacked potency as an inhibitor of these activities: a very high concentration (700 μM) only inhibited calcium loading and Ca-Mg ATPase activity of sarcoplasmic reticulum by 40% and failed to inhibit the proton pump (Mg ATPase) of mitochondria or the Na-K ATPase of sarcolemma.[160,167] Thus, initially, doxorubicinol, by inhibiting both Ca-Mg ATPase of sarcoplasmic reticulum and ATP synthesis by mitochondria, could block loading of Ca into the sarcoplasmic reticulum-releasable pool. Later, as concentrations of doxorubicinol increased intracellularly, ion transport systems such as Ca-Mg ATPase, Na-K ATPase, or Na/Ca exchange may be inhibited.

Although it is unclear how doxorubicinol inhibits these enzyme activities, it is probably unrelated to free radical production because doxorubicin and doxorubicinol have near equivalent activities to generate free radicals in cardiac sarcosomes and mitochondrial NADH dehydrogenase preparations.[170] Furthermore, doxorubicinol inhibits the above-mentioned enzyme activities more rapidly than can be accounted for by a free radical mechanism (unpublished data).

D. Metabolite Hypothesis of Cardiotoxicity

Any hypothesis should account for the cumulative dose and time-dependent nature of cardiotoxicity and explain why heart is particularly susceptible to the toxicity. The hypothesis should also account for histopathologic changes in sarcoplasmic reticulum and mitochondria, which are conceivably the earliest manifestations of anthracycline cardiotoxicity. Also, alterations of both systolic and diastolic function that occur following multiple injections of doxorubicin should be explicable.

How can information about anthracycline metabolites be integrated into a hypothesis of doxorubicin cardiotoxicity? One possible scenario to explain anthracycline cardiotoxicity could be as follows. After an injection of doxorubicin, a fraction of the dose is taken up by the heart, causing transient cardiac dysfunction (acute toxic effect). This dysfunction improves as doxorubicin diffuses out of myocytes. Concurrently, some doxorubicin is metabolized to doxorubicinol via cardiac anthracycline reductase enzymes. Doxorubicinol, being more

polar and less likely than doxorubicin to diffuse out of myocytes, actually accumulates in the heart with subsequent injections of doxorubicin. After a cumulative dose in the 200 to 300 mg/m^2 range, myocardial doxorubicinol levels become sufficiently high to cause subclinical toxicity (e.g., histology lesions on subendocardial biopsies and diastolic dysfunction). The heart becomes compromised and more susceptible to a variety of toxins, including doxorubicin and doxorubicinol. Diastolic dysfunction worsens as calcium uptake into the sarcoplasmic reticulum is further impaired and mitochondrial ATP production diminishes. As the total cumulative dose approaches 550 mg/m^2, elevated intracardiac concentrations of doxorubicin and doxorubicinol act synergistically to markedly compromise cardiac function. At this point, doxorubicinol inhibits the proton pump and doxorubicin interferes with electron transport (cytochrome c oxidase), thereby preventing mitochondria from meeting the increased energy demands imposed by a cytoplasmic calcium overload. Systolic dysfunction worsens as doxorubicin depletes the calcium pool of the terminal cisternae by opening SR release channels and doxorubicinol prevents repletion of this pool by inhibiting the pump that transfers calcium from the cytoplasm to the sarcoplasmic reticulum (i.e, Ca-Mg ATPase). Because only a small amount of calcium is available to the myofibrillar apparatus for active contraction, heart failure results.

X. CONCLUSION

In conclusion, anthracycline cardiotoxicity appears to result from an alteration of calcium homeostasis through a mechanism that remains to be clarified. The free radical hypothesis is a popular explanation, but free radical scavengers have not been uniformly effective in preventing doxorubicin cardiotoxicity. A speculative and intriguing hypothesis is based on the following data and reasoning: (1) doxorubicinol is more potent than doxorubicin as an inhibitor of systolic and diastolic function in isolated cardiac preparations; (2) doxorubicin lacks potency as a cardiotoxin *in vitro*; conceivably, the high concentrations or long durations of exposure required to produce cardiotoxicity *in vitro* may be dependent upon intracardiac metabolism of doxorubicin to doxorubicinol; (3) concentrations of doxorubicinol generated by incubating cardiac preparations with doxorubicin nearly abolish calcium loading and markedly inhibit the ATPase activities of isolated preparations of sarcoplasmic reticulum, mitochondria, and sarcolemma; and (4) doxorubicinol accumulates in heart to a greater extent than in other tissues, which may explain the high susceptibility of heart to doxorubicin toxicity. The metabolite hypothesis needs to be thoroughly evaluated; it is testable and encourages experiments with both *in vivo* and *in vitro* models of anthracycline cardiotoxicity. Ultimately, the value of this hypothesis will be determined by its utility to enhance the safety of anthracycline therapy.

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Chapter 4

CARDIOTOXICITY OF CATECHOLAMINES

Gordon L. Todd

TABLE OF CONTENTS

I. INTRODUCTION

Catecholamines, which represent a subclass of sympathomimetic amines, are ubiquitous throughout the body in their distribution in sympathetic nerves, adrenal gland, or selected regions of the central nervous system.[1] They are neurotransmitters and neuroendocrine hormones that are essential for the regulation of most body functions because of the presence of adrenergic receptors in essentially all organ systems. Despite their essential role and widespread occurrence, both the naturally occurring and synthetic catecholamines can accumulate to such levels that they become toxic agents. Catecholamine excess can undoubtedly affect a number of organ systems, but its most profound effect is on the cardiovascular system. One of the first reports of the toxic effects of catecholamines was by Pearce in 1906 who characterized the histopathologic changes as a form of myocarditis.[2] In 1907 Josue reported hypertrophic effects from chronic administration of epinephrine (E).[3] It was not until more than 50 years later in 1958 that reports appeared that clearly demonstrated the cardiotoxic effects of norepinephrine (NE).[4-6] The following year Rona and his colleagues began an extensive study extending over the next 30 years on the cardiotoxic effects of catecholamines, particularly the synthetic catecholamine isoproterenol (ISO).[7-9] Other pioneers in this emerging field included Raab[10-12] and Selye[13,14] who promoted the importance of the role of catecholamines in a variety of cardiovascular diseases, especially stress-induced cardiovascular disease.

This review focuses on the current status of the cardiotoxic effects of the major sympathomimetic compounds. While there are features in common with the various catecholamines, there are also profound differences which emphasize the need to examine the underlying multifactorial pathogenesis.[7] The chapter includes a summary of the voluminous older literature on experimental catecholamine-induced necrosis and more recent information on the cellular aspects of this catecholamine stimulation in an effort to elucidate the relationship between human and experimental myocardial disease and the pathophysiologic mechanisms responsible for the cardiotoxic effects.

II. TOXICITY

Like most drugs, the cardiotoxicity of catecholamines is primarily related to a dose-dependent effect on their pharmacological properties. It can involve physiological, electrophysiological, biochemical, and structural responses. Additionally, cardiac toxicity can be significantly modified by predisposing factors, which include acute and chronic responses, species differences, drug interactions, underlying disease conditions, and environmental factors. The predominance of previous studies involved very high pharmacological levels of catecholamines at which significant responses were produced. A recent editorial has suggested that massive release of catecholamines may result locally in very high levels thereby justifying this use of large doses in experimental studies.[15] At the other extreme, there is a real paucity of the more clinically useful information on the threshold levels for each of these parameters.

The median lethal dose of a single subcutaneous injection of *l*-epinephrine in rats was reported to be 6.1 mg/kg, *l*-arterenol (NE) was 8.6 mg/kg, and ISO was 581 mg/kg.[16] While ISO is close to 100 times less toxic than E or NE, doses as low as $^1/_{512}$ of the LD_{50} were still capable of producing cardiac necrosis.[16,17] Doses of ISO of 340, 170, and 85 mg/kg for two days produced deaths of 90, 50, and 10%, respectively, in rats. Doses above 170 mg/kg also produced lesions outside the heart.[8] Animals treated with 85 mg/kg on two consecutive days exhibited the most severe cardiac necrosis and enlargement, as well as more consistency in their lethal responses. This became the standardized regimen most used by Rona and other investigators.

Physiologically, ISO, over a broad dose range, produced reasonably similar increases in rats of both heart rate (HR) and force of contraction, but more varied decreases in blood pressure.[18,19] Balazs and Bloom[20] reported in dogs a 30% decrease in systolic blood pressure and a 72% decrease in diastolic pressure with a pharmacological dose of ISO (2.5 μg/kg/min). At this same dose, we observed in dogs a 52% increase in heart rate, a 26% decrease in systolic pressure, and a 52% decrease in diastolic pressure.[21] With NE treatment there was only a 30% increase in heart rate, but more significant increases in blood pressure with an 83% increase in systolic pressure and a 96% increase in diastolic pressure. While heart rate remained elevated throughout the 60 min of infusion, blood pressure increased only transiently and commonly returned toward baseline levels by 60 min.

Electrocardiographically, subcutaneous injections of ISO (100 μg/kg) in rats resulted in ECG changes of both ST segment depression and ventricular arrhythmias including premature ventricular contractions (PVCs) and fibrillation. In conscious dogs, pathological ECG changes reported after 3 consecutive daily s.c. injections of ISO (2.5 μg/kg) consisted of ST segment depression and negative T waves with some ventricular and supraventricular ectopic beats that persisted for up to 24 h.[19] In a comparative study of ISO (2.5 μg/kg/min) and NE (4.0 μg/kg/min) at doses that produced comparable degrees of pathological

necrosis, remarkable differences were seen in the ECG responses.[22] ISO resulted in ST segment depression but limited ectopic activity whereas NE exhibited no ST segment change but a significant increase in ventricular ectopic activity with PVCs and runs of ventricular tachycardia. These responses with NE were generally transient with a return to a sinus tachycardia after 10 to 15 min. There was no evidence with any of the catecholamines that the arrhythmias developed into fatal ventricular fibrillation.[21-23]

There are also differences in toxicity when based on morphologic criteria. In rats, ISO was more cardiotoxic than either E or NE.[24] The natural catecholamines required doses closer to their LD_{50} to elicit myocardial necrosis.[16,17] Balazs and Bloom[20] reported that as little as $^1/_{1000}$ of the lethal dose of ISO can produce focal myocardial lesions. The toxic effects of ISO, which were more predominant in the subendocardial region because of decreased perfusion pressure, were 1000 times less in smaller and/or younger rats (200 g) than in larger rats (500 g).[20]

In addition to direct toxic effects, the relative toxicity of catecholamines can be influenced by several predisposing or external factors. There are substantial species differences as exemplified by a dose of ISO of 2.6 mg/kg in the rat which caused only mild cardiac lesions but in the dog caused severe cardiac necrosis.[19] The LD_{50} after a single s.c. injection in the dog was less than 10 mg/kg.[19] However, an intratracheal route of administration of as much as 75 mg of ISO in dogs was not lethal. It did, however, produce significant increases in heart rate (HR), ventricular arrhythmias, and ST segment depression.[25] I.v. infusions of ISO as low as 0.15 mg/kg produced significant hemodynamic changes but also irreversible pathological lesions.[26,27] The overall cardiotoxicity of catecholamines are also influenced by environmental factors such as social interaction with a median s.c. lethal dose of ISO below 50 mg/kg in isolated rats but greater than 800 mg/kg in group-caged rats.[28]

III. VASCULAR EFFECT

On a systemic basis, E is different from other catecholamines because it produces a mixture of vasodilatation and vasoconstriction.[29,30] It increases cardiac output and, therefore, raises peripheral blood pressure despite its concurrent vasodilatation.[7] Norepinephrine causes pure vasoconstriction while ISO produces pure vasodilatation.[9,29,30] These peripheral vascular differences have been used to support the qualitative histological and ultrastructural differences between the various catecholamines.[7,9,31-33] E was reported to induce mostly reversible cardiac cell injury while NE and ISO produced irreversible injury.[30] It does not explain, however, the similarity of the key morphologic feature of myocardial contraction band lesions in spite of their markedly different hemodynamic, ECG, and metabolic parameters.[21-23,26,34]

Catecholamines also exert independent effects on the coronary circulation which have been debated for a number of years. It is generally agreed that the primary response is dilatation,[35] yet, there have been numerous reports of

decreased coronary flow.[36] The inotropic effect of ISO results in increases in both intramyocardial and intracavitary pressures. However, these myocardial changes when coupled with the peripheral hypotension, lead to a decrease in coronary blood flow.[37,38] The decrease in coronary flow has also been ascribed to an overall depressor effect of ISO which leads to coronary dilatation, but which is not able to adequately compensate for both the directly and indirectly stimulated metabolic demand.[39] Using radioactive microspheres, our group demonstrated an overall increase in coronary blood flow in response to ISO, but it was not uniform across the wall.[26] There was a reduction in the endocardial/epicardial ratios and increased vulnerability of the endocardial third of the wall, which correlated with the transmural gradients in metabolic parameters.

Differences in responses of the coronary vasculature have also been reported with the other catecholamines, NE and E. In a study in isolated coronary vessels, the small coronary arteries consistently exhibited dilatation mediated exclusively by beta receptors.[40] The large coronary arteries exhibited either contraction or relaxation mediated by both alpha and beta receptors. Utilizing a different approach with the ultrastructural tracer, horseradish peroxidase (HRP), Boutet et al.[41] reported striking differences between the three catecholamines in the uptake and passage of HRP through the microvasculature. They ascribed these differences to their diverse effect on the coronary microvasculature. The combination of factors leading to decreased coronary blood flow and perfusion pressure with ISO, especially in the subendocardial layers,[26,42] resulted in a decrease of capillary endothelial transport of HRP.[7] The tracer was readily transported with NE where it appeared within myocardial cells after only 10 minutes of infusion.[41,43]

In contrast to the above reports of differences in receptors or imbalances between demand and supply, Ostadal et al.[44,45] and Handforth[46] reported that mechanical or dynamic obstructions of the coronary circulation may be a contributing factor in catecholamine-induced injury. However, few of the more recent studies report any obstructions of the coronary circulation raising questions about the functional significance of such obstructions. Possible exceptions to such a conclusion are the clinical reports of vasospasm (see discussion below).[47]

IV. MYOCARDIAL EFFECTS

A. Comparative Terminology

The high incidence of coronary heart disease and myocardial infarction and the presence of features in common with catecholamine-induced necrosis has led on occasion to the classification of pathologic features of catecholamine-induced necrosis as synonymous with the generic term myocardial necrosis. Most standard textbooks of pathology classify cell death or necrosis as one of three general categories: (1) coagulative necrosis, (2) liquefaction necrosis, or (3) caseous necrosis,[48] while more specialized textbooks of cardiac pathology and scientific publications expand this to a host of additional terms.[49]

In an extensive series of autopsy studies and reports, Baroldi has characterized three different types of myocardial necroses based on their acute features, functional state, and chronic resolution.[50-52] The most common form of irreversible damage to the myocardium is ischemic or coagulation necrosis, pathognomonic for myocardial infarction. It is characterized by early nuclear and mitochondrial changes and loss of contractility with death of the cell in an atonic state.[50,52,53] Except for the elongation and wavy appearance as a result of the loss of contraction,[54] the myofibrillar changes are delayed until later stages when there is an early exudation and centripetal polymorphonuclear leukocytic infiltration followed by granulation tissue and fibrotic repair.[50,53,55,56]

Another form of myocardial necrosis described by Baroldi is that found with cardiac failure and certain forms of cardiomyopathies, particularly alcoholic. This form of death of the cell occurs in a progressively failing state and is termed colliquative myocytolysis.[50,53] The cells are characterized by an early intracellular edema, vacuolization, and dissolution of the myofibrils without the formation of contraction band lesions. There is minimal nuclear change during the acute phase and little inflammatory infiltration during the chronic repair phase.

A third pattern, completely different both acutely and chronically from the other two forms of myocardial necrosis, has been defined by Baroldi as coagulative myocytolysis.[50,53] In this category the myocardial cell dies in a hypercontracted state with coagulation of the myofilaments to form contraction bands interspersed with areas of myofibrillar rhexis. Except for some nuclear clumping there are no early nuclear changes and there is little or no polymorphonuclear leukocytic infiltration. This form of myocardial necrosis is associated with clinical cases of pheochromocytoma, the stone heart syndrome, and clinical and experimental cases of catecholamine excess. The characteristic feature of the acute phase is the *coagulation* of the contractile proteins into contraction band lesions. The chronic phase is characterized by the *myocytolysis* of individual myocardial cells without the associated inflammatory infiltration and fibrosis.

Catecholamine-induced myocardial necrosis represents a spectrum of pathologic changes which over the years has been described by different investigators under a number of terms to emphasize particular features. Schlesinger and Reiner[57] employed the term myocytolysis to characterize the selective, focal dissolution and disappearance of myocardial cells without the typical leukocytic infiltration. Similar features were described by Reichenbach and Benditt[58-60] under the term of myofibrillar degeneration. There is a strong resemblance to the morphological changes seen in "Zenker hyaline degeneration" of skeletal muscle.[61] The most striking feature of Baroldi's coagulative myocytolysis is the presence of contraction band lesions, yet most of the terms that have been used in the past, including the more common terms myocytolysis[57] or myofibrillar degeneration,[58,59] are non-specific or only provisionally descriptive. Ferrans used the more descriptive term of "necrosis with contraction bands".[62] However, our group together with Baroldi felt that the general term coagulative myocytoly-

sis was a more descriptive term to characterize the predominant features of both the acute and chronic phase of the necrosis.[21,34] Under the collective term of coagulative myocytolysis, Baroldi and Todd have further defined two subcategories of the predominant feature of the acute lesion based on the specific type of contraction band lesion: "paradiscal" and "holocytic" (see descriptions below).[21,34] The light microscopic appearance of the paradiscal lesions have not been described in any detail or frequency previously. The holocytic contraction band lesions resembled the numerous previous reports under the variety of terms that include myocytolysis,[57] myofibrillar degeneration,[58] or necrosis with contraction bands.[62]

These different histopathologic features of coagulative myocytolysis suggest that there may be different pathogenetic mechanisms with different underlying biochemical derangements. As stated by Baroldi[53] in an editorial, recognition of the pathologic features of the variant forms of myocardial necrosis is essential before reaching an understanding of the natural history and underlying mechanisms. To facilitate a better understanding of the terminology, chronology, morphologic features, and pathophysiology of catecholamine-induced myocardial necrosis found in the literature, comparative terms and acute features have been listed in Table 1.

B. Morphology

1. Myofilaments

Much of our current knowledge of the cardiotoxicity of catecholamines began with Rona and his colleagues. In 1959, they characterized and clearly established the "infarct-like" description of myocardial necrosis produced by ISO.[8,17,19,65] The lesions with ISO consisted primarily of homogenization of the muscle fibers and interstitial edema.[17] During the refinement of his observations since that early report, Rona has emphasized the distinct features of ISO-induced necrosis from those associated with the natural catecholamines.[7] In a more recent report, Rona and Bier[30] reported that the early changes with ISO were irregular myofilament contraction and contraction band lesion formation, in contrast to NE which produced lysis of the myofilaments. In a series of histochemical and electron microscopic studies of several sympathomimetic agents, Ferrans et al.[31-33,66] explained that the qualitative differences in the ultrastructural features were the consequence of their diverse pharmacological effects. The myofilament changes were limited to thickening and increased density of the Z bands as early as 30 min after s.c. injections of ISO. Obvious myofibrillar disintegration was a later development. He also characterized other ISO-induced myofibrillar changes as hypercontraction lesions with shortened sarcomeres paralleled by an increased thickening of the Z lines.[31] These features were ascribed to an exaggerated demand for oxygen as a result of the cardiac stimulation.[31,33] Other investigators described the primary ISO-induced changes as rupture and disintegration of the myofibrils.[67]

Despite these diverse observations in the literature, a comprehensive com-

TABLE 1
Comparative Terminology and Chronology of Acute Catecholamine-Induced Myocardial Necrosis

Term	Description	Ref.
Focal myocytolysis	Selective, focal disappearance of cells	57
Focal myocarditis	Focal lesions with degeneration of myofibrils, edema and infiltration, hemorrhage	6
Infarct-like myocardial necrosis	Focal to confluent lesions with hyaline necrosis or granular disintegration, edema	8
Zonal lesions	Hypercontracted area confined to intercalated disc without electron-dense band	63
Focal myocytolysis with major contraction bands	Minor contraction bands of dark, wide Z-disc, major contraction bands of sarcomeres contracted into single mass interspersed with pale areas	71
Myofibrillar degeneration	Early clumping and disorganization of myofibrils with dense transverse banding alternating with light staining zones	60
Zenker hyaline degeneration	Cloudy swelling with obscuration of striations with fragmentation into horizontal segments	61
Necrosis with contraction bands	Extreme shortening and disruption of sarcomeres and displacement of myofilaments	62
Coagulative myocytolysis	Cells die in hypercontracted state with early myofibrillar damage of clumping of sarcomeres to form dense, anomalous, acidophilic cross bands alternating with stretched and broken myofibrils	50
Paradiscal and holocytic contraction bands	Paradiscal with single transverse band adjacent to intercalated disc and holocytic with entire cell disrupted with transverse bands interspersed with area of myofibrillar rhexis	21

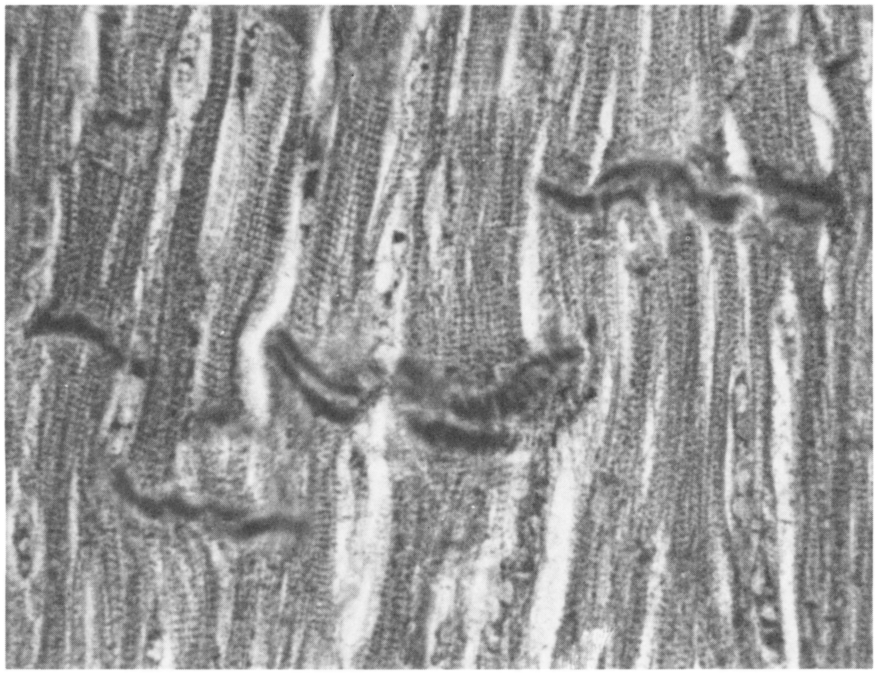

FIGURE 1. Photomicrograph of cluster of paradiscal contraction band lesions in dog myocardium infused with ISO (2.5 μg/kg/min) for 60 min. The two lesions in the center that appear as double lines are actually paradiscal lesions in the ends of two adjoining cells separated by the intercalated disc. Phosphotungstic acid-hematoxylin. (Magnification × 486.)

parative study of the morphological responses to ISO and NE clearly demonstrated a spectrum of changes but in which two distinct forms of contraction band lesions were the predominant histopathologic feature with both catecholamines.[21] At the minimal end of the spectrum of myofibrillar changes were individual cells which exhibited hypercontraction but with the sarcomeres and Z lines maintained in register. The first characteristic form of contraction band lesion, paradiscal, had not been described previously as a prominent feature in association with catecholamines.[21,34] There was usually a bulge in the transverse diameter of the cell opposite the paradiscal lesions (Figure 1). These lesions consisted of a single transverse band located adjacent to the intercalated disc.[21,34] What appeared with light microscopy as double lines (Figure 1) with electron microscopy consisted of single paradiscal lesions in the adjoining ends of two myocardial cells separated by the intercalated disc.[21] There did not appear to be any damage to the disc region,[21] although Rona's group did report an occasional herniation of material through the disc.[68] Mitochondria in the vicinity of the transverse band were displaced into dense clusters surrounding the transverse band at the end of the cell (Figure 2). The remainder of the cell exhibited a normal

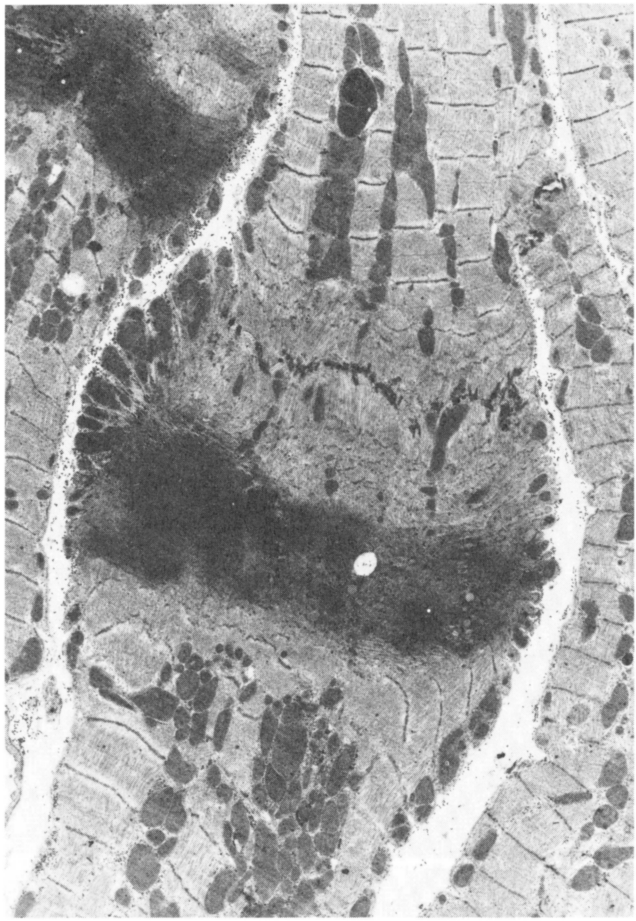

FIGURE 2. Electron micrograph of paradiscal contraction band lesions in two adjacent myocardial cells in a dog infused with ISO (2.5 μg/kg/min) for 60 min. The lesion is at the end of the cell adjacent to the intercalated disc with displacement of mitochondria to the periphery in the vicinity of the lesion. The remainder of the cell exhibits a normal appearance. Uranyl acetate and lead citrate. (Magnification × 6,100.)

architecture. To emphasize the involvement of only a portion of the cell and the characteristic association with the intercalated disc regions, we termed these lesions "paradiscal" contraction band lesions. Their limited size and difficulty of resolving in routine histological sections has undoubtedly contributed to the lack of identification of these possibly reversible lesions by previous investigators. They were reported only as incidental observations in subjects dying after cardiac surgery,[60] experimental catecholamine-induced necrosis,[69] or what was considered the pathognomonic lesions of hemorrhagic shock, zonal lesions.[63,64] While they did resemble the zonal lesions at the light microscopic level, at the ultrastructural level the electron-dense transverse band was not described as a feature of the zonal lesions following hypovolemic shock.[63,64] The ultrastructural appearance of a light-stained zone of hypercontracted sarcomeres in the reports on zonal lesions[64] did not match the histologic appearance of a single, dark-stained transverse band. In our morphologic study with catecholamines, lesions were observed that resembled the ultrastructural appearance of the so-called "zonal lesions", but more frequently paradiscal lesions exhibited an electron-dense transverse band that more closely correlated with the light microscopic appearance.[21] The consistent association with the intercalated disc would suggest a possible selective alteration in some portion of the disc, but to date there has been no evidence to substantiate such a hypothesis. Furthermore, it remains unknown at this time whether these paradiscal contraction band lesions are reversible, since the majority of the cell appears intact.

The larger form of contraction band lesion, holocytic, is similar to what a number of other investigators have reported.[33,57,60,62,70-73] These lesions involve the complete disruption of the normal striation pattern throughout the entire cell (Figure 3). They were given the term "holocytic" to emphasize this involvement of the entire cell and to differentiate them from the smaller and possibly reversible paradiscal lesions. Holocytic lesions ranged from a minimal pattern of fragments of hypercontracted contractile proteins and small areas of early rhexis to coagulation of large blocks of contractile proteins into prominent acidophilic bands extending across the myofiber.[21] The transverse bands were interspersed with more extensive areas of myofibrillar rhexis of stretched and broken myofilaments (Figure 4). In many cases remnants of the Z-line material could be identified and varied from very faint to very thickened material. In some cases the number of sarcomeres clumped together to form these electron-dense bands were small and did not extend all the way across the fiber. At a light microscopic level this pattern would appear as a more granular pattern than the typical prominent transverse bands.

2. Mitochondria

The most consistently reported mitochondrial response to catecholamine excess was the calcium accumulation and subsequent formation of electron-dense granular deposits. Csapo et al.[69] reported calcium influx through altered sarcolemma membrane and the subsequent sequestering in mitochondria as an

FIGURE 3. Photomicrograph of holocytic contraction band lesions (single arrows) together with the dark-stained hypercontracted cell (lower right) and paradiscal contraction band lesion (double arrow). See Figure 1 for additional details. (Magnification × 486.)

early ultrastructural feature. Bloom and Cancilla[71] have reported mitochondrial calcification occurring as early as 2 min after ISO exposure. Other mitochondrial changes that have been reported include structural damage to the cristae[67] and swelling.[71] By two h following s.c. injections of ISO, Ferrans et al.[31,33] observed mitochondrial swelling, vesiculation, and cristolysis. In our light and electron microscopic study, the mitochondria were displaced from their normal intermyofibrillar location to densely packed clusters scattered throughout the cell.[21] In paradiscal contraction band lesions the displacement was limited to a small area adjoining the disc region and the transverse band (Figure 2). Otherwise, the mitochondria appeared normal without any evidence of deposits or swelling. In contrast, the holocytic contraction band lesions exhibited extensive displacement of the mitochondria into densely packed clusters. They also contained two types of deposits: electron-dense, granular deposits which were the more common form and the less electron-dense, amorphous deposits (Figure 5). The mitochondria also were variably swollen, but only infrequently exhibited any breakdown of the cristae.

3. Other Organelles

There is considerable discrepancy in the literature as to which subcellular

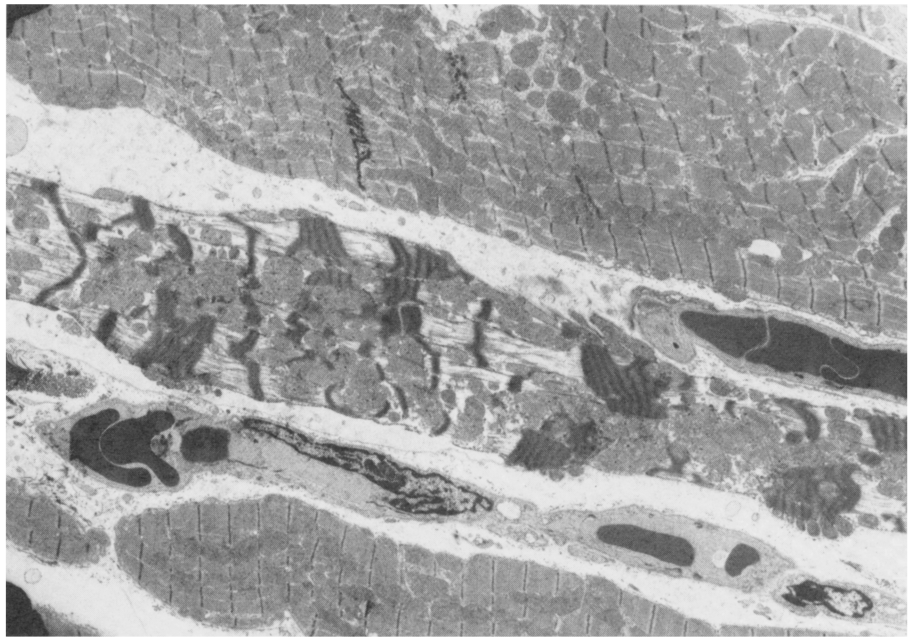

FIGURE 4. Low-power electron micrograph of a single holocytic contraction band lesion surrounded by normal myocardial cells and patent capillaries. The myofilaments are coagulated into electron-dense clumps throughout the entire cell interspersed with clearer areas of stretched and broken myofilaments. See Figure 2 for additional details. (Magnification × 2,940.)

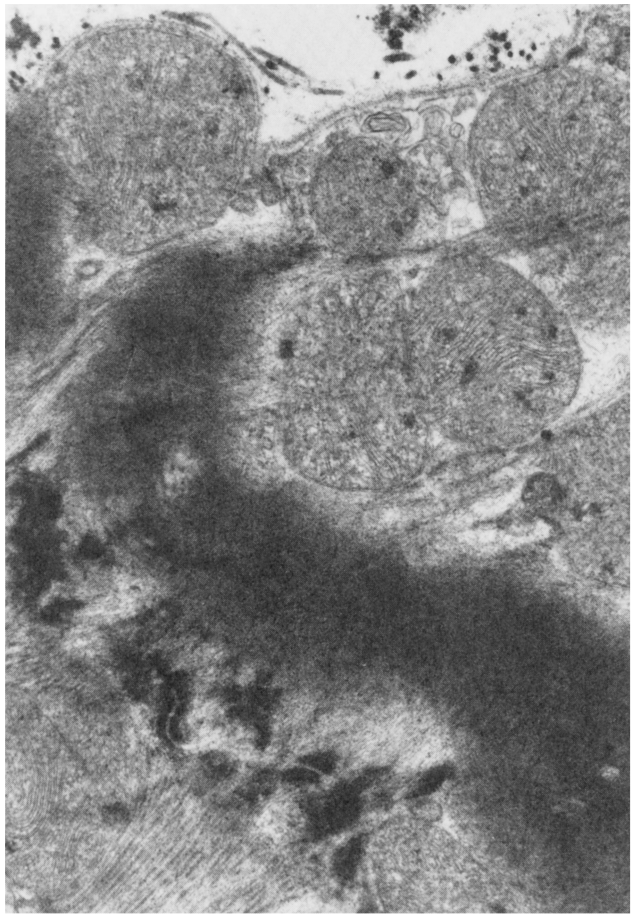

FIGURE 5. Higher-power electron micrograph of a portion of a holocytic contraction band lesion. The electron-dense band represents the coagulation of myofilaments of a number of sarcomeres. The mitochondria in the holocytic contraction band lesion contain electron-dense granules presumed to be calcium deposits while the mitochondria in the adjoining cell are free of deposits. See Figure 2 for additional details. (Magnification × 38,000.)

organelles are affected by catecholamine excesses and the extent of damage. In a series of studies of the various catecholamines, Ferrans et al.[31-33,66] reported lipid droplet formation by 2 h following s.c. injection of ISO. This was accompanied by dilatation of the sarcoplasmic reticulum in the absence of contraction band lesion formation. With infusions of either ISO or NE in our studies, there was a complete destruction of the sarcotubular system in the holocytic contraction band lesions but only minimal damage in the paradiscal lesions.[21,34] After 60 min, some of the damaged holocytic contraction band lesions showed some clumping or margination of the nuclear chromatin material,[21] but little of the nuclear rhexis characteristic of the early stage of myocardial ischemia.[74] There was extensive folding of the sarcolemmal membrane in the vicinity of the transverse bands in both types of contraction band lesions. The membrane appeared intact, but, as demonstrated by others[41,75] and in our isolated perfused heart study,[76] the tracer molecule, HRP, was concentrated in those myocardial cells with contraction band lesions and on a few occasions in cells with an otherwise normal-appearing internal architecture. It was suggested that these normal-appearing cells may represent an early stage of the necrotic process, which adds support to the concept of changes in membrane permeability as an early step.[75,76]

Catecholamine stimulation has been shown biochemically to produce a generalized depletion of glycogen stores.[77,78] With histochemical and ultrastructural studies, Ferrans et al.[31,33] reported selective depletion of glycogen in the subendocardial region. In a combined biochemical, histochemical, and cytochemical study, we reported a dose-dependent depletion of glycogen stores in a transmural gradient which was reinforced with the histochemical studies.[79] Cytochemical localization revealed a cell-to-cell heterogeneity with the contraction band lesions completely devoid of glycogen granules, yet substantial amounts retained in neighboring cells.

4. Conduction System

Under *in vivo* conditions with ISO, a reflex bradycardia initially develops in response to the adrenergic stimulation, which leads to a decrease in sinus dominance, thereby facilitating the appearance of ectopic foci.[80] I.v. infusions or inhalation therapy with ISO have both been shown clinically to produce ST segment depression in 8 patients.[81] Our own experience in laboratory animals suggests only a minor effect on ectopic activity with ISO but a rapid and extensive development of PVCs in the early stages of NE stimulation.[22] Rather than the conduction system, Papp and Szekeres[82] claim direct arrhythmogenic changes due to catecholamines-mediated activation via the beta-adrenergic receptors of the heart.

5. Interstitium and Inflammatory Response

The peripheral vascular effects of the various catecholamines would be expected to exert differential effects on the interstitium. For example, NE

produced prominent diffuse interstitial edema within 10 min, E produced only focal edema, and ISO did not produce any significant edema for several hours.[30] The differential effects on the passage of HRP through the microvasculature and interstitium correlated with these differences.

Some investigators observed little inflammatory response with catecholamine stimulation, which is in contrast to a marked leukocytic infiltration as the hallmark feature of myocardial infarction. Others reported a different inflammatory response with ISO characterized by a complete absence of the typical polymorphonuclear leukocytic infiltration that is the key component of the inflammatory response associated with myocardial ischemia.[83] Instead, infiltration of macrophages dominated, even at the early stages. Unlike the localization of necrotic cells in a contiguous ischemic zone, the catecholamine-induced necrotic cells are diffusely distributed throughout the myocardium with only a few cells per contiguous foci.[21] This distribution pattern with patent coronary arteries may help explain this lack of a significant infiltration.

The loss of contractility and subsequent pulling action of the surrounding normal myocardium with myocardial ischemia led Bouchardy and Majno[54] to characterize waving and elongation of cells as an early pathognomonic feature of myocardial ischemia. In the catecholamine-induced form of myocardial necrosis, a somewhat different pattern of waving of myocardial cells was observed.[21] The hypercontraction of one cell or a cluster of a few cells distorts the surrounding normal cells into a wavy pattern until the hypercontracted cells relax or the connective tissue filaments relax. Generally, the extent of wavy fibers is considerably larger with ischemia, but the important distinction is that waviness of fibers should not be considered pathognomonic of myocardial ischemia.[21]

C. Time Course

While many of the studies of ISO involved two s.c. injections on consecutive days, Bloom and Cancilla[71] reported ISO-induced morphological changes as early as 2 min with hypercontraction and mitochondrial swelling. They reported a progressive increase in hypercontraction and subsequent development of what they termed "major contraction band lesions" and calcification of mitochondria. The inflammatory response did not begin to develop until after 8 h with the peak at 48 h. A time course study with i.v. infusions of ISO (2.5 μg/kg/min) revealed significant numbers of the small paradiscal contraction band lesions by 5 min and the large holocytic contraction band lesions after 15 min of continuous infusion.[23] The number of lesions continued to increase with increasing infusion times up to the 60 min studied. Clearly, catecholamine-induced necrosis is a rapid process developing irreversible changes in a matter of seconds to minutes.

D. Quantitation

In the characterization of the animal model of catecholamine-induced myocardial necrosis, Rona et al.[8,19] developed a grading system to provide semiquantitative space evaluation of the severity of myocardial necrosis. They were able

to show a close correlation between the dose of ISO and the severity of myocardial damage.[16,84] There have been only a few other attempts to quantitate the degree of myocardial necrosis in relation to either the dose or duration of exposure or regional distribution of the lesions. These attempts have included a variety of methods: (1) a histologic grading scale,[8,85] (2) weighing cards with necrotic areas drawn on them,[86] (3) point counting morphometric techniques,[87-89] (4) uptake of radioactively labeled compounds,[90-92] and (5) direct quantitation of contraction band lesions.[21,23,76,93] In a comprehensive quantitative study from our laboratory on the production of contraction band lesions with both ISO and NE, doses of ISO as low as 0.1 μg/kg/min for 60 min were capable of producing a significant number of paradiscal contraction band lesions.[21] The production of a significant number of holocytic contraction band lesions required a higher dose of ISO (1.0 μg/kg/min). The number of either type of contraction band lesion at the highest dose of ISO (2.5 μg/kg/min) was comparable to a dose of NE at 4.0 μg/kg/min.[21] Even when considering the total dose of ISO at 150 μg/kg or NE at 240 μg/kg/min, this is considerably below the milligram levels reported in rats and in dogs.[19]

E. Regional Distribution

The distribution of the myocardial lesions can be characterized by both the regional distribution following i.v. or s.c. injections and the associated distribution in various disease states. Relatively few of the previous studies have examined the regional distribution of catecholamine-induced necrotic changes. The strong inotropic action of ISO increases the intramyocardial and intracavitary pressures which is reported to increase the vulnerability of the ventricular subendocardium.[94] An increased papillary muscle involvement has been reported as confirmation of a greater vulnerability of this layer.[25,33] A more severe subendocardial depletion of glycogen[79] and transmural gradients in other metabolic parameters correlated with the transmural distribution of necrosis with ISO.[26,95] The same was not true for NE where there was not a significant decrease in ATP levels across the wall at doses that produced similar morphologic damage to that with ISO.[96] Quantitatively, the semblance of a transmural gradient in contraction band lesions was the only significant regional distribution pattern.[21] There were more of the larger, more severe, holocytic contraction band lesions in the papillary muscle and endocardial third with ISO than with NE.

There have been only a few reports of other aspects of the regional distribution of myocardial damage. Ferrans et al.[33] have reported more extensive necrosis with ISO in the left ventricular free wall than the interventricular septum. We found no statistically significant difference in distribution in any of the circumferential blocks around the left ventricle.[21] There have been occasional reports of increased necrosis in the apex of the heart with ISO.[88] Our quantitative study revealed fewer paradiscal lesions in the apex compared to the mid or base regions with ISO but more with NE.[21] The larger holocytic lesions were fairly uniformly distributed with ISO but were increased in the apical region with NE.

Guilt by association has frequently characterized the association of contraction band lesions with particular disease states or as a simple artifact. The holocytic contraction band lesions are distributed along the border of permanent myocardial ischemia and occasionally in the normal myocardium surrounding the ischemic zone.[50,58,97] With reperfusion the contraction band lesions are located throughout the ischemic zone,[74,97-101] and depending on the duration of the ischemic period may represent an accentuation of the damage.[102] In disease states such as stone heart, pheochromocytoma tumors, a high percentage of cases of sudden cardiac death, or experimental conditions with high levels of catecholamines with intact coronary arteries, the lesions are diffusely distributed throughout the myocardium.[21] This difference in distribution can be explained on the basis of an initial insult or injury to myocardial cells, but the development of contraction band lesions only in the areas where there is continued supply of substrate and calcium ions and perhaps catecholamines themselves.[99-101,103] They can develop as an artifact of such conditions as myocardial biopsy. However, the distribution is limited to a small zone (<1 mm) from the cut edge and, therefore, can be excluded by carefully monitoring the collection of tissue.[21]

V. EXPERIMENTAL AND CLINICAL MANIFESTATIONS

A. Myocardial Ischemia

The long-held view of an occlusive mechanism in the evolution of coronary heart disease and myocardial infarction has been challenged with reports of the lack of correlation between coronary atherosclerosis, the number of vessels involved, the presence or absence of an acute occlusion, or the size of infarction.[104] With sudden coronary death, coagulation necrosis was observed in only 18.6% of the cases of sudden *expected* death with a prior history of coronary heart disease.[51] In this same study of 102 cases, coagulative myocytolysis was the most frequent acute myocardial lesion (74.9%). As stated by Baroldi, "...coagulative myocytolysis is the histologic 'hallmark' of a primitive 'sympathetic' disorder not necessarily associated with an infarction."[51] This conclusion of the fatal event, especially in cases of sudden cardiac death, being the consequence of a disorder leading to ventricular fibrillation is also supported by studies of the release of catecholamines or by treatment with beta blocking agents. One minute of coronary occlusion produces a rapid activation of the sympathetic nervous system and by 60 min has produced a 10-fold increase in plasma E and a 3-fold increase in NE.[105] "Release of catecholamines is one of the final and crucial steps in ischemic myocardial injury in experimental animals."[106]

There was a significant increase in the number of myocardial cells with coagulative myocytolysis in coronary-occluded dogs developing ventricular fibrillation.[93] The incidence of lesions and frequency of ventricular fibrillation was reduced by treatment with the beta blocker, propranolol. The similarity of the histopathologic features of myocardial infarction and ISO-induced changes lead Rona et al. to characterize this form of necrosis as "infarct-like".[8] There are

numerous morphological, biochemical and physiologic features in common between these two pathological entities so that many of the subsequent studies of catecholamines attributed the changes to myocardial ischemia. However, upon closer examination, there are biochemical changes and structural characteristics of ischemia and catecholamine-induced lesions that are quite different, particularly with the natural catecholamines. While the large holocytic contraction band lesions are seen in both conditions, their distribution is quite different under these two conditions. The lesions are diffusely distributed throughout the myocardium with catecholamines, while they are limited to the border zone around an area of permanent ischemia or throughout the ischemic zone with occlusion and reperfusion. Catecholamines can also aggravate the effect of ischemia, as demonstrated by the beneficial effect of beta blocking agents with myocardial ischemia. Such studies help reinforce the notion that the presence of some features in common does not imply a similar pathophysiology.

B. Sudden Cardiac Death

One of the most difficult clinical entities for which to unravel the pathophysiologic basis is sudden cardiac death. There has been considerable controversy concerning the actual pathologic findings and its relationship to coronary heart disease. Contributing to these controversies are differences in the criteria for selection of cases, methods of examination, definition of the time interval, and premortem symptomatology. Friedman et al.[107] have further tried to differentiate between instantaneous death (seconds to minutes), which is largely due to a primary arrhythmia, and sudden death (<24 h), which usually follows a new acute thrombosis.

For many years the scientific opinion was held that sudden "coronary" death was the consequence of varying degrees of ischemia.[108] In an extensive series of autopsy studies of sudden coronary death (>700 cases), Baroldi and co-workers[50-52] have questioned the classic pathogenic view that sudden death and myocardial infarction are synonymous. In contrast to the expected appearance of coagulation necrosis of myocardial ischemia, the most frequent acute lesion (82%) was the functionally and morphologically different coagulative myocytolysis. More importantly, coagulative myocytolysis was the unique lesion in 69 to 72% of the cases of sudden cardiac death.[51,52] It was speculated that the presence of contraction band lesions of coagulative myocytolysis in the border zone of myocardial ischemia was due to release of catecholamines to reflexly compensate for the loss of contractility.[50] The unique presence of coagulative myocytolysis in cases of sudden death strongly suggest a primary role of catecholamines in the cell death and may represent a histologic hallmark of sympathetic overdrive.[51] Reichenbach et al.[109] have also emphasized a resemblance of sudden death to catecholamine-induced necrosis. Similar features and presumed mechanisms are believed to be responsible for concentric hemorrhagic necrosis and associated contraction band lesions as a result of reperfusion injury (see following).

C. Stress

Numerous studies over the past 30 years have documented that catecholamine-induced myocardial injury is a classic example of stress cardiomyopathy.[13] Klein[83] was one of the early investigators to document that endogenous catecholamine release was capable of inducing significant myocardial necrosis consisting of acute and chronic subendocardial contraction band necrosis. Raab[11] reported that specific emotional responses elicit a selective increase in a particular catecholamine. Thus, a predominance of E is evident under conditions of anxiety while NE predominates during periods of aggressiveness. Release of one or the other of these catecholamines was reported by Raab "to be the common denominator of all stresses in the usual sense of the term".[110] The sympathetic overactivity then leads to myocardial hypoxia and associated metabolic degenerations.[111] More recently, Cebelin and Hirsch[112] classified certain features of the myofibrillar degenerative form of myocardial pathology with contraction bands as a "stress cardiomyopathy". Eighty-eight percent of 15 assault cases supported their hypothesis that susceptible individuals when subjected to stress release excessive amounts of catecholamines which are capable of characteristic pathological changes.

D. Reperfusion Injury

Coronary occlusion of sufficient duration leads to myocardial ischemia, but even reperfusion under certain conditions fails to protect the ischemic myocardium and can even produce a deleterious effect resembling the necrotic changes seen with catecholamine excess.[100,113-115] Upon reperfusion, substrates and calcium and to a varying degree catecholamines are again available and bound to or readily taken up by the viable cells. In partially damaged cells there is excessive influx of calcium presumed to be responsible for the explosive damage upon reperfusion.[99,100] The degree of structural damage or degree of potential recovery after coronary occlusion and reperfusion is dependent on the duration of the initial ischemic period.[98,102] The cell death seen following reperfusion is more rapid in onset and with a completely different pattern than that seen following permanent ischemia which suggest different mechanisms for these two conditions.[116] This difference is further supported by studies with the fine structural tracer, HRP. In permanently ischemic cells there was little affinity of HRP for the relaxed myofilaments while in the reperfusion-injured cells there was strong binding of HRP to the hypercontracted myofilaments.[117]

E. Stone Heart Syndrome and Concentric Hemorrhagic Necrosis

There are two clinical syndromes associated with cardiac surgery that exhibit extensive myocardial necrosis with holocytic contraction band lesions as a hallmark feature. The first category was termed the "stone heart" when it was first described as a small, irreversibly contracted ventricle.[118] This postoperative ischemic contracture occurs in less than 2% of the cases and consistently presents

with hypertrophy and extensive fibrosis. Additionally, the syndrome is characterized by massive contraction band necrosis.[73,119,120] The massive necrosis with large foci of contraction band lesions clearly exhibits a morphological resemblance to catecholamine-induced myocardial necrosis.[120] The proposed mechanisms have centered on an accelerated form of ischemic injury or extreme form of reperfusion injury.[73,118] Because of the histopathologic similarities, Lie and Sun[73] added the possibility of ischemic-triggered release of myocardial catecholamines. At the cellular level in an experimental model of the stone heart, Hearse et al.[121] has proposed that it represents the accumulation of rigor complexes as a consequence of ATP depletion.

The other category is the clinical condition of concentric hemorrhagic myocardial necrosis. This condition also has a strong morphological resemblance to catecholamine-induced necrosis.[101] Unlike the stone heart, this condition is associated with the death of patients undergoing cardiopulmonary bypass with a low cardiac output and large, overdistended ventricles.[122] Microscopically, the condition is located subendocardially around the circumference of the ventricular wall and consists of prominent contraction band lesions in association with concentric subendocardial hemorrhagic necrosis as well as the typical ischemic pattern of coagulation necrosis and granulation repair tissue.[122] This form of necrosis has been hypothesized to be the result of transient hypoxemia during bypass followed by reperfusion and accentuated hemorrhagic necrosis which did not follow the distribution of the coronary arteries.[101,122] The morphologic similarities of these two clinical conditions with reperfusion injury and catecholamine-induced necrosis[101] suggests some commonality in underlying mechanisms, but whether or not catecholamines play a major role in any of them remains to be determined.

F. Pheochromocytoma

As an experiment of nature, pheochromocytomas represent a clinical condition that closely resembles the myocardial damage seen in experimental catecholamine-induced lesions.[123] Pheochromocytomas are catecholamine-secreting tumors, which predominantly secrete NE and exhibit hypertension.[124,125] There have been reports of plasma levels of NE below 1000 pg/ml, but most subjects exhibit levels from 3000 to excesses of 12,000 pg/ml compared to normal levels of 400 pg/ml.[125] One of the first reports of clinically significant myocardial lesions by endogenous catecholamines associated with these tumors was by Klein in 1961.[83] Four patients were reviewed and exhibited extensive acute and resolving subendocardial contraction band necrosis which was described as patchy "degeneration" of myofibrils. There have been a few reports since then that have described similar lesions in this surgically curable condition.[126-128] Ultrastructural studies have confirmed the myofilament involvement along with the deposition of calcium granules in the mitochondria.[126] Furthermore, the condition differs from typical myocardial infarction in the absence of a polymorphonuclear leucocytic infiltration.[128]

G. Vasospasm

Suggestions have been made that endogenous release of catecholamines can cause coronary artery spasm.[15] Maseri et al.[47] have reported on the clinical experience of vasospasm. Postmortem studies have confirmed this hypothesis with the production of vascular smooth muscle contraction bands that reportedly result in regional ischemia and subsequent necrosis.[129, 132] In a recent report of myocardial injury following a drowning, it was hypothesized that the development of both myocardial and coronary artery smooth muscle contraction band lesions were the result of toxic levels of catecholamines.[91] These authors described this condition as a "sympathetic storm". Unfortunately, detailed biochemical and ultrastructural studies to validate the ischemic component of the myocardial lesion are still lacking. Furthermore, there is a lack of pathologic evidence of contraction band lesions in catecholamine-induced vasoconstriction of isolated muscular-sized arteries.[130]

VI. MECHANISMS

A. Relative Ischemia

One of the early investigators, Handforth,[46] reported that ISO-induced injury was the consequence of mechanical or dynamic hindrance of coronary circulation and, therefore, a true infarct and not just infarct-like. The similarities between ISO-induced necrosis and myocardial infarction made the acceptance of a noncoronarogenic mechanism more difficult. In the early studies on ISO it was proposed that the necrosis was due to relative ischemia or hypoxia as a result of direct stimulation of the myocardium and secondary reductions in perfusion pressure. Only at a later date were distinct mechanisms of ISO-induced damage as opposed to NE- or E-induced damage emphasized.[7,9,131] Balazs and Bloom[20] considered that the $beta_2$ agonists, including ISO, produced severe enough peripheral vasodilation to induce hypotension. This response combined with a secondary baroreceptor-mediated tachycardia can lead to adverse cardiac effects in a dose-related fashion. Winsor et al.[129] reported a more regional effect of ISO with only subendocardial ischemia that they described as the result of "subendocardial steal."

The diverse coronary microcirculatory effects of ISO vs. NE and E are the key factors in explaining why ISO is the only catecholamine capable of infarct-like necrosis.[9,115,131] The unique combination in ISO of strong inotropic and chronotropic stimulation and peripheral vasodilatation leads to a decrease in coronary blood flow and resultant relative ischemia, whereas the pressor response and compensated coronary vasodilatation of NE increases coronary flow and exhibits only a focal myocarditis.[9,115,131] Based on these findings, it is appropriate to use the term "infarct-like" but not the entity myocardial infarction because (1) the ISO-induced necrosis is not true ischemia with coronary occlusion, but relative ischemia; (2) it exhibits a different inflammatory response and distribution of the lesions; (3) in a hypermetabolic state without circulatory

stasis there is only a decrease of nutrients without accumulation of metabolic intermediates;[20] (4) regional ischemia is followed by cessation of myocardial contraction reflected by excessively relaxed sarcomeres as opposed to the hypercontracted sarcomeres with catecholamine-induced necrosis;[55] and (5) there is an absence of granular mitochondrial deposits since calcium delivery is restricted.[55] This differentiation is further supported by vasodilating antihypertensive drugs which are capable of producing areas of necrosis in papillary muscle and subendocardium.[133] Additional distinctions between true ischemia and catecholamine-induced relative ischemia include (1) the presence of both types of contraction band lesions in significant numbers in intact dogs with both ISO and NE despite markedly different perfusion pressures[21,34] and (2) the presence of both types of contraction band lesions in isolated heart preparations with ISO but perfused at constant pressures.[76] This raises the question that possibly factors other than ischemia are involved in the pathogenesis of ISO-induced necrosis.

B. Calcium Overload

The pioneering work of Fleckenstein and colleagues clearly established a role for calcium overload in the pathogenesis of catecholamine-induced cardiotoxicity.[134,135] Uptake of $^{45}Ca^{++}$ into the myocardium following isoproterenol administration increased 6- to 10-fold within 6 h accompanied by decreases in ATP and creatine phosphate and the development of cell necrosis.[136] These effects could be reversed by prior treatment with calcium channel blocking agents. According to his scheme, an initial influx of Ca^{++} led to calcification of the mitochondria and depletion of ATP through activation of Ca-ATPases. This, in turn, led to a breakdown of normal membrane permeability and a further influx of Ca^{++}. Interference with energy-dependent processes has been implicated in the necrosis that results from calcium repletion after a period of calcium-free perfusion, the calcium paradox.[137] The necrotic changes, interestingly, resemble the catecholamine-induced contraction band lesions, except that there is both separation of the basement membrane from the sarcolemma and separation of opposing sides of the intercalated disc. In the calcium paradox, there is initially an energy-dependent loss of intracellular calcium during the depletion phase followed by an energy-independent massive influx of calcium during repletion. Similarities also exist with the necrotic changes following hypoxia and reoxygenation (oxygen paradox) or ischemia and reperfusion. However, fine differences exist in the nature and progression of the damage of contraction band necrosis with the calcium paradox, oxygen paradox and ischemic reperfusion and have been reviewed by Ganote.[138] Whether these similarities or differences extend to catecholamine-induced myocardial changes remains to be determined.

Balazs and Bloom[20] suggested that the ISO-induced hypercontraction is a reflection of Ca^{++} influx without the associated proton accumulation and subsequent acidosis. The calcium influx through an altered sarcolemma and calcification of mitochondria is substantiated by the appearance of electron-dense gran-

ules.[21,34,42,71,139,140] The earliest detectable changes were swelling of mitochondria, but deposits appeared within 2 min after ISO administration.[71] Sufficient sequestering of calcium by mitochondria is considered as a major determinant of irreversibility of myocardial injury.[139]

Rather than an increased Ca^{++} influx and accumulation, studies with isolated sarcolemmal vesicles suggest that catecholamines may exert a direct inhibitory effect on Ca^{++} efflux through Na^{+}-Ca^{++} exchange transport across the sarcolemma.[141] While acknowledging the calcium influx, Lehr and co-workers have proposed that this electrolyte redistribution is actually a secondary phenomenon.[67,142,143] In fact, Mg^{++} depletion has been suggested as a common denominator of a number of disease processes.[142]

Despite the acknowledged involvement of Ca^{++} influx, there is still debate in the literature as to whether the calcium overload is the cause or the result of the catecholamine-induced toxicity. This catecholamine-induced calcium influx through the slow channels has been characterized as a direct toxic effect leading to myocardial necrosis.[144] On the other hand, Fleckenstein et al. have suggested that calcium ions alone are cardiotoxic, and by facilitated breakdown and depletion of high-energy phosphate compounds membrane exchange pumps and normal permeability are lost, allowing further influx of calcium.[134,135,145] To carry this a step further, it has been suggested that it is actually this influx of Ca^{++} that is a final common pathway for a number of toxic agents and not specific to catecholamine excess.[146] Meerson[147] has described a calcium "triad" as the final link in the necrotic process. It consists of (1) disturbances of relaxation, (2) activation of phospholipases and proteases which alter the Z disc proteins, and (3) mitochondrial calcification which interferes with oxidation and phosphorylation. It is clear that more biochemical and cellular studies are essential before a more definitive sequence of events and mechanisms can be determined.

C. Membrane Permeability Alterations

Membrane permeability alterations go hand in hand with the calcium overload. Ultrastructural studies with macromolecular tracers have been able to document an increase in permeability as an early event in the evolution of catecholamine-induced cardiac muscle injury.[30,41] NE represents an example of direct cardiotoxic effect as demonstrated by early membrane permeability alterations.[101] ISO, on the other hand, more closely resembles ischemically induced changes with minimal membrane damage.[101] With restitution of more normal blood pressure levels, ISO is capable of inducing more severe damage, similar to that seen with NE and reperfusion injury. However, our time course study with ISO demonstrated numerous irreversible contraction band lesions as early as 5 to 15 min.[23] The contraction band lesions were accompanied by ST segment depression and metabolic changes mimicking those commonly associated with myocardial ischemia, but without the restoration of blood pressure.

As rapidly as 10 min following NE infusion, membrane permeability tracers can be localized in selected myocardial cells that exhibit an otherwise normal

architecture.[30,41,75] It took 60 to 90 min for HRP to penetrate myocardial cells following ISO treatment because of the hypotension.[41] The delayed time or return of blood pressure to normotensive levels was required for the coronary endothelial transport of the tracer and passage through the interstitium to reach the cardiac cells.[9,41,75] These factors along with increased contractility and overstimulation of myofilaments may each contribute to the necrosis.[148] In isolated perfused hearts with ISO, HRP was primarily concentrated in those isolated cells exhibiting contraction band lesions and absent from the majority of the surrounding normal cells.[76] HRP was occasionally seen in cells that otherwise exhibited a normal internal architecture and may represent an early stage of the necrotic process reflecting cells with altered membrane permeability prior to the development of myofibrillar or other organelle changes.[41,76]

While the changes in permeability as demonstrated by membrane tracers or accumulations of electrolytes is well established, the precise mechanisms responsible for the alterations are not known. Lipid peroxidation and free radical formation have emerged as major factors in a variety of cell injuries and may well be active in catecholamine-induced necrosis. Lipid peroxidation produced by catecholamine stimulation has been suggested as a causative agent in the membrane injury[149,150] as well as responsible for the formation of free radicals.[150] The free radicals, by further promoting lipid peroxidation, may significantly increase membrane permeability leading to the cardiac injury.[151,152] Studies to counteract the effect of hypothetical free radicals with such agents as antioxidants like vitamin C either offered protection against ISO-induced lipid peroxidase activity[151] or gave equivocal results.[153] Research by the Russian pathologist Meerson[147] led to a proposed chain of events following catecholamine excess. The initial step in his scheme is activation of lipid peroxidation of the sarcolemma, followed by labilization of lysosomes and subsequent damage to the sarcolemmal membranes followed by the calcium accumulation and terminal series of events described as the calcium triad (see Section VI.B). While this research is suggestive, clearly more needs to be done to understand the influence of free radicals and the sequence and mechanisms whereby they alter membrane function.

D. Oxidation Products

Rather than demonstrating that the catecholamines themselves act as the toxic agent, Dhalla and his colleagues have published a number of studies which emphasize that oxidation products of the catecholamines were also capable of inducing myocardial injury. Oxidized ISO,[154,155] as well as adrenochrome, an oxidation product of E metabolism,[156,157] exerts significant pathological effects on myocardial cells, presumably acting via toxic effects on the cell membrane. Its effect on myocardial contractile function was both dose- and time-dependent.[158] This was followed later in time by damage of mitochondria, sarcotubular system, and contractile apparatus. A discussion of some of the issues related to the findings and conclusions reached by Dhalla and his colleagues concerning

the necrogenic role of the oxidation products of catecholamines has been reported by Rona, who argued against a primary role of these compounds.[7]

E. Platelet Aggregation

The concept of catecholamine-induced platelet thrombi as a major factor in the pathogenesis of myocardial necrosis has been advanced by Haft et al.[159,160] As further support for this concept, agents which inhibit platelet aggregation offered some protection from epinephrine-induced myocardial necrosis.[161] Unfortunately, there are few confirming studies from other laboratories of the significance of the thrombi and the histopathologic consequences.

F. Catecholamine Storage and Receptors

Defects of endogenous catecholamine storage in sympathetic nerve endings may be a contributing factor in catecholamine-induced injury.[162] In addition to an actual defect, Mueller et al.[163] has also considered increased turnover of cardiac NE as a contributing factor. Myocardial ischemia induces a release of NE from myocardial nerve terminals,[164] and is also reported to promote the formation of additional beta receptors in the myocardium as well as peripheral alpha receptors.[165] ISO stimulation in an ischemic milieu has been reported to stimulate beta-receptor formation, resulting in an increase in cAMP and phosphorylase activity.

Enhanced responses and even toxic levels of NE can be produced in the vicinity of sympathetic nerve endings as the result of blockade of reuptake of NE back into the nerve endings. At lower concentrations, cocaine interferes with the neuronal reuptake of NE which can lead to excessive local and circulating levels of catecholamines.[166] Toxic effects of cocaine with its presumed imbalances of catecholamine levels were recently reported in 34 autopsy cases.[167] The most significant pathologic finding was the presence of myocardial contraction band lesions. These authors reported a dose-response relationship between the severity of the myocardial necrosis and the level of cocaine in urine and blood. Unfortunately, catecholamine levels were not determined which could substantiate the presence of catecholamine excess as a possible toxic agent in these autopsy cases.

G. Metabolic Factors

A number of metabolic factors and biochemical changes have been implicated in the pathogenesis and evolution of catecholamine-induced necrosis. Andrieu et al.[168] found that ISO at a dose of 1 μg/kg/min produced a decrease in glycogen and a rise in lactate. They also reported a slower decrease in free fatty acids (FFA) which was confined to subendocardium, but no change in creatine phosphate or ATP. This is in contrast to Pieper et al.[26] who showed a decrease in high-energy phosphates with ISO at a dose of 2.5 μg/kg/min. Others have reported more general changes in FFA with an increased mobilization in

response to the catecholamine stimulation,[169] which would then lead to the increased serum levels that have been reported.[170,171] Clearly, these intermediary metabolites and substrates may be a contributing factor, especially with ISO, in the development of the myocardial necrosis.[172-176]

Catecholamine-induced increases in myocardial cAMP content itself has been reported and considered as a potential contributing factor in the myocardial injury.[177-179] This is supported by the production of lesions similar to those found with ISO that have been induced in both rats and rabbits with treatment with dibutyryl cAMP.[180]

A deranged electrolyte milieu has been cited as a primary factor in catecholamine-induced injury.[143,181,182] Between 3 and 6 h following ISO, there are decreases in Mg^{++} and phosphate together with increases in Ca^{++} and Na^{+}.[67] Lehr advanced the theory that these electrolyte alterations are the result of ionic transfers at the plasma membrane and selected subcellular sites such as mitochondria. The result is an ionic imbalance that contributes to the initiation of a state of irreversible failure of key cell functions.[67] During the course of these studies, he has emphasized a key role of Mg^{++} depletion which may be a common biochemical denominator in the early pathogenesis of a variety of myocardial diseases.[142,183]

Catecholamine stimulation increases oxygen demand and, when coupled with ISO-induced hypotension, is unable to adequately compensate. Inefficient O_2 utilization has been proposed as a possible contributing factor to relative ischemia[184-186] rather than the consequence of it.

H. Environmental Factors

Finally, a host of environmental factors undoubtedly influence the character and degree of catecholamine toxicity. It has been shown that changes in diet can accentuate the severity of the lesions.[187] As discussed above, various forms of stress in animals can lead to the excess production of catecholamines and the subsequent production of myocardial necrosis. Cardiac lesions develop in rabbits when subjected to overcrowding that is severe enough to produce death in close to 50% of the animals within one month.[188] The opposite condition of isolation did not appear to produce any cardiac necrosis directly, but was reported to significantly increase the cardiotoxicity of E.[189]

There is a clinical syndrome, largely familial, known as malignant hyperthermia which is triggered by anesthesia such as halothane or by stress. The condition in severe cases exhibits myocardial necrosis with contraction band lesions.[190] A similar condition known as the porcine stress syndrome found in certain strains of pigs which have a genetically bred stress susceptibility was first characterized by Topel et al.[191] Like malignant hyperthermia, the disease process affects skeletal muscle as well as cardiac muscle with the acute production of contraction bands and hyalinization, followed by chronic myocytolysis and phagocytosis.[192] The common denominator of myocardial contraction band lesions in these two swine conditions and the experimental studies with catechol-

amines should stir continued interest in the characterization of this and other animal models of catecholamine toxicity and sudden cardiac death.

VII. CONCLUSIONS

Catecholamines do play an essential role in homeostasis but are also capable of a cardiotoxic role. The resultant catecholamine cardiomyopathy is a complex, multifactorial entity in its own right. There are clearly established hemodynamic, ECG, neuroendocrine, biochemical, and morphological responses. However, the development of the precise sequence of events at the cellular level and the interaction of these alterations in the pathophysiologic mechanisms are not totally understood. As stated by Boor,[15] "Many of these hypothetical mechanisms are based on older studies...". Clearly more experimental work with the benefit of newer technology needs to be done to elucidate these final pieces of the puzzle. During the process, however, investigators and clinicians have gained important insights into the role catecholamines play in normal and disease processes. Only through continued investigation of mechanisms and identification of the conditions and associated disease states in which catecholamines may assume a toxic role will the development of preventive measures and appropriate therapy be possible.

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Chapter 5

THE TOXIC EFFECTS OF COCAINE ON THE HEART

Allison Anne Welder

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I. INTRODUCTION

A. Cocaine Abuse: An Epidemic in the 1980s and 1990s

It has been reported that in the 1990s we find ourselves in an epidemic of stimulant abuse whch began in the 1980s. Cocaine is the major stimulant of abuse and the denoted "drug of greatest national public health concern".[1,2] Although cocaine has been used for its euphoric effects for thousands of years, in the last 100 years there has been a cyclic pattern of epidemic abuse occurring in the 1890s, 1920s, 1980s, and continuing into the 1990s.[1-5]

The explosion of cocaine abuse in the 1980s which has continued into this new decade has instigated the interest and concerns of the National Institute on Drug Abuse (NIDA), the media, politicians, and the scientific community as to the psychological and physiological dangers of the drug.[1,2] In the last 15 years there has been an upsurge in the number of emergency room visits and deaths due to cocaine abuse.[1,2,5] Currently, in the U.S., there are more cocaine addicts than heroin addicts.[1,2] Cocaine use is prevalent in young, healthy individuals. It has been estimated that approximately 50% of those individuals in the age group of 25 to 30 years old have experimented with cocaine at least once.[1,2] In this decade, it is estimated that 3 to 5 million Americans use cocaine on a regular basis.[1-3,5]

Cocaine was once thought to be a relatively safe and nonaddicting euphoriant.[1-5] It is now well documented that cocaine dependence and withdrawal symptoms occur with repeated use of the drug.[1-5] Individuals seek the use of cocaine for the pharmacologic neurochemical magnification of euphoria and pleasure.[1-3] Most abusers usually self-administer low doses of cocaine in the beginning but quickly discover that higher doses intensify the pharmacologic euphoria of the drug.[1-3] With cocaine dependence, the user becomes focused on the intense feelings that cocaine produces and the user may become socially isolated. High-dose binging can occur with the user experiencing "a kind of cerebral masturbation" in which the euphoria and pleasure derived from the cocaine enables the user to ignore the ensuing personal disaster or death.[1-5] Similar self-destructive behavior has been measured in the laboratory setting.[1,6] For example, with laboratory rats, it was demonstrated that the intense desire for the pharmacologic effects of cocaine was higher than that demonstrated for heroin, a well-documented drug of addiction.[6] Unlike the rats exposed to heroin, during 30 d of self-administration of cocaine, the rats stopped their grooming behavior and lost 47% of their original body weight. After the 30-d period, there was a dramatic 90% mortality in the group of rats that self-administered cocaine compared to 36% in the heroin group.[6] The reported behavioral patterns of human cocaine abuse combined with the documented high mortality rates in animals self-administering cocaine allude to the serious implications of cocaine abuse.

B. History

Historically, cocaine is one of the oldest abused drugs, and it was the first local anesthetic to be discovered.[5,7] Archaeological evidence demonstrates that co-

caine was used as early as 3000 B.C.[5] Cocaine is the primary alkaloid of the plant *Erythroxylon coca,* which was widely cultivated and chewed for its euphorant effects in South America in the regions of the Andes and Amazon.[4,5,7] Incan and pre-Incan Andean civilizations also enjoyed the stimulant effects of cocaine.[5]

The first reported medical use of cocaine was in 1596 by a Spanish physician, Nicholas Monardes.[3] The drug was introduced in the 16th century into Europe, where it was isolated and named by Albert Nieman.[3-5,7] Its local anesthetic properties were discovered by Von Anrep in 1880.[7] It was during the 1880s that Sigmund Freud and Karl Koller, Viennese physicians, began using cocaine clinically.[7] Freud was intrigued by the central actions of cocaine and used it in the treatment of depression, gastric disorders, asthma, and cachexia.[3-5,7] He also frequently prescribed cocaine as an aphrodisiac and for the treatment of morphine and alcohol addiction.[3-5,7] In fact, Freud was successful in weaning one of his colleagues from morphine but at the same time producing one of the first known cocaine addicts.[7] Koller introduced cocaine into the field of ophthalmology as a local anesthetic.[7]

At the turn of the century, cocaine was extremely popular for both its stimulant and local anesthetic properties and was used in many patented medicines.[3,4] It was added to tonics and sodas such as Coca Cola until it was excluded in 1903 when it was first recognized as dangerous in that it was a potential drug of abuse and was capable of producing dependence.[3-5] Finally, it was classified as a narcotic and included in the Harrison Act of 1914, which banned its sale and regulated it as a potentially addicting drug.[7,8]

C. Individuals Who Abuse Cocaine

There is no clearly defined personality for cocaine users.[1,3] In general, the common denominator for cocaine abuse by individuals is the desire to enhance pleasure and their self-image or simply to add excitement to their lives.[1,3] However, it is interesting that the average cocaine user has been reported to be approximately 32 years of age, employed, and highly educated with an approximate $800 (6 to 8 g) -a-week habit. All routes of administration such as injection, inhalation, and oral are used by cocaine abusers with the ready-to-smoke "crack" becoming more and more popular.[1,4,5]

D. Social Implications

Contrary to previous beliefs, cocaine abuse is now accepted as a medical and social problem in the U.S.[1-5] Its use promotes dependence, addiction, and withdrawal symptoms. Furthermore, the street cocaine is becoming more affordable and available.[1,4] Cocaine is particularly popular with individuals in glamorous occupations such as entertainers, musicians, and athletes.[4] Therefore, it is not surprising that the widespread media coverage of the deaths of two popular athletes, Len Bias and Don Rogers, in 1986 brought to the attention of our nation the possible cardiotoxic effects of cocaine.[8-14]

II. CLINICAL MANIFESTATIONS OF COCAINE CARDIOTOXICITY

A. Case Reports

Since 1978, there have been an increasing number of clinical case reports temporally relating both habitual and recreational use of cocaine to life-threatening cardiac events.[15-42] Even more alarming is the fact that most of the individuals in these reports were under 40 years of age, and many had no previous history of heart disease. These cocaine-induced cardiac events were not dependent upon ingestion of large amounts of cocaine. Furthermore, they were precipitated by parenteral means as well as by smoking and sniffing cocaine. Collectively, these studies suggest that cocaine is potentially toxic to the cardiovascular system. The cellular causal mechanisms of this cardiotoxicity remain to be determined.

B. Theories of Cocaine-Induced Cardiotoxic Mechanisms

Recent published reviews have discussed the specific clinical manifestations of cocaine-induced cardiac events in detail.[43-47] Several theories have emerged relating these life-threatening cardiac disorders to the pharmacologic effects of cocaine. Cocaine inhibits the re-uptake of norepinephrine released into the synaptic cleft by the sympathetic neurons, resulting in an excess of this neurotransmitter at the postsynaptic site. Thus, the effects of the elevated amounts of this sympathomimetic agent to produce untoward cardiac events (e.g., vasoconstriction, vasospasm, tachycardia, hypertension, increased arterial pressure, aortic rupture, cereberal vascular hemorrhage, increased myocardial oxygen consumption, contraction band necrosis, and a predisposition to ventricular arrhythmias) can be partially explained on this basis. By virtue of its local anesthetic effect, cocaine can physically block the sodium channel and effectively decrease depolarization of atrial, ventricular, and Purkinje cells, resulting in an increased PR interval and QRS prolongation of the electrocardiogram. Ultimately, the pacemaker cells would be depressed, leading to sinus bradycardia and asystole. Extremely high doses of cocaine have been postulated to be directly cardiotoxic, resulting in cardiac arrest. Finally, the etiology of the coronary thrombus formation related to cocaine use is not understood but it may be related to a cocaine-induced increase in platelet aggregation.

III. EXPERIMENTAL MANIFESTATIONS OF COCAINE CARDIOTOXICITY

A. Autonomic Nervous System Involvement

1. Human Cardiac Responses to Cocaine Administration

Cocaine administered to human volunteers ranging in age from 21 to 45 years has repeatedly been demonstrated to induce alterations in cardiovascular parameters.[48-54] Heart rate and blood pressure were consistently increased in a

dose-dependent manner after cocaine administration.[48-54] Unlike their prototype, cocaine, two other synthetic local anesthetics, lidocaine[51] and procaine,[52] did not significantly alter cardiovascular parameters from controls. Later, it was demonstrated that an acute, transient, cardiovascular tolerance to cocaine can develop in humans.[49] For example, the mean increase in heart rate after i.v. administration of 32 mg of cocaine given 1 h after an inhaled placebo was 32 beats per minute. In contrast, the heart rate increased only 12 beats per minute for those individuals who had inhaled 96 mg cocaine 1 h previously.[49] Even though effects were more variable, similar acute tolerance effects were observed for blood pressure responses to cocaine.[49]

In contrast to the acute tolerance which was demonstrated after the pretreatment of a single i.n. dose of cocaine prior to i.v. administration,[49] there was a consistent increase in pressor effects in individuals who were allowed to self-administer 96 mg of cocaine by inhalation once every 35 min in 6-h sessions.[53] The cocaine-induced pressor effects of heart rate and blood pressure were so dramatically elevated that five out of the six experimental sessions had to be terminated because of consistent elevated diastolic blood pressure. These individuals had a mean cocaine blood level of 437± 78.9 ng/ml (approximately 1.3×10^{-6} *M*) in sessions which lasted approximately 218.3 min. All three cardiovascular indices measured (mean arterial blood pressure, diastolic pressure, and heart rate) continued to increase steadily during the sessions with heart rate reaching an asymptote. It was speculated that the pressor response may be a mechanism for increased cardiotoxicity in the cocaine users.

Most recently, cardiovascular responses to cocaine administration were evaluated under resting and nonresting "stressful" conditions.[48,54] Nonresting conditions consisted of having the individuals perform behaviorally demanding tasks. In both studies, the individuals were seated before an Apple IIe computer and monitor and asked to perform a modified acquisition task.[48,54] Inhalation of 4, 48, and 96 mg of cocaine alone significantly increased heart rate and blood pressure in a dose-dependent manner.[54] These dose-dependent increases in heart rate and blood pressure were further increased by task performance.[54] The data in these studies demonstrate that heart rate and blood pressure are significantly elevated in response to the combination of cocaine administration and task performance. Furthermore, these increases were greater than that induced by either activity alone and were most likely related to inhibition of norepinephrine uptake by cocaine.[48,54] These provide preliminary evidence that the cardiotoxic potential of cocaine may be related significantly to environmental parameters.[48,54] Furthermore, these studies support the theory that the fatal cardiac events of athletes such as Len Bias and Don Rogers in the summer of 1986 may be related to a cocaine/exercise stress interaction.[8-14]

2. Canine Cardiac Responses to Cocaine Administration

The *in vivo* canine animal has been popular in evaluating effects of cocaine on cardiovascular parameters such as electrophysiology,[57-60] hemodynamics,[57,58]

and pharmacologic properties of selected drugs.[55,56,60-65] When *in vivo* systemic evaluations were made of the electrophysiological responses of the heart after cocaine administration, it was found that cocaine increased electrocardiographic intervals in a dose- related manner.[57] The PR, QRS, and QT electrocardiographic readings increased by 14, 12, and 6%, respectively.[57] However, cocaine did not alter diastolic pacing threshold, intraventricular conduction, or repetitive ventricular responses to programmed ventricular stimulation or rapid overdrive pacing.[58] Electrophysiological studies have also confirmed earlier studies[60-65] demonstrating that cocaine induces a cardiac supersensitivity to infused norepinephrine but not to ansa subclavia nerve stimulation.[60] It was concluded that in the normal canine heart cocaine may alter electrocardiographic intervals but does not result in sustained spontaneous or induced ventricular arrhythmias.[57] Yet, in dogs infused with norepinephrine, cocaine potentiates the development of ventricular tachycardia after acute myocardial infarction.[60] The mechanism for this cocaine-induced supersensitivity to infused norepinephrine but not to sympathetic nerve stimulation remain to be determined.

Cocaine administration in dogs has been demonstrated to alter hemodynamic parameters of the cardiovascular system.[55-59] Blood pressure increased after cocaine administration by 19[69] and 24%[66] with corresponding plasma levels of cocaine at 1969 ± 959 ng/ml (5.8×10^{-6} *M*) and 1272 ± 800 ng/ml (3.752×10^{-6} *M*), respectively. In a more recent study, not only did cocaine significantly increase mean aortic pressure, left ventricular systolic pressure, left ventricular end-diastolic pressure, and mean circulatory filling pressure, but it also induced a 58% increase in systemic vascular resistance and a 32% decrease in arterial compliance.[59] It was concluded from this study that i.v. cocaine can have a vasoconstricting effect on both arterial and venous circulations without evidence of inducing vasospasms.

These increased blood pressure responses of the dog heart to cocaine administration can be blunted[55] or enhanced[56] by other pharmacologic agents. For example, pretreatment with hexamethonium significantly depressed a cocaine-induced increase in blood pressure whereas anesthesia with pentobarbital virtually abolished the pressor response.[55] In contrast, pretreatment with atropine or yohimbine alone or the combination of atropine and yohimbine significantly enhanced the cocaine-induced pressor response.[56] Collectively, these studies demonstrate that the cardiotoxic effects of cocaine may be partially dependent upon the control of the central nervous system on peripheral sympathetic tone and presynaptic alpha-adrenergic and cholinergic modulation of norepinephrine release.

3. Rodent Cardiac Responses to Cocaine Administration

In the last year, it was demonstrated for the first time that cocaine acts directly on the rat heart as an antimuscarinic agent.[66] [^{3}H]quinuclidinyl benzilate exhibited high-affinity, saturable binding characteristics in the heart preparations indicative of a homogeneous population of receptor sites.[66] However, when

cocaine was added to the incubation medium, there was concentration-related increase in K_D of [^{3}H]quinuclidinyl benzilate for muscarinic sites of the heart tissue with no effect on the B_{max}. The cyclic nucleotide 5′-quanylylimidodiphosphate did not shift the competition curve, suggesting that cocaine is a muscarinic antagonist. It was concluded from these data that the direct antimuscarinic effects of cocaine on the heart could prevent vagal inhibition of cocaine-enhanced sympathomimetic effects.

B. Central Nervous System Involvement

It has recently been demonstrated that there is a central nervous system component in the cardiovascular responses to cocaine.[52,56,57] For example, both pulse rate and blood pressure were elevated in human male volunteers that were injected with a cocaine placebo after prior conditioning with cocaine.[52] These investigators suggested that central nervous system stimulus substitution was involved in such a classical isodirectional conditioning response.[52] Furthermore, when dogs were generally anesthetized with sodium pentobarbital, blood pressure responses to cocaine were virtually abolished.[56,57] This confounding effect of general anesthesia on cardiovascular responses to cocaine strongly suggest that agents affecting expression of the central nervous system may also affect biochemical endpoints evaluated after cocaine administration. Therefore, the central nervous system variable in cocaine-induced cardiovascular effects should be taken into account when interpreting data from investigations where there has been prior conditioning to cocaine administration or general anesthesia during cocaine administration.

C. Direct Mechanisms of Cocaine Cardiotoxicity

Although the toxic cardiac effects of cocaine have traditionally been attributed to sympathomimetic stimulation, it has recently been demonstrated that cocaine is directly cardiotoxic.[67] The direct cardiotoxic effects of cocaine on beating activity, morphological status, and lactate dehydrogenase (LDH) release were evaluated in primary rat cardiac muscle cell cultures, which is an *in vitro* experimental model devoid of hepatic metabolites and sympathetic and hormonal influences. The cultures were exposed to cocaine concentrations of 1×10^{-7},1×10^{-5}, and 1×10^{-3} *M* for 1 to 24 h. Beating activity was completely inhibited in approximately 50% of the cells after 1-h exposure to 1×10^{-5} *M* cocaine. With those cells that maintained activity, the contractions were weak and asynchronous. In addition, morphological alterations were visible in the primary cell cultures. When compared to the untreated controls (Figure 1), vacuoles (clear inclusions) started to appear after 1 h of exposure to 1×10^{-3} *M* cocaine. By 4 h, vacuolization was abundant and prominent around the nuclei (Figure 2). Most of the vacuoles appeared as dark granules after the 24-h exposure of the cardiac cells to the cocaine (Figure 3). Finally, after 24-h exposure to the highest concentration of cocaine, significant LDH release was observed in the cardiac cells demonstrating sarcolemmal membrane damage.

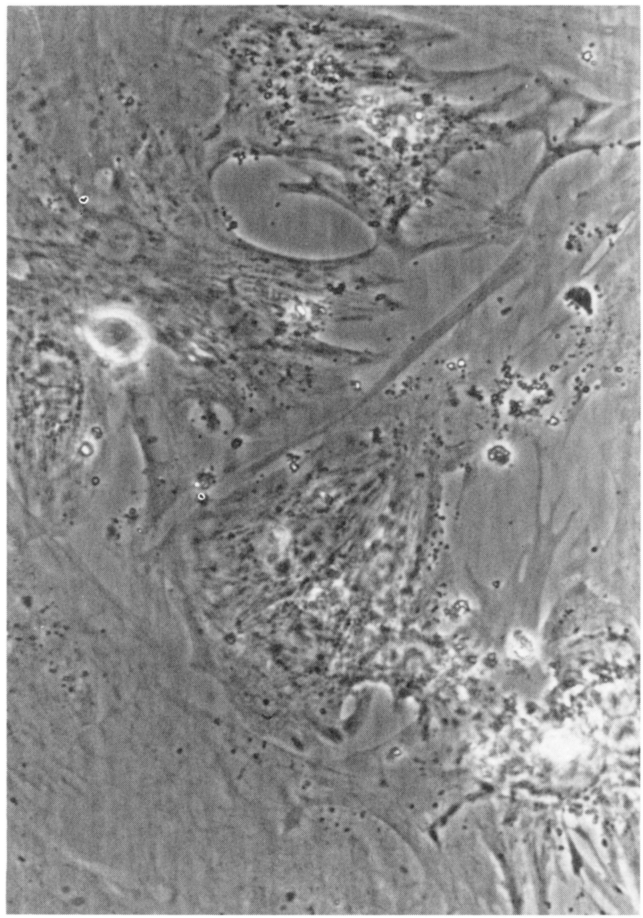

FIGURE 1. Phase-contrast photomicrographs of untreated (control) primary myocardial cell cultures maintained for 4 d. (Magnification × 100.) (From Welder, A. A., Smith, M. A., Ramos, K., and Acosta, D., *Toxicol. in Vitro*, 2(3), 205, 1988. With permission.)

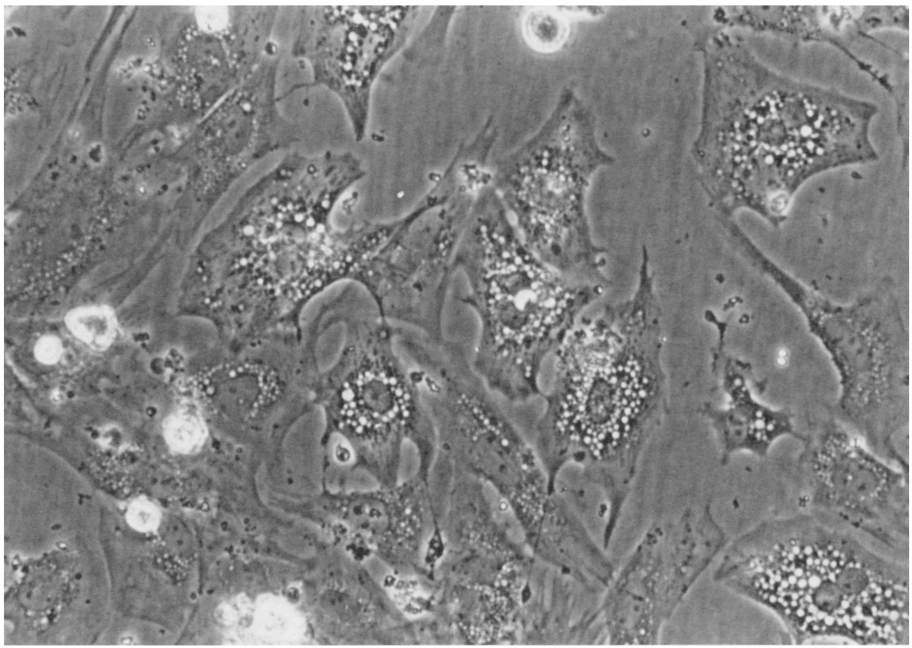

FIGURE 2. Phase-contrast photomicrographs of primary myocardial cell cultures maintained for 4 d (magnification × 100) and then exposed to 1×10^{-3} *M* cocaine for 4 h, showing disruption of the monolayer, pronounced vacuolization around the nuclei, and pseudopodia.

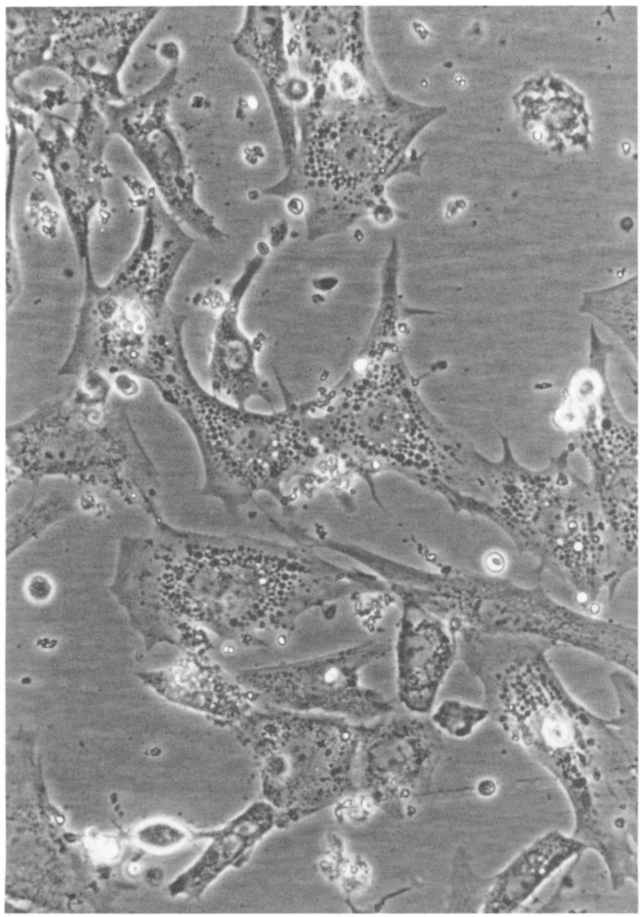

FIGURE 3. Phase-contrast photomicrographs of primary myocardial cell cultures maintained for 4 d (magnification × 100) and then exposed to 1×10^{-3} *M* cocaine for 24 h, showing disruption of the monolayer, pronounced granulation around the nuclei, and pseudopodia.

From these data, a temporal- and concentration-dependent direct cardiotoxic effect of cocaine was established in an *in vitro* cellular model. The cocaine concentrations used in this acute *in vitro* study were based on the postmortem blood concentrations of cocaine reported for "street cocaine" fatality victims.[46] Furthermore, for the first time, a delineation of temporal direct toxic effects of cocaine to cardiac muscle cells, as opposed to *in vivo* toxic effects enhanced by catecholamines and/or metabolites, was illustrated. These data suggest that exposure of the myocardium to cocaine alone is sufficient to induce beating irregularities, morphological alterations, and the release of cytoplasmic enzymes which have previously been associated with cocaine-induced cardiac events *in vivo*. Therefore, primary myocardial cells in culture appear to be an attractive investigative tool for evaluating the cellular and subcellular mechanisms of cocaine cardiotoxicity.

D. Cocaine Hepatic Metabolite-Induced Cardiotoxicity

Cocaine is hepatotoxic in humans and most investigators believe that the free radical norcocaine nitroxide is responsible for this hepatotoxicity.[68,69] Approximately 90% of ingested cocaine is metabolized by hydrolysis and hydrolytic esterases in the plasma and liver to benzoylecgonine, ecgonine methyl ester, and ecgonine. The rest of the cocaine is metabolized by oxidative processes and demethylation to form norcocaine which is rapidly oxidized to *N*-hydroxynorcocaine. It is this *N*-hydroxynorcocaine which is metabolized to the free radical hepatotoxin, norcocaine nitroxide. The levels of certain cocaine hepatic metabolites released into the system and their ability to induce significant cellular target organ toxic effects on the cardiac tissue remain to be determined.

IV. INTERACTIONS WITH OTHER ACTIVE DRUGS OR AGENTS

A. Agents Which Enhance Cocaine Cardiotoxicity

One of the complicating factors in evaluating the toxic effects of cocaine on the heart is the fact that other active ingredients are usually ingested along with the cocaine.[3,32,46] In other words, cocaine is seldom used alone. The other active ingredients can be mixed with cocaine by drug dealers before it is passed on to the user, or they can be ingested at the same time as the cocaine by the drug user. For example, codeine, Librium, norpropoxyphene, propoxyphene, theophylline, lidocaine, nordiazepam, diazepam, morphine, amphetamine, methadone, alcohol, aspirin, and methamphetamine were found in the blood and urine upon autopsy in 30 cases of individuals with cocaine-associated deaths.[32,46] The most prevalent drugs found in conjunction with cocaine in the cocaine-associated deaths were methamphetamine and alcohol found in 33 and 53% of the cases, respectively.[32,46] Currently, there is a paucity of information on the effects and contributions that these drugs might have on a cocaine-induced cardiac event.

However, there is evidence to suggest that alcohol[48] and morphine[70] interact

with cocaine to affect the cardiovascular system. There was a recent case report in which a healthy 31-year-old man developed severe retrosternal pain and circulatory failure after ingesting 150 g of alcohol and a large amount of cocaine.[71] His electrocardiogram demonstrated supraventricular tachycardia and myocardial ischemia.[71] It was concluded that cocaine and alcohol have an additive or synergistic toxic effect on the cardiovascular system.[71] In another study, nine research volunteers were administered various doses of i.n. cocaine and oral ethanol after which heart rate and blood pressure were monitored while resting and performing a serial acquisition task.[48] It was demonstrated that the combination of ethanol, cocaine, and task performance produced greater increases in blood pressure and heart rate than that observed when either variable was administered alone or when cocaine and ethanol were administered together.[48] Finally, the cardiovascular and lethal effects of i.v. cocaine were compared in guinea pigs pretreated with morphine.[70] Although, morphine did not alter the pattern of cocaine cardiotoxicity, it did potentiate the lethality.[70]

B. Agents Which Protect Against Cocaine Cardiotoxicity

Currently, there is no uniform treatment for abnormal cardiac events precipitated by cocaine use.[2,45,47] However, clinical treatment strategies have been suggested for cocaine-induced cardiovascular emergencies which include coronary vasodilators,[2,45] beta-adrenergic blocking agents,[2,45,72] calcium channel blockers,[2,45] amitriptyline,[2,73] aspirin,[2] and antiarrhythmics.[2] Furthermore, experiments evaluating the protective effects of the two calcium channel antagonists nitrendipine[74-76] and verapamil[77] and the tricyclic antidepressant amitriptyline[73] on cocaine-induced cardiac events and lethality have been conducted on animals.

In the isolated perfused rat heart, exposure to 1×10^{-7} to 1×10^{-4} *M* cocaine induced tachycardia and tachyarrhythmias.[74,75] Irreversible cardiac injury and arrhythmias occurred with cocaine concentrations in excess of 1×10^{-4} *M* cocaine.[75] When nitrendipine was simultaneously administered with cocaine, it suppressed the increases in heart rate and regularized the rhythm.[57-58] Propranolol did not alter the cocaine-induced effects.[75] Similarly, when nitrendipine and cocaine were administered together i.a. to Sprague-Dawley rats, cocaine-induced arrhythmias were suppressed, and the survival time and mean lethal dose of cocaine were significantly increased.[74-76] It was also demonstrated by histological examination of sections from the hearts of these rats that nitrendipine protected the heart from acute cocaine-induced morphological lesions.[75,76]

In a recent study conducted with conscious dogs, it was demonstrated that the calcium channel antagonist verapamil can prevent cocaine-induced ventricular fibrillation during myocardial ischemia.[77] When 13 dogs were exercised after occlusion of the left circumflex coronary artery, none of the dogs developed cardiac arrhythmias. The exercise test was repeated after cocaine administration (1.0 mg/kg). Cocaine significantly elevated heart rate and left ventricular systolic pressure, and it elicited ventricular fibrillation in 11 of the 13 dogs. Verapamil attenuated these cocaine-induced cardiac abnormalities.

Finally, it was demonstrated with rats that a single injection of the tricyclic antidepressant amitriptyline provides long-lasting protection against sudden cardiac death from cocaine.[73] Of those animals injected with cocaine (35 mg/kg), more than 50% died virtually instantaneously. However, in the group of rats treated with amitriptyline 24 h prior to cocaine, treatment only one of the six animals died whereas complete protection was afforded to those animals treated with one 10-mg/kg dose of amitriptyline 10 d before cocaine treatment.

V. SUMMARY

Collectively, the studies presented in the discussion above clearly provide abundant evidence that examining the toxic effects of cocaine on the heart is a complicated and challenging issue for investigative scientists to address. These data suggest that there are direct and indirect components to cocaine-induced cardiac events, the direct component consisting of a direct toxic effect of cocaine itself to the cardiac tissue which may be due to its local anesthetic properties. The indirect effects consist of three separate toxic components: (1) an autonomic and central nervous system component which may involve the pharmacologic property of cocaine to block reuptake of catecholamines, (2) a metabolic component which may involve the hepatic metabolite target organ cardiotoxicity, and (3) an ischemia-related component which may involve cocaine induction of coronary artery spasms or toxicity to the endothelium of the coronary arteries, resulting in the reduction of nutrient, oxygen, and blood supply to the heart. In addition, environmental stress such as exercise and other active ingredients such as alcohol, which can be, and usually are, ingested with the cocaine, may enhance cocaine-induced cardiotoxicity. Therefore, a delineation must be made as to the separate contribution of each of these components to the overall cocaine-induced cardiotoxic event. Then synergistic and additive toxic effects of the components must be defined.

Once specific mechanisms of cellular cocaine cardiotoxicity are delineated, the stage will be set for the continued investigation of specific protective compounds for cocaine-induced cardiac events. Currently, there is no standard defined treatment for abnormal cardiac events induced by cocaine. These data discussed from the above investigations emphasize the importance and possibilities of using certain classes of drugs for the prevention and protection of cocaine-induced cardiac events. Therefore, it is important that data from both *in vitro* and *in vivo* experimental models of investigation be used and compared to focus on elucidating cellular mechanisms of cocaine-induced cardiotoxicity and protection.

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Chapter 6

CELLULAR AND MOLECULAR BASIS OF XENOBIOTIC-INDUCED CARDIOVASCULAR TOXICITY: APPLICATION OF CELL CULTURE SYSTEMS

Kenneth Ramos

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I. INTRODUCTION

The cardiovascular system consists of the heart and a diverse vascular network which includes arteries, capillaries, and veins. These divisions form a circular transport system that delivers oxygen and other essential substrates to cells throughout the body and removes the waste products of metabolism. Although circulatory function is carried out in a highly coordinated fashion, major structural and functional differences characterize the various cell types found within the cardiovascular system.

The wall of the heart consists of three distinct layers of mesenchymal origin.[1] The epicardium is the external layer originating from visceral pericardium and consisting primarily of connective tissue. The middle layer is referred to as the myocardium and consists of muscle cells that form the myofibrillar network responsible for contractile function. The innermost layer of the heart is the endocardium, which is formed by a thin sheet of endothelial cells that extend from the intimal layer of coronary vessels to line the chambers and valves of the heart. Coronary blood vessels traverse the epicardium to reach the myocardium and supply oxygenated blood. Cardiac endothelial cells are morphologically and functionally distinct from macro- and microvascular endothelial cells found elsewhere.[2] Muscle cells contract in response to electric or pharmacologic stimuli. Stimulatory signals are transduced from the cell surface to the interior by second messenger systems which modulate ionic gating mechanisms. Excitation of cardiac muscle cells results in a marked increase of intracellular calcium, which in turn interacts with target proteins to mediate muscle contraction. While the primary physiologic role of cardiac myocytes is to support contractile function, some cardiac cells specialize for the generation and/or conduction of electric impulses.

The mammalian blood vessel wall consists of three layers of relative cellular homogeneity.[3] The innermost layer is known as the intima and represents a single layer of endothelial cells. These cells act as barriers to prevent the interaction of blood-borne substances with deeper regions of the vessel wall. Endothelial cells are also involved in the regulation of the structural and functional integrity of the vessel.[4,5] As with endothelial cells of cardiac origin, vascular endothelial cells exhibit a high degree of heterogeneity depending upon their location throughout the body.[6] The medial layer consists of several sheets of smooth muscle cells dispersed in an extracellular matrix consisting primarily of collagen and elastin. The outermost layer consists of fibroblastic cells which serve to provide structural support to the vessel wall and contribute to the regulation of parenchymal cell function.[7]

II. HISTORICAL PERSPECTIVE

The systematic assessment of cardiovascular toxicity using *in vitro* cell

culture techniques was first introduced by Wenzel and colleagues in the early 1970s. Taking advantage of major advances in cardiac cell culture, these investigators developed a primary culture system of synchronously beating cardiac myocytes.[8-10] Their experimental approach emphasized the separation of muscle from non-muscle cells and the use of sensitive indices of cytotoxicity to define cell-specific cardiotoxic responses.[11,12] These studies paved the way for the development and refinement of cell culture systems as experimental models to evaluate xenobiotic-induced cardiovascular toxicity.

A detailed description of the advantages and limitations of cell culture methodology in toxicity testing has been previously presented.[13] Some of the most salient features include (1) accurate control of the physical and chemical environment of target cells, (2) focus on single or multiple cell types, (3) examination of the toxic response in the absence of neurohumoral or rheologic factors, (4) study of transmembrane movements of ions and macromolecules without major diffusional barrriers, (5) simultaneous evaluation of multiple toxicologic endpoints in the course of cellular damage, and (6) application of a reasonable level of biologic variability when cell batches obtained from different animal populations at different times are compared.

III. RELEVANT TOXICOLOGIC INTERACTIONS

A. Primary vs. Secondary Culture Systems

Fetal, newborn, or adult cells of cardiovascular origin from a wide variety of animal species can be successfully established in primary culture. Since the preparation of primary cultures is as much art as science, standardization of the conditions employed to isolate and establish cells in culture is essential to ensure the uniformity of individual cell batches. Specific details for the preparation of primary rat myocardial[14] or vascular[15] cell cultures have been previously described.

The dissociation of myocardial tissue yields a heterogeneous cell suspension which can be separated into relatively pure populations of muscle and non-muscle cells. Non-muscle cells attach to the dishes by 3 h, whereas muscle cells remain in suspension for 16 to 19 h. In contrast, the anatomic distribution of cells within the mammalian vasculature allows the preparation of relatively pure muscle and non-muscle cell suspensions. One must consider, however, that the separation of cell types from vessels of non-mammalian species, such as the avian aortae, may present complications due to a high degree of medial heterogeneity.[16]

Isolated cell suspensions are plated onto a substratum and allowed to attach and flatten out. Various substrates are now available to select for specific cell populations. The substratum may also influence the phenotypic expression of cells in culture.[17,18] Cells are grown to confluence in a medium consisting of vitamins, glucose, minerals, and growth factors. Although media from commercial sources are readily available, special attention must be given to the composi-

tion since slight qualitative or quantitative differences may have major influences on cellular behavior and, thus, toxicologic responsiveness.

Cardiac muscle cells in primary culture establish cell-to-cell contact in the course of cultivation and form sheets of cells that beat synchronously. These cells appear thick and refractile with a granular cytoplasm and uninucleolate nucleus. At the ultrastructural level, large numbers of mitochondria which align parallel to the myofibrills and a dense sarcoplasmic reticulum can be easily identified. Muscle cells have a thick membrane that surrounds the nucleus and gives the appearance of a double nuclear membrane.

Cardiac non-muscle cells constitute approximately 50 to 65% of the cellular suspension prepared by enzymatic dissociation of ventricular tissue.[2] Five non-muscle cell types including endothelial cells, fibroblasts, pericytes, smooth muscle cells, and macrophages have been identified. Endothelial cells constitute the largest subpopulation of non-muscle cells present in this suspension. Unlike endothelial cells of vascular origin, cardiac endothelial cells lining the internal wall of the ventricle are extremely flat and exhibit unique surface morphology and shape. Cardiac fibroblasts resemble fibroblasts in other tissues and are mostly found scattered among the muscle cells within the myocardium. These cells contain a well-developed rough endoplasmic reticulum, abundant free ribosomes, and highly organized perinuclear heterochromatin. Pericytes are structurally similar to fibroblasts and typically found on the periphery of blood vessels. Smooth muscle cells are relatively fewer in number than other non-muscle cell types. These cells are derived from the wall of medium caliber vessels within the heart and can be easily identified by the presence of myofilaments. Although macrophages are found in different regions of the heart, a small number of cells are strongly attached to muscle cells. Macrophages are characterized by filopodia, large numbers of lysosomes, and a highly developed Golgi complex.

The growth pattern of rat vascular endothelial and smooth muscle cells in culture exhibits marked differences.[15] Freshly isolated endothelial cells are small and round. Cells begin to attach and flatten out within the first 24 h. These cells differentiate into oval-shaped cells that proliferate to form small islands of cells within 48 to 96 h. Endothelial cells become more closely packed as a function of time and change gradually from oval to polygonal in shape. At confluence, the endothelial cell monolayer exhibits a "cobblestone pattern".

At plating, smooth muscle cells are round. Cellular differentiation is not observed until approximately 48 h after plating. At this time, smooth muscle cells appear narrow, elongated, and ribbon shaped. The cell boundaries do not exhibit the spiky projections characteristic of fibroblastic cells. Smooth muscle cells usually band together to form a meshwork of growth. At confluency, cells are elongated, spindle-shaped and form a meshwork that displays a "hill-and-valley" pattern.

Vascular endothelial cells grow as a monolayer of polygonal cells with a squamous epithelial cell morphology, whereas smooth muscle cells grow as a

multilayer of elongated cells that align into specific patterns leaving gaps in the meshwork of growth. An extracellular matrix is secreted by smooth muscle cells, which coats the boundaries of the structures formed. These morphologic features have been used to distinguish between endothelial and smooth muscle cells. However, morphologic examination alone is not sufficient to identify multiple cell types in culture. Antigen-specific immunoreactivity and cell-specific enzyme markers are considered more accurate indices of cell identity.[19,20] Ultimately, parameters which assess the functional integrity of the cell should be preferentially employed.

Parallels between cardiovascular cells isolated from different vessels or animal species must be established with caution. For example, the synthesis and secretion of basement membrane components such as collagen and fibronectin by endothelial cells in culture varies according to the source and species.[4] In the case of laminin, another component of the basement membrane, its production rate varies depending upon the level of culture confluence.[21] Prostacyclin is not the major arachidonic acid metabolite synthesized by all endothelial cells.[22] *In vivo*, endothelial cells synthesize tissue plasminogen activator while *in vitro* both tissue and soluble plasminogen activators are found.[23] Subconfluent endothelial cells in culture bind and internalize, low-density lipoproteins, whereas confluent cells bind, but do not internalize, this lipoprotein.[24] As exemplified by these observations, structural and functional differences expressed *in vivo* are likely to be expressed *in vitro* and must be considered in the design and execution of toxicity studies.

The use of primary cell culture systems to study the cardiovascular toxicity of xenobiotics is advantageous because these cells retain the functional characteristics of parent cells. Thus, the responses to toxic insult of cells in primary culture are likely representative of those observed *in vivo*. Unfortunately, the application of cellular and molecular biology techniques in toxicology often demands the availability of large sample pools. Because only a limited number of cultures can be established in primary culture, efforts are being directed towards the development of subcultured systems of cardiovascular cells that can be maintained under stable conditions for long periods of time.

The development of a permanent cell line from cardiac muscle tissue which retains differentiated features has not yet been accomplished.[25] Likewise, no reports describing the subculture of cardiac endothelial cells are available. In contrast, as exemplified by the work of Kan et al.[26] and Knodle et al.,[27] both vascular endothelial and smooth muscle cells are now routinely established in secondary culture. Because differentiated smooth muscle cells retain the ability to divide *in vitro*, serially passaged cultures are easily propagated. Reports describing subcultured smooth muscle cells which retain functional angiotensin II receptors, mobilize calcium in response to pharmacologic stimulation, and express surface alpha-adrenergic receptors are readily found in the literature.[28-30] However, under most circumstances caution must be exercised because the preponderance of myofilaments and the molecular expression of muscle-

specific proteins is often compromised as a function of age in culture.[15] Smooth muscle cell dedifferentiation is reflected by the loss of contractile responsiveness to physiologic or pharmacologic agonists. A similar problem is encountered with subcultured endothelial cells since functional integrity is rapidly lost upon dedifferentiation.[31]

B. Phenotypic Modulation

The use of cell culture systems in toxicity testing has emerged as a powerful tool to evaluate the responses of target cells at the cellular and molecular level. However, as described above, one of the intrinsic pitfalls of culture systems is that cells undergo varying degrees of dedifferentation. From a toxicologic viewpoint, the extent of cellular dedifferentiation must be carefully defined since multiple phenotypic states may exhibit different toxicologic responsiveness and the phenotypic expression of cells in culture may itself be the target of toxic insult. This concept is supported by recent studies in our laboratory which show that allylamine depletes glutathione levels in primary, but not secondary, cultures of vascular smooth muscle cells.[32] The significance of these observations is further emphasized by a recent report which shows that cell lines from different organs do not recognize organ-specific toxins in a predictable and reliable manner.[33]

As a function of time in culture, cardiac myocytes dedifferentiate to resemble more primitive cells which exhibit poorly defined contractile structures and are unable to contract spontaneously.[34] Factors which influence the degree of differentiation of cardiac myocytes include the geometry and age of the culture and the serum content of the medium.[35,36] Under most experimental conditions, cultured cells do not lose their intrinsic potential for differentiation unless they are transformed by intrinsic or exogenous mechanisms.[37]

In recent years, our laboratory has been interested in the development, characterization, and validation of cell culture systems of aortic smooth muscle cells, as well as endothelial cells, from several species to evaluate the cellular and molecular basis of xenobiotic-induced vascular toxicity. Although our approach has emphasized the use of primary culture systems which more closely resemble vascular cells *in vivo*, recent efforts have been made towards the development of phenotypically stable subcultured cell systems. Dedifferentiated cells retain the genetic makeup to respond to appropriate signals in the regulation of phenotypic expression. Evidence is available to support the concept that manipulation of *in vitro* culture conditions can be successfully used to prolong the expression of differentiated features and prevent the regression towards a more primitive state.[38-40]

Morphologic and metabolic differences have been documented among vascular smooth muscle cells as a function of the degree of phenotypic differentiation.[41-43] Under normal conditions, most arterial smooth muscle cells exhibit a well-defined myofilament network and contract in response to physiologic or pharmacologic agonists. These cells are believed to be present in a "contractile

phenotype" which serves to ensure the maintenance of vascular tone. Alternatively, smooth muscle cells may express a different phenotype characterized by enhanced proliferative and synthetic capabilities. These cells are believed to have modulated from a contractile to a "synthetic phenotype". Synthetic smooth muscle cells are characterized by the lack of an organized myofilament structure, as well as the preponderance of rough endoplasmic reticulum and lysosomes and a large Golgi complex. This enhanced proliferative behavior and loss of contractile responsiveness in smooth muscle cells has been considered a feature of dedifferentiated cells. However, differentiation implies a terminal state of phenotypic expression and as such it represents an inaccurate description of the phenotypic profile of smooth muscle cells *in vivo*. Instead, contractile smooth muscle cells retain a limited degree of synthetic function essential for the preservation of the structural and functional integrity of the vessel wall.

The modulation of smooth muscle cell phenotype from a contractile to synthetic state has been achieved under a variety of experimental conditions. Spontaneous alterations from contractile to synthetic phenotypes have been observed in primary cultures of smooth muscle cells isolated from normal arterial vessels.[15,44] Contractile smooth muscle cells isolated by enzymatic digestion remain, if seeded densely (>300 cells/mm^2), in the contractile phenotype for the first 2 to 3 days in culture. After 3 d, the smooth muscle cells undergo a distinct structural transformation and begin to proliferate. Upon reaching confluence, smooth muscle cells revert to the contractile phenotype. Previous studies in our laboratory have shown that plating density modulates the phenotypic expression of smooth muscle cells in primary culture.[15] More recently, preliminary studies have suggested that the cellular growth of smooth muscle cells in secondary culture is also dependent upon culture density.[45]

Chemically induced injury of smooth muscle cells is associated with an increase in synthetic capabilities and loss of contractile responsiveness.[46,47] These changes are thought to represent the phenotypic modulation of smooth muscle cells. Modulation has also been observed following mechanical injury *in vivo*.[48] Injured smooth cells retain their synthetic characteristics *in vitro*. Thus, in contrast to smooth muscle cells isolated from normal arterial tissue, modulated smooth muscle cells *in vivo* remain synthetic throughout the culture period.

IV. CARDIAC TOXINS

A. Doxorubicin

Doxorubicin (adriamycin) is an anthracycline antineoplastic agent widely used in the treatment of malignant neoplasms. Its clinical use is often limited by cumulative, dose-dependent cardiotoxicity. Although the cellular and molecular bases of adriamycin-induced myocardial injury are not yet clear, cardiotoxicity has been related to oxidative stress leading to peroxidative injury of cellular

constituents;[49] alterations in myocardial energetics;[50] disturbances in calcium homeostasis;[51] and/or inhibition of DNA, RNA, and protein synthesis.[52,53]

Seraydarian and Artaza[54] have shown that a short exposure of cultured myocardial cells to adriamycin causes pronounced inhibition of cellular growth. Longer durations of exposure (>12 h) are required before alterations in rhythmic activity and morphologic changes are evident.[55,56] Newman et al.[57] have shown that intracellular enzyme leakage is observed in cardiac, but not skeletal, myocytes in culture upon exposure to toxic concentrations of adriamycin. These observations are consistent with the cardiospecificity observed *in vivo* upon exposure to adriamycin. In agreement with these observations, Lampidis et al.[58] have shown that adriamycin accumulates at faster rates and to a greater extent in cardiac muscle vs. non-muscle cells. While adriamycin-induced structural and functional alterations in the myocardium are thought to be mediated intracellularly, evidence is available suggesting that intracellular drug deposition is not essential for the manifestation of cardiotoxicity *in vitro*.[59] The significance of these observations is not yet clear.

Adriamycin-induced cardiotoxicity is often associated with progressive loss of contractile function that ultimately results in congestive heart failure. Although the molecular basis of this response is at present unknown, recent studies have shown that adriamycin selectively decreases the synthesis of alpha actin, a muscle-specific protein.[60] Consistent with these observations, Rabkin and Sunga[61] have shown that adriamycin disrupts the structural integrity of microtubules and inhibits microtubule reassembly in cultured chick embryonic ventricular cells. Since microtubules are intimately associated with structures involved in the regulation of contractile function, these actions may account for, or contribute to, the contractile derrangements induced by adriamycin.

Acute exposure of cultured myocardial cells to adriamycin is associated with a dose-dependent increase in calcium uptake.[62] This increase appears within 1 min, subsides within 30 s, and is reestablished 10 min after chemical exposure. Since both phases of calcium uptake are reduced in a dose-dependent manner by verapamil (a calcium channel antagonist), these results implicate a voltage-dependent calcium channel process. This interpretation is consistent with the work of Azuma et al.[63] who showed that adriamycin increases slow channel-mediated calcium influx in the chick heart.

B. Isoproterenol

Isoproterenol is a potent stimulant of beta-1 and beta-2 adrenergic receptors. Beta-1-receptor activation is associated with guanine nucleotide binding protein (G-protein) -mediated stimulation of adenylate cyclase.[64] Binding of isoproterenol to the receptor promotes the binding of GTP to oligomeric, stimulatory G proteins. The binding of GTP causes the dissociation of the G protein into at least three individual subunits; one of which interacts with the catalytic subunit of adenylate cyclase to cause an increase in enzyme activity. The resultant increase in cAMP levels is associated with activation of cAMP-dependent protein kinases

which are thought to phosphorylate calcium channel proteins to cause an increase of the slow inward current.[65] This results in a marked increase of free intracellular calcium levels which in turn mediate the positive inotropic and chronotropic actions of the drug.

The mechanisms by which isoproterenol and other catecholamines cause myocardial injury have been summarized in a preceding chapter. This discussion focuses on some of the cellular and molecular alterations associated with toxic insult in cultured myocardial cells. The toxicity of isoproterenol and related amines has been associated with the accumulation of abnormally high calcium levels. However, the mechanism by which alterations in calcium homeostasis are evoked remains the subject of controversy.

The myocardial calcium overload observed upon exposure to toxic concentrations of isoproterenol may be due to excessive stimulation of beta-1-adrenergic receptors. As such, the accumulation of calcium would represent a calcium channel-mediated transport process. Calcium overload in turn leads to failure of calcium regulatory mechanisms, induction of contracture, increased oxygen requirement, excessive ATP breakdown, and activation of degradative proteinases and phospholipases.[66] This concept is supported by studies which show that agents capable of interfering with receptor-mediated calcium transport modulate the expression of toxicity.[67,68] However, a number of studies have been published which argue against this interpretation.[69,70]

Alternatively, Dhalla and co-workers have proposed that oxidative byproducts and/or activated oxygen species play an important role as mediators of isoproterenol-induced myocardial toxicity.[71,72] This proposal is based on the observation that oxidized, but not fresh, solutions of isoproterenol cause necrosis and hypercontractility of the isolated rat heart. Although isoproterenol undergoes oxidation at slower rates than other catecholamines, oxidation at measurable rates under physiologic conditions has been documented.[73] The formation of reactive oxidative byproducts could result in damage to critical cellular constituents. Such alterations may in turn contribute to the alterations in calcium translocation associated with myocardial insult.[74] Rona has consistently emphasized that major differences exist between the toxicologic profile of adrenochrome, an oxidative metabolite of epinephrine and isoproterenol.[67] Such differences are not surprising when comparisons between preformed oxidative metabolites of catecholamines and oxidizing solutions containing multiple oxidation by-products are attempted.

The pattern of calcium uptake induced by toxic concentrations of isoproterenol in cultured myocardial cells shows a biphasic response.[75] The initial phase of calcium uptake is present when cardiac cells are exposed to nontoxic concentrations of isoproterenol and is blocked by agents which prevent calcium channel-mediated transport. In contrast, the second phase of calcium uptake is associated with progressive accumulation of calcium and is only observed in the presence of toxic drug concentrations. This phase is only partially antagonized by propranolol or verapamil, agents which antagonize channel-dependent

mechanisms, but prevented by antioxidants such as ascorbic acid and sodium bisulfite. These observations suggest that oxidative processes play an important role in isoproterenol-induced myocardial injury.

Oxidative damage to membranes has previously been shown to increase calcium permeability.[76] Such alterations are due to increased calcium translocation, deterioration of calcium regulatory mechanisms, and/or enhanced passive calcium permeability in favor of the concentration gradient. However, the ability of oxidative by-products to cause cardiac injury is influenced to a significant extent by the status of endogenous and exogenous antioxidant defense systems. This concept is consistent with previous studies which show that isoproterenol decreases the glutathione content of heart cells *in vivo*[77] and *in vitro*[78] and increases H_2O_2 production *in vivo*.[79]

V. VASCULAR TOXINS

A. Allylamine

A limited number of studies have been reported describing the use of cell culture systems to elucidate the cellular and molecular basis of toxicant-induced vascular injury.[80-82] The application of *in vitro* approaches to assess xenobiotic-induced vascular toxicity is particularly advantageous in light of the high degree of nonspecificity characteristic of most vasculotoxic agents. In this regard, studies which focus on allylamine as a prototypical toxin can be useful in identifying vascular-specific responses since this compound acts selectively to cause atherosclerotic-like lesions in several animal species *in vivo*.[83]

Much of our understanding of the mechanism of allylamine-induced cardiovascular toxicity has been made possible by the contributions of Boor and Nelson. These investigators first proposed that allylamine toxicity results from bioactivation of the parent compound to a toxic aldehyde by benzylamine oxidase.[84] This proposal has since been confirmed and validated using cultured cell systems of cardiovascular cells to evaluate the cellular and molecular basis of the toxic response.[80,81]

Because the expression of vasculotoxic responses is often a multifactorial phenomenom characterized by long latency periods, an *in vivo/in vitro* approach is currently used in our laboratory to evaluate the toxicity of selected xenobiotics. Upon completion of defined dosing regimens *in vivo*, aortic smooth muscle cells are isolated and established in culture. Such a strategy allows the interaction *in vivo* of multiple variables in the expression of toxic insult while facilitating the study of chemically induced alterations at the cellular and molecular level.

Recent studies in our laboratory have shown that smooth muscle cells isolated from animals exposed subchronically to allylamine grow more rapidly in culture than cells isolated from control animals.[85] At confluence, the morphology of cells isolated from allylamine-treated rats is distinct from that of control cells. Smooth muscle cells obtained from treated animals are broad and round and exhibit prominent nucleoli, whereas cells isolated from control animals are

elongated and spindle-shaped. The morphologic pattern of cells cultured from allylamine-treated rats suggest that these cells have modulated from a contractile to a more synthetic state. These observations have been confirmed and extended by recent studies designed to examine the proliferative, synthetic, and functional capabilities of smooth muscle cells upon allylamine exposure.[86] Similar observations have been reported by Thyberg[46] using cultured smooth muscle cells upon repeated exposure to nicotine *in vitro* and by Yarom et al.[47] using smooth muscle cells isolated from animals exposed to T-2 toxin *in vivo*.

Cells isolated from allylamine-treated rats undergo a morphologic reversion from a synthetic to a contractile state upon exposure to dibutyryl cAMP (0.2 m*M*) and theophylline (0.1 m*M*) for 72 h. These observations suggest that elevated intracellular levels of cAMP promote the differentiation of smooth muscle cells *in vitro*. This proposal is supported by previous studies which show that prostacyclin, a cyclooxygenase product known to stimulate adenylate cyclase in various cell types, inhibits DNA synthesis in smooth muscle cells from atherosclerotic aortae.[87] Although the significance of these observations is at present unclear, one can speculate that chemically induced alterations in the proliferation/differentiation mechanisms of smooth muscle cells may be of significance in the development of atherosclerotic lesions.

B. Benzo(a)pyrene

The polycyclic aromatic hydrocarbons benzo(a)pyrene, 3-methylcholantrene, and 7,12 dimethylbenz(a)anthracene cause vascular lesions of atherosclerotic etiology in several animal species.[88,89] Furthermore, the spontaneous development of lesions in avian species can be markedly enhanced by acute[90] or chronic[91] exposure to these agents. Collectively, these observations have led investigators to propose that polycyclic aromatic hydrocarbons play an important etiologic role in human atherogenesis. Because polycyclic aromatic hydrocarbons may require metabolic activation to exert their carcinogenic or atherogenic effects, several studies have been conducted to evaluate the capacity of vascular cells to bioactivate the parent compounds.

Aortic cells catalyze cytochrome P-450-dependent monooxygenation and bioactivation of polycyclic aromatic hydrocarbons.[92,93] Since the major hydroxylated metabolite of benzo(a)pyrene in both hepatic and aortic tissues is 3-hydroxy-benzo(a)pyrene, similar mechanisms may be associated with the initiation and/or promotion of the carcinogenic/atherogenic process. Yang et al.[94] have conducted a series of studies to evaluate the capacity of cultured aortic smooth muscle cells to form sulfated metabolites. These studies have shown that aortic enzymes effectively sulfoconjugate 3-hydroxy benzo(a)pyrene and suggest that aortic sulfotransferases play an important role in reducing local cellular concentrations of toxic phenolic metabolites. Thus, the ultimate toxicity of polycyclic aromatic hydrocarbons in vascular tissue is likely determined by the balance between bioactivation and detoxication reactions.

An initiation-promotion sequence using dimethylbenz(a)anthracene acutely

followed by chronic treatment with methoxamine produces aortic lesions.[95] However, multiple regional specificities for promotion vs. initiation of vascular lesions have been identified. Abdominal segments of aorta are preferential sites for promotion, whereas initiation is typically observed in the thoracic region. In this regard, previous studies have shown that glucuronidation of 3-hydroxybenzo(a)pyrene is specific to cultured vascular smooth muscle cells, but not endothelial cells.[95] The rate of glucuronidation is, however, more rapid in abdominal than thoracic segments and can be induced with phenobarbital. The differential distribution of glucuronyltransferase activity may account for differences in the responses of abdominal vs. thoracic regions to benzo(a)pyrene. This concept is further supported by studies which show that glucuronidation decreases the mutagenic and carcinogenic effects of polycyclic aromatic hydrocarbons in the Ames test,[96] but increases the generation of reactive intermediates in other preparations.[97]

VI. CONCLUDING REMARKS

Because the cardiovascular system is characterized by marked structural and functional heterogeneity, significant variations in toxicologic responsiveness are often encountered. To date, most attempts at elucidating the mechanisms of xenobiotic-induced cardiovascular toxicity have failed to consider cytologic and functional heterogeneity in the assessment of toxic insult. The application of cell culture methodology provides a unique opportunity to specifically address this deficiency.

Continued technologic growth will further advance the evolution and refinement of cell culture systems as models to evaluate the cellular and molecular basis of toxic insult. Ultimately, the data generated can be used to design strategies aimed at reducing the incidence and/or severity of xenobiotic-induced cardiovascular disease.

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