# ANGIOTENSIN II RECEPTOR BLOCKADE: PHYSIOLOGICAL AND CLINICAL IMPLICATIONS

# PROGRESS IN EXPERIMENTAL CARDIOLOGY

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# ANGIOTENSIN II RECEPTOR BLOCKADE
# PHYSIOLOGICAL AND CLINICAL IMPLICATIONS

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This book is dedicated to Dr. Arnold Naimark, who in his capacity as President of the University of Manitoba, promoted the development of the Institute of Cardiovascular Sciences at the St. Boniface General Hospital Research Centre. His extraordinary vision, enthusiastic support, invaluable advice, and superb leadership have helped in making Winnipeg headquarters for the International Society for Heart Research during 1972–1989 and the International Academy of Cardiovascular Sciences (1996–Present).

## A TRIBUTE TO ARNOLD NAIMARK

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The accomplishments of Dr. Arnold Naimark are legion in number and boundless in breadth. Surely they are the issue of genius—a gift most difficult to define. Over a hundred years ago, Henri-Frédéric Amiel came close when he wrote: "Doing easily what others find difficult is talent; doing what is impossible for talent is genius". With an exceptional intellect, enormous energy, and a talent for practicality, he cast a mantle over medicine, science, administration, research, philosophy, education, and business. He is a renaissance man in the age of molecular biology.

Dr. Naimark was born and educated in Winnipeg. He achieved great distinction during his undergraduate studies, winning medals, scholarships and prizes. As an undergraduate he enrolled in a special program of research and study leading to the degree of Bachelor of Science in Medicine, which was awarded concurrently with the Doctor of Medicine degree in 1957. During his undergraduate studies, Dr. Naimark became interested in pursuing an academic career in medicine and physiology. He was greatly influenced by the unique approach to medical education fostered by his mentor Professor Joseph Doupe. The "Doupe school" is regarded by many leaders in scientific medicine, in Canada, and abroad, as the seminal influence in their careers.

Following graduation, Dr. Naimark alternated periods of advanced research training with specialist training in internal medicine. His scientific interests turned to respiratory physiology and the work leading to his Master of Science degree won the Prowse Prize in Clinical Research. A residency in Internal Medicine was followed by research fellowships at the Cardiovascular Research Institute at the

University of California in San Francisco where he undertook studies on the respiratory and metabolic changes during intensive physical work. In 1962, a McLaughlin Travelling Fellowship took him to the Royal Postgraduate Medical School at the Hammersmith Hospital in England where he served as a Registrar in Medicine and Visiting Scientist and participated in pioneering studies on blood flow in the lung.

On returning to the University of Manitoba, Dr. Naimark joined the Departments of Physiology and Internal Medicine and the medical staff at the Winnipeg General Hospital. In 1964, he was admitted as a Fellow of the Royal College of Physicians and Surgeons of Canada. He began an intensive process of reorienting the Department of Physiology from its clinical emphasis to a focus on basic research and graduate training, which resulted in it becoming one of the leading departments in the world. In the hospital, he jointed Dr. Reuben Cherniack in developing a leading laboratory for the investigation of respiratory disease and the first clinical unit in the world devoted exclusively to intensive respiratory care. In undergraduate education, Dr. Naimark spearheaded a major reform of the undergraduate curriculum. In 1967, he was appointed Professor and Head of the Department of Physiology.

In addition to his local professional activities, Dr. Naimark participated actively in national and international societies. As President of the Canadian Physiological Society, he fostered links with other scientific societies, stimulated new programs, and launched a new journal, Physiology Canada. He also participated actively in the Canadian Society for Clinical Investigation and during his Presidency of the Society, initiated a major organizational reform to provide for broader disciplinary and regional representation.

Following his appointment to the Deanship of Medicine in 1971, Dr. Naimark reorganized the Faculty into four major divisions. In addition, a Department of Family Medicine and the Northern Medical Unit were established, new research and teaching facilities were constructed, extramural funding increased significantly, and the Faculty's full-time clinical professoriate was greatly expanded. Affiliation agreements with the major teaching hospitals and several community hospitals were executed. On the national scene, he was elected President of the Association of Canadian Medical Schools. Under his leadership, the structure and mission of the Association were significantly altered, and it became more heavily involved in national policy and issues affecting biomedical research and education. During this period, Dr. Naimark was elected the Sir Arthur Sims Commonwealth Professor and undertook an extended period as a visiting professor in Australia and New Zealand. His interest in international development included the establishment of exchange programs with the National University of Columbia and Ben-Gurion University of the Negev.

While Dean of Medicine, Dr. Naimark was elected to the Board of Governors by the Senate of the University of Manitoba, which marked the beginning of his active involvement in issues affecting higher education generally. In 1981, he was appointed President and Vice-Chancellor of the University. During his tenure, major

new academic programs were established in a variety of disciplines. The University expanded its links with other institutions, became a prominent participant in national networks of centres of excellence, and greatly expanded its links with the community. Several prominent new buildings were added to both the Fort Garry and Bannatyne campuses, and the University's endowment was increased nearly sixfold.

Dr. Naimark not only devoted his energies to his own university but also has been active in university affairs nationally. As a Board Member of the Association of Universities and Colleges of Canada, Dr. Naimark had special responsibility for national research policy. He is past-Chairman of the Council of Western Canada University Presidents, President of the AUCC, Chairman of the Association of Commonwealth Universities, Member of the Research Council of Canadian Institute for Advanced Research, Canadian Vice-President of the Inter-American Organization for Higher Education, and Director of the Corporate-Higher Education Forum. He is currently the founding Chairman of the Canadian Health Services Research Foundation. Dr. Naimark's greatest strength lies in his ability to comprehend several diverse arguments, identify the major issue, and state the problem in simple but most eloquent words. His memory of events, choice of words, and compassionate approach have earned him great respect from both medical and lay people.

Dr. Naimark has published extensively in his scientific discipline and produced many articles of a more general nature. He has been a visiting professor and invited speaker on many occasions, has served on the editorial board of several journals, and has been a consultant to universities and government agencies in the field of medical research and education. He was appointed by the Government of Manitoba to chair the Health Advisory Network. He was a Governor of the University of Manitoba and was a Director of the Health Sciences Centre and St. Boniface General Hospital of Winnipeg and continues to serve on a variety of national committees of both governmental and nongovernmental bodies and agencies concerned with health care, higher education, and research. He is a director of several private corporations, and as founding Chairman of the North Portage Development Corporation, he spearheaded the largest urban redevelopment program in Manitoba's history.

Dr. Naimark has received several awards and distinctions including appointment as an Officer of the Order of Canada, the award of honorary degrees, the Ben Gurion University of the Negev Distinguished Service Award, the G. Malcolm Brown Award of the Medical Research Council and the Royal College of Physicians and Surgeons of Canada, the Sir Arthur Sims Professorship of the Royal College of Surgeons of England, the Osler Award of the Canadian Society of Internal Medicine, the Queen Elizabeth Silver Jubilee Medal, and the 125th Anniversary of Confederation Medal.

## PREFACE

The relationship between angiotensin II and hypertension was established in 1898 when angiotensin II was shown to modulate systemic blood pressure. Over the intervening decades, a complete characterization of the renin-angiotensin system (RAS) has been achieved, and our understanding of its biochemistry and physiology has led to the directed development of agents such as ACE inhibitors and receptor antagonists capable of controlling hypertension. More recently, it was shown that angiotensin II is secreted within certain tissues and that these tissue-specific systems operate independently of the systemic RAS. The novel concept that angiotensin II regulates a number of cardiovascular processes that are unrelated to blood pressure has renewed the interest of both basic and clinical scientists in angiotensin II. The association between angiotensin II and cardiac growth, in particular, has indicated that therapies currently in use for hypertension may have direct application to the treatment of heart failure.

The Manitoba Cardiovascular Forum on Angiotensin Receptor Blockade in Winnipeg was convened October 18–20, 1996 to examine the clinical and basic aspects of angiotensin receptor biology as they apply to hypertension and heart failure. In addition, the potential treatment of these conditions using specific angiotensin receptor antagonists was addressed within the context of their immediate therapeutic application and future potential. Three distinct concepts were highlighted within the framework of this conference: (1) With respect to clinical application, it was generally agreed that angiotensin receptor blockade presents a viable alternative to ACE inhibition for the treatment of both hypertension and

heart failure. The universality of this approach is still questionable; however, since clinical trials using losartan have yet to provide an evaluation of the long-term effects of its use. Nevertheless, its use is recommended in cases where the side effects of ACE inhibition are severe. (2) It has been clearly established that local production of angiotensin II in the heart and vasculature is an important factor in tissue remodeling. In particular, it has become evident that angiotensin II mediates the changes in cell phenotype that are associated with hypertension and heart failure, as well as the synthesis of extracellular matrix that accompanies these conditions. (3) Significant movement has been made with respect to our understanding of the intracellular signaling pathways that are activated by the $AT_1$ and $AT_2$ receptor subtypes. These pathways include mediators such as PI3-kinase and the JAK/STAT proteins, which indicate there is substantial overlap with those systems previously associated solely with tyrosine kinase receptors. While the functional significance of the $AT_1$ receptor in cardiac and vascular tissues has been understood for a number of years, an increased awareness that the $AT_2$ receptor also contributes to cardiovascular physiology was demonstrated.

The goal of the Manitoba Cardiovascular Forum was to promote an exchange of ideas among basic scientists, clinicians, and practitioners, with the aim of improving our understanding of the processes that lead to the development of hypertension and heart failure. This book, which is a collection of papers based on the presentations made at this conference, deals with the most recent developments in the molecular biology, cellular physiology, and structure-function relationships of angiotensin II and its receptors. These papers also discuss the current therapeutic uses for angiotensin receptor antagonists and consider their potential future applications. It is our hope that this book will be informative to the students, scientists, and practicing clinicians who are attempting to extend our knowledge in the field of hypertension and heart failure and are devoted to improving cardiovascular health.

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# A. PHARMACOLOGICAL INTERRUPTION OF RENIN-ANGIOTENSIN IN HYPERTENSION

# PHARMACOLOGICAL INTERRUPTION OF THE RENIN SYSTEM AND THE KIDNEY: LESSONS FROM COMPARATIVE PHARMACOLOGY

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***Summary.*** Pharmacological interruption of the renin system has played a crucial role in our understanding of this system's contribution to normal physiological processes and in the pathogenesis of disease. Blocking the system pharmacologically is a crucial line of investigation, a function ordinarily played by glandular ablation and hormone replacement. The pharmacological agents, on the other hand, are often plagued by actions which contribute to ambiguity. In the case of the renin system, the angiotensin-converting enzyme (ACE) inhibitors have multiple additional actions beyond blocking angiotensin (Ang) II formation, including kinin formation and consequent promotion of nitric oxide and vasodilator prostaglandin release. The development of Ang II antagonists and renin inhibitors has provided pharmacological alternatives and improved information on specificity of action. Although concern has been expressed that the alternatives to ACE inhibition may lack some therapeutic features, it is suspected that blockade at the renin or the Ang II receptor levels will block the system more effectively, and thus may provide a positive alternative. The renin inhibitor is more effective because the interaction of renin with its substrate is a rate-limiting step in the process. The possibility that non-renin—non-ACE dependent Ang II generation contributes to blocking the Ang II receptor is real and will be the subject of substantial investigation in the near future.

## I. WHY THE SPECIAL ROLE FOR PHARMACOLOGIC INTERRUPTION OF THE RENIN SYSTEM?

The rules of logic discovered by Koch in the Nineteenth Century made it possible to create order from the chaos that followed recognition that microorganisms are ubiquitous and can cause disease [1–3]. The application of these rules of logic made

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it possible to prove beyond reasonable doubt that a specific organism could be responsible for a specific disease. It was all too easy to suggest—based on the presence of a microorganism—that the microorganism might be the cause of a disease. These rules of logic apply equally to a wide variety of biological phenomenon, including hormones and their actions. Haber pointed out that the fundamental evidence linking a hormone to its possible actions involved the ablation: replacement experiment [4]. If removal of a hormone led to a constellation of findings and replacement of the hormone reversed that constellation, powerful support is lent to the possibility that the hormone was responsible for the action. But what if extirpation of the organ leads to rapid demise, or the hormone action is autocrine or paracrine? The ablation experiment cannot be applied easily. It is here, Haber pointed out [4], that interruption of the system by pharmacological or immune mechanisms has come to replace the ablation experiment in fulfilling Koch's postulates. The case of the renin system and the kidney provides an excellent example. The kidney is not only the source of renin, but it is also a major responding organ; nephrectomy removes not only the source of the hormone but also the response.

Pharmacological interruption of locally acting paracrine systems has proven so important in medicine and biology that it is difficult to imagine our attempting to function without the information it provides. Identification of receptor subtypes for histamine, serotonin, dopamine, acetylcholine, catecholamines, and most recently angiotensin (Ang) II and the development of specific blockers has proven pathogenesis and has led to new approaches to treatment. On the other hand, pharmacological agents never enjoy the specificity that the ablation: replacement experiment provides. In the case of the renin system, the problem of specificity was recognized early: The Ang II antagonists available in the 1970s were not competitive antagonists, but rather were partial agonists with substantial residual angiotensin-like activity [5]. As a consequence, they were useful in defining the direction of a response, but not the magnitude Angiotensin-contenting enzyme (ACE) inhibitors were also complicated, because they blocked a major pathway involved in kinin degradation. Kinin accumulation could lead to vasodilatation, thereby mimicking the effect of Ang II disappearance [6]. Issues related to the specificity and the magnitude of the renal response to pharmacological interruption continue to this day [7].

## II. THE KIDNEY: PHYSIOLOGICAL LESSONS FROM ACE INHIBITION

Before the development of ACE inhibitors, it was recognized that restriction of dietary sodium intake resulted in renal vasoconstriction, a fall in renal blood flow, and a change in the pattern of the intrarenal distribution of blood flow. However, the mediator responsible for these effects remained obscure [8]. The exquisite sensitivity of the renal blood supply to Ang II made the renin-angiotensin system (RAS) an attractive candidate [9,10]. When pharmacological interruption of the RAS became possible, studies in animals quickly confirmed that Ang II played a

major role in the renal vascular response to restriction of sodium intake [11]. In the first study reported, Freeman, et al. [11] employed the partial agonist, saralasin, to explore the control of the renal circulation in the dog. In dogs ingesting liberal sodium and potassium intake, saralasin did not increase renal perfusion. In dogs in which a volume contraction had been achieved, either by combining a low-salt diet with a diuretic or by partial occlusion of the thoracic inferior vena cava, saralasin increased renal blood flow. In a series of studies that followed shortly, a strategy was adopted to deal with the lack of specificity and limitations of the two classes of drugs available. An increase in renal blood flow was documented in both the dog and the rabbit when either class of agent, an ACE inhibitor and an Ang II antagonist, was employed [5,12,13]. Perhaps the least equivocal study among the several reported was that performed by Kimbrough, et al. [14]. They studied unanesthetized, trained dogs to avoid the complicating effects of anesthesia and infused the Ang II antagonist and ACE inhibitor directly into the renal artery to avoid the complicating drop in blood pressure. They documented a virtually identical 29% increase in renal perfusion in response to both classes of blocker, with a smaller but still substantial increase in glomerular filtration rate, but only when the agents were administered to the dog in balance on a reduced sodium intake. A striking increase in sodium excretion also occurred. Neither agent influenced renal perfusion, glomerular filtration rate, or sodium excretion in animals ingesting a high-sodium intake. The essentially identical vascular and functional influence of a converting enzyme inhibitor and the angiotensin analog made it very likely that the renal vascular and functional response reflected Ang II entirely. Thus in the simplest and most gentle model of volume deficit—that induced by restriction of salt intake, without complicating diuretic use—the entire renal response was attributed to the direct action of angiotensin on renal blood supply.

Information became available rapidly on the role of Ang II in the renal response to larger volume deficits. Studies in animal models documented an enhanced response to pharmacological interruption in a variety of states, including partial occlusion of the thoracic inferior vena cava, hemorrhage, and congestive heart failure [14–17]. Where examined, the increase in renal perfusion induced by ACE inhibitors or Ang II antagonists was associated with an increase in glomerular filtration rate, urine flow rate, and sodium excretion. Angiotensin clearly had contributed to the overall renal response, including sodium retention. In general, it appeared that pharmacological interruption had not entirely reversed the renal response in these settings. It was likely, under these circumstances, that an additional effector system or systems may have contributed a direct action of the sympathetic nerves on the renal blood supply, or it may be a reflection of reduced arterial perfusion pressure during pharmacological interruption of the renin system.

Studies in man quickly confirmed observations from animal experimentation. Saralasin and ACE inhibition with teprotide produced an identical increase of approximately 20% in renal blood flow in normal human subjects on a low salt diet [18,19]. Since the only shared action involved Ang II, the only likely conclusion to be drawn from these studies was that angiotensin-mediated vasoconstriction had

been reversed. The studies in normal man, however, differed from those in animals in one important way. Whereas in animals on a high-salt diet, ACE inhibition did not alter renal blood flow [5,11–13], in some humans, renal blood flow rose despite suppression of the RAS by a substantial sodium intake [18]. This was an unexpected and somewhat puzzling observation.

Further investigation has provided insight into why some normal humans respond with an increase in renal blood flow to ACE inhibition despite a high salt diet, whereas others do not. Individuals whose renal blood flow increases with ACE inhibition differ from those whose renal blood flow is unresponsive in one important way; the responders have a parent who has hypertension, presumably essential in origin [20]. The offspring of hypertensives respond not only to ACE inhibition but also to calcium channel blockade [21] in a quantitatively similar fashion. This is unlikely to be a nonspecific influence, since the offspring of hypertensives do not show a potentiated renovascular response to acetylcholine, a vasodilator that does not depend on reversal of the impact of endogenous vasoconstrictors for its action [21]. These observations in the normotensive offspring of hypertensives, as it turned out, shed new light into the pathogenesis of a group of patients that Gordon Williams and I have been studying since the 1960s—the nonmodulators [22]. In brief, normal individuals show a large, reciprocal shift in renal vascular and adrenal responsiveness to Ang II with changes in salt intake. The normal modulation process in healthy individuals also upregulates the adrenal response on a low-salt diet and enhances the renal vascular response on a high salt diet [22]. Thus an increase in angiotensin-mediated control in these systems reflects not only an increase in the plasma concentration but also a shift in response to any plasma Ang II concentration. When this normal bit of physiology had been worked out, it became apparent to us that a group of essential hypertensives that we had been studying failed to show this normal modulation process. Hence, we called them "nonmodulators". Not surprisingly, given the important role that aldosterone and renal hemodynamics play in normal renal sodium handling, these individuals cannot handle a salt load normally, will retain more sodium at any given sodium intake, and have salt-sensitive hypertension. ACE inhibition corrects this abnormality in renal and adrenal control, returns renal sodium handling to normal, and controls blood pressure in these patients. As to the mechanism, multiple lines of evidence suggest that the problem is familial and, indeed, genetic [22]. A family history of hypertension in multiple family members is striking; there is remarkable concordance in the renal vascular response to Ang II in siblings and evidence of similar alterations in the offspring of hypertensives, as reviewed above.

Although there has been considerable inconsistency, polymorphisms involving the angiotensinogen (AGT) gene have provided the most promising leads in recent research on the genetics of hypertension [23–26]. The polymorphisms identified to date involve a location in the gene that is unlikely to be functionally significant: The M235T and T174M loci may be markers in disequilibrium with variants that influence AGT production or efficacy as a substrate, and there might be more than one etiologic mutation at this locus. Unfortunately, we are still ignorant as to

regulation of AGT gene expression, but a number of lines of investigation have suggested that tissue expression will be more important than AGT plasma concentration as a functional determinant. This possibility is strongly supported by a recent study [23] on the relationship between AGT genotype and the renovascular response to Ang II in caucasians. Plasma AGT did not vary with genotype, but renal vascular responsiveness did. Specifically, normal subjects and patients with hypertension who are homozygous for threonine at codon-235(TT) showed a blunted renovascular response to Ang II despite a high salt diet, which is indicative of nonmodulation. This polymorphism can account for the unusual responses to ACE inhibitors and calcium channel blocking agents in the normotensive offspring described above. This field, then, has moved a long way from two decades of confusion about the determinants of responsiveness.

## III. THERAPEUTIC IMPLICATIONS OF ACE INHIBITION

One approach employed by historians to highlight unanticipated involves asking a rhetorical question: What might have been reasonably expected on a certain date? In 1972, when ACE inhibition was first emerging, surely no one would have guessed that 25 years later the ACE inhibition story would involve as many therapeutic triumphs as it has. Difficult hypertension requiring three or more drugs for hypertension control was identified early as a reasonable target [27–30]. Targets that followed were scleroderma renal crisis [31,32], congestive heart failure [33–36], myocardial remodelling after myocardial infarction [37,38], diabetic nephropathy [39], and in all likelihood, other forms of progressive renal injury [40].

The experience with treatment of "difficult" hypertension in the late 1970s and early 1980s is instructive to review, since it is easy to forget how remarkable the advent of ACE inhibition was. Hypertension clinics of any size rapidly accumulated a series of patients in whom hypertension is difficult to treat. The process in these patients is known as refractory or resistant hypertension. Such patients generally had very high blood pressure, often required three or more drugs to achieve even partial blood pressure control, and often had abnormalities of renal excretory function—either as the primary reason for hypertension or as a consequence of prolonged, substantial blood pressure elevation. In such patients, activation of the RAS is often evidenced. The report by Studer, et al. in 1981, among the first on the subject, was instructive [20]. Using captopril, they treated 19 patients in whom standard triple therapy—a combination of large doses of a beta blocker, vasodilator, and diuretic—had been unsuccessful. Despite triple drug therapy, mean systolic pressure remained above 180 mm Hg and diastolic pressure remained at 110 mm Hg. Among the patients with essential hypertension, more than 80% achieved goal blood pressure, and mean diastolic pressure was reduced to below 100 mm Hg. The regimen was associated with a sharp reduction in symptoms related to prior drug therapy, general well being, and well maintained renal and cardiac function. Treatment was simplified, and difficult or refractory hypertension had to be redefined.

Although far less common, the problem of scleroderma renal crisis provided an even more dramatic example. The first report describing the maintenance of renal

function and control of blood pressure in two patients with unequivocal scleroderma renal crisis treated with captopril [31] was followed by a series of reports describing twenty-three patients [32]. Of the twenty-three, seven lost sufficient renal function and required maintenance hemodialysis, two died, and the remainder stabilized [32]. This is a remarkable contrast to earlier literature in which few patients survived more than a few weeks, and survival beyond six months demanded bilateral nephrectomy and hemodialysis. Although no controlled evaluation has ever been performed and despite difficulty in performing a controlled study in patients with a rare disease, there is no question that ACE inhibition has changed the natural history of disease associated with a rapid downhill course.

Current interest in the specific underlying pharmacological mechanisms by which ACE inhibition achieved therapeutic goals reflects not only the pleasure that we share in understanding a process, but also a more specific and practical issue. With the emergence of alternatives for blocking the renin system, we have a new choice and an unambiguous need to understand its mechanisms. Calling the responsible enzyme "ACE" reflects our narrow, provincial view [6]. The enzyme is a peptidyl dipeptide hydrolase and has a wide range of substrates, among them bradykinin, as pointed out above. Thus it engages prostaglandin release and the nitric oxide pathway. Much of the energy invested to this point on mechanisms has reflected this fact, and a strong suspicion is held by many that the kinin pathway makes a very substantial contribution to the therapeutic efficacy of ACE inhibition. Should that be the case, alternatives for blocking the renin system via renin inhibition or competitive Ang II antagonists would have limited therapeutic application.

Another view is possible. No pharmacologist examining the renin cascade would have chosen to block the ACE step. The first step—the renin : angiotensinogen interaction—is rate limiting, and remarkably specific, which would have made it a much more attractive alternative. The Ang II receptor provides a second attractive target [7]. Because Ang II formation can be catalyzed by a number of serine poteases, which are ubiquitous, pharmacological interruption at the Ang II receptor level has the distinct potential advantage of blocking the action of Ang II, whatever the pathway of its formation. The fact that ACE inhibition came along before the two alternatives was not a product of a planned program, but rather was an unanticipated by-product of snake toxin pharmacology. This was a happy accident, but an accident nonetheless.

## IV. THERAPEUTIC IMPLICATIONS OF PHARMACOLOGIC ALTERNATIVES

With its specificity and efficacy, renin inhibition would have been a very attractive approach to pharmacological interruption of the renin system. The interaction of renin with its substrate, angiotensinogen, is the rate-limiting step. However, because of limited bioavailability of the agents developed, cost of synthesis, and rapid development of a pharmacological alternative—the Ang II antagonist class—attempts to develop renin inhibitors for clinical application appear to have ceased.

The Ang II antagonists, on the other hand, have gained substantial momentum. Several dozen $AT_1$ receptor antagonists were developed by several dozen pharmaceutical firms. Presently two are marketed in Western Europe or the U.S.A., and at least another eleven agents are under active clinical investigation [41]. During development of these agents, there have been several focuses of interest beyond blood pressure reduction.

The first focus directly involved the possibility that the lack of specificity of ACE, and thus of ACE inhibition, contributed to one of its more frequent, annoying, and disruptive side effects, cough. One widely held concept was that cough reflected bradykinin accumulation or accumulation of other cough-promoting factors, such as substance P [42]. A study designed to address this issue found that the incidence of cough in losartan-treated patients was essentially identical to cough in thiazide-treated patients: As the patient population was selected for the study not only because they were believed to have suffered from ACE inhibitor-induced cough, but also because they showed cough on rechallenge with an ACE inhibitor, the finding was especially persuasive [43]. The $AT_1$ receptor antagonists had passed their first important test beyond blood pressure efficacy with this successful study.

Interest in the kidney and the possibility that $AT_1$ receptor antagonists will prevent nephropathy is greater than interest at the same stage in the evolution of ACE inhibitor therapy [40]. There are a number of reasons. Evidence of the potential efficacy of these agents in animal models of renal injury and in humans with proteinuria appeared early [44,45]. Although end-stage renal disease (ESRD) is far less common than acute myocardial infarction and other cardiovascular endpoints, it is enormously costly. Identification of stages, especially the earliest stages, and assessment of progression is relatively easy. All make renal injury an attractive therapeutic target with implications that may go well beyond the specifics of ESRD prevention. For these reasons, many of our colleagues have urged the pharmaceutical firms involved in $AT_1$ receptor antagonist development to pursue studies on the kidney, and many of these companies have shown interest. All have had to address an issue raised in the piquant title of a recent review [46] which is, "will Ang II receptor antagonists be renoprotective in humans?" This essay reflects a substantial body of current thinking.

The analysis was based on a range of considerations, the majority of which seem to favor ACE inhibition as these considerations suggest that angiotensin is not the primary determinant [46]. ACE inhibitors block alternative pathways that influence extracellular matrix protein degradation and the rate of development of glomerulosclerosis. Macrophage infiltration, thought by many to contribute to the pathogenesis of nephropathy, is also ACE inhibitor responsive. Blocking the $AT_1$ receptor opens the short feedback loop, thereby leading to renin release and increased Ang II formation. With the $AT_1$ receptor blocked, this sequence could lead to unopposed activation of the $AT_2$ receptor, with unknown but potentially negative consequences. Although all of these considerations favor ACE inhibition over Ang II $AT_1$ antagonist action, each is a construct based on a slim database, generally obtained in vitro. Perhaps the most important consideration in Ichikawa's

analysis involved glomerular hemodynamics, especially glomerular capillary pressure, which for many goes beyond the construct level. In brief, this analysis suggests that much of the ACE inhibitor-dependent improvement in natural history reflects the salutary effect of ACE inhibition to reduce glomerular capillary pressure via bradykinin-mediated efferent arteriolar dilatation. Thus, the kininase action of ACE inhibitors is crucial, and the reduction in Ang II formation is less important or perhaps even irrelevant.

On the other hand, one can make an equally compelling argument for greater potential efficacy of Ang II antagonists, based on more effective blockade of the renin system [7]. Moreover, much of the most important data reviewed above were obtained in vitro or in small animal models. If studies in rats never predicted responses in humans, we would probably never do studies in rats. If studies in rats always predicted what would happen in humans, we could not justify studies in humans. Is this an area in which there might be important species differences?

This issue was addressed specifically in a recent editorial on ACE inhibition and the kidney [7]. To isolate species differences, one has to apply an essentially identical protocol to multiple species. Such studies have been done. Bradykinin antagonists blunted the renal vasodilator response to ACE inhibitors in the dog and in the rat, but not in the rabbit. In accord, an Ang II antagonist somewhat blunted ACE inhibitor-induced renal vasodilation in the rat and dog but completely blocked it in the rabbit. ACE inhibition increased prostaglandin release in rat and canine kidneys, but not that of the rabbit. In an elegant study Roman, et al. [47] showed that in the rat, it was medullary perfusion that was primarily kinin dependent. Thus, apparent species differences may be primarily anatomical, reflecting the relative contribution of medullary perfusion to total renal blood flow: In this feature, humans resemble the rabbit far more than they do the rat or dog [7]. We cannot extrapolate from studies in a limited range of species, especially the rat, to control mechanisms in humans, even in health and much less so when disease is superimposed.

What about information on the control of the renal circulation in humans and the mechanisms by which ACE inhibition might influence the renal circulation? Although there are powerful limitations in the approach that can be employed in humans, several lines of evidence provide an answer. The striking influence of salt intake on the renal vasodilator response to ACE inhibition reviewed earlier supports a dominant role for the Ang II mechanism. More recently, comparative pharmacology has strengthened that conclusion substantially. If the renal vasodilatation induced by ACE inhibitors in humans included a substantial component because of bradykinin, prostaglandins, or nitric oxide, one would anticipate that the renovasodilator response to renin inhibitors would be substantially less. To our surprise the renal vasodilator response to a renin inhibitor, enalkiren, exceeded expectations from early experience with ACE inhibitors [7]. To address this issue, we performed a range of follow-up studies. To ascertain whether the observation represented an idiosyncracy of one renin inhibitor, we studied a second and an identical result. Because of the notorious risk of employing historic controls, we performed a study in which patients received an ACE inhibitor, a renin inhibitor, or a vehicle during

the same week. This study was coded and double blind. To avoid an idiosyncracy of one ACE inhibitor, we employed three, each at the top of the dose-response curve. The findings all provided support for a surprising but unambiguous conclusion. Although our original premise was reasonable and supported by a wealth of information in animal studies, the renovasodilator response to renin inhibition is approximately 140 ml/min/1.73 $m^2$, substantially larger than the response to ACE inhibition, typically in the 90–100 ml/min/1.73 $m^2$ range.

Although the fundamentals of pharmacology would favor more effective pharmacological interruption of the renin system at the rate-limiting step as the explanation, and thus would favor our drawing that conclusion from these data, there is an alternative interpretation. The two renin inhibitors were structurally related, as most drugs in a class are, and it is possible that they share a renovasodilator action through a mechanism unrelated to a reduction in Ang II formation. In the case of the renin cascade, we have the potential for a "tie breaker". If, indeed, the renin inhibitors operated via this cascade, one would anticipate a similar or larger renovasodilator response to Ang II antagonists when the studies were performed in the same way. This is precisely what we found in studies performed with an identical model, protocol, and technique. Two Ang II antagonists induced a renovasodilator response that matched or slightly exceeded the response to renin inhibition in healthy humans on a low-salt diet [48,49]. From this observation we would draw several conclusions. The renal hemodynamic response to ACE inhibition has underestimated, systematically, the contribution of Ang II to renovascular tone. The effectiveness of renin inhibition suggests that this response represents interruption of primarily renin-dependent, additional non-ACE-dependent pathways. In healthy humans, there might be a small contribution from proteolytic pathways that bypass both renin and ACE. In disease, on the other hand, the latter pathway may provide a more substantial contribution.

The final conclusion is that therapeutic trials with Ang II antagonists offer far more promise than did ACE inhibitors, despite the gloomy predictions. They are more effective blockers.

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# THE BRAIN RENIN-ANGIOTENSIN SYSTEM AND SALT-SENSITIVE HYPERTENSION

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***Summary.*** An intrinsic tissue renin-angiotensin system (RAS) has been described in the brain. This review provides an overview of the localization of the enzymes, peptides, and receptors of the brain RAS and the organization of angiotensinergic pathways involved in cardiovascular regulation. Centrally administered exogenous angiotensin (Ang) II increases sympathetic neuronal activity, decreases the gain of the baroreflex, and induces vasopressin release. Ang II generated by the brain can cause similar changes through effects in nuclei from the forebrain to the brainstem. In salt-sensitive hypertension, both brain ouabain-like compounds ("ouabain") and the brain RAS appear to play an essential role. Both central and high sodium intake activate brain "ouabain" followed by stimulation of the brain RAS and sympathoexcitatory and hypertensive responses. The actual pathways involved have not yet been established, but appear to involve the ventral anteroventral third ventricle region, the anterior hypothalamic area, and the paraventricular nucleus of the hypothalamus.

## INTRODUCTION

In 1961, Bickerton and Buckley first reported that circulating Angiotensin (Ang) II is able to act on the central nervous system to increase blood pressure [1]. Since then a number of Ang II sensitive sites in the brain have been demonstrated. Moreover, besides the classical circulatory renin-angiotensin system (RAS), intrinsic tissue RASs have emerged, including that in the heart and brain. The brain RAS and central actions of the circulatory RAS are involved in central cardiovascular regulation and body fluid homeostasis, cyclicity of reproductive hormones, sexual behavior, and perhaps neuronal development and differentiation, and learning and

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memory [2–4]. This review (1) provides an overview of the localization of the brain RAS and the organization of angiotensinergic pathways, and (2) describes the role of brain Ang II in salt-sensitive hypertension.

## LOCALIZATION OF THE BRAIN RAS

All components of the RAS have been identified in brain tissue, including angiotensinogen, the precursor for angiotensins, the protease renin, which cleaves angiotensinogen to the decapeptide Ang I; and the angiotensin-converting enzyme (ACE), which converts Ang I to the octapeptide Ang II. Expression of the mRNA for angiotensinogen, renin and ACE has been demonstrated in the brain of several species, including rats, and is consistent with the concept of a local brain RAS [5,6]. Incubation of brain homogenates with renin generates Ang I, implying that the precursor angiotensinogen is present locally [7]. In addition angiotensinogen has been identified immunohistochemically [8]. Following initial reports on central renin activity [7,9], the presence of brain renin that is independent of circulating renin, distinct from other proteases, active in vivo and inhibited by renin specific antibodies was confirmed [10,11]. Brain ACE is similar to peripheral ACE with respect to molecular weight, optimum pH, chloride dependency, and inhibition by various inhibitors [12]. However, ACE isozymes with different molecular weights have been demonstrated in the brain [13,14]. Brain ACE is ubiquitous in distribution and, like that of peripheral sources, is nonspecific in action, i.e., in addition to converting Ang I to Ang II, ACE of central origin degrades kinins and neuropeptides such as substance P.

Four main angiotensin receptor subtypes have been described [15,16]. Three of these—namely, the $AT_1$, $AT_2$, and $AT_4$ receptors—are distributed in the brain as well as in peripheral tissue. Central areas involved in cardiovascular regulation, body fluid homeostasis, and neuroendocrine function exhibit a predominance of $AT_1$ receptors [17–19], which bind Ang II with high affinity.

Angiotensinogen, detected immunocytochemically, is predominantly located in astrocytes and ependymal cells [20], and angiotensinogen mRNA detected by in situ hybridization is localized mainly in astrocytes [21]. However, angiotensinogen immunoreactive neurons have also been identified [22], and the presence of angiotensinogen has been demonstrated in CSF as well [23]. The site of synthesis of brain angiotensins is as yet unresolved. Bunnemann et al. [21] suggested that angiotensinogen may be produced in astrocytes and converted to Ang I by renin in the extracellular fluid or alternatively may be taken up by neurons and converted intraneuronally. Renin and ACE activity have been colocalized in synaptosomes, supporting the concept of intraneuronal synthesis [24]. However, renin has also been detected in oligodendrocytes, and ACE has been detected extracellularly [2,25]. After conversion of Ang I to Ang II by ACE, Ang II is further acted on by aminopeptidases to form the heptapeptide Ang III, which is converted to the hexapeptide Ang IV. Ang II is the first biologically active molecule in this cascade and acts as a neurotransmitter/neuromodulator. An alternate pathway exists whereby

Ang I is converted to the nonapeptide des-Asp[1], Ang I which is acted on by ACE to form Ang III. The presence of these angiotensin peptides in the brain despite the blood brain barrier and their presence in nephrectomized rats [26] indicate that the peripheral RAS does not contribute to these peptides. Angiotensin peptides that have also been isolated from neuronal cell cultures further strengthen the concept of an intrinsic brain RAS [27]. Details of studies demonstrating the presence of RAS peptides and enzymes in the brain have been extensively reviewed elsewhere [28,29].

The components of the brain RAS are distributed in areas that mediate cardiovascular regulation and body fluid homeostasis, including septal nuclei, the preoptic region, hypothalamic nuclei, the midbrain, and nuclei of the medulla oblongata [21]. The presence of the brain RAS in other discrete regions such as the basal ganglia suggests a role in other functions. We briefly describe the localization of the components of the brain RAS in areas that regulate cardiovascular function and fluid homeostasis.

Central areas with a cardiovascular regulatory role in which all components of the RAS are present include the median preoptic area; the supraoptic, paraventricular, dorsomedial, and ventromedial hypothalamic nuclei; the midbrain preriaqueductal gray; the locus ceruleus; the nucleus tractus solitarius; the dorsal motor nucleus of the vagus; and, in very low concentrations, the cerebral cortex [21]. Among these areas the highest concentrations of angiotensinogen immunoreactivity and Ang II nerve cell body and terminal immunoreactivity were found in the paraventricular nucleus (PVN) and the supraoptic nucleus (SON) [8]. In the preoptic area high levels of angiotensinogen immunoreactivity were found to be concentrated in glia with no neuronal angiotensinogen present [21].

In the circumventricular organs (CVOs) an apparent topoloigcal mismatch is observed [21]. Angiotensinogen immunoreactivity is absent in CVOs that are implicated in cardiovascular and neuroendocrine function, such as the subfornical organ (SFO), area postrema (AP), and the organum vasculosum laminae terminalis (OVLT). However, angiotensinogen mRNA is demonstrable in the rostral part of the SFO, the AP, and the median eminence. Ang II receptors in CVOs of rat brain have been characterized by $^{125}$Iautoradiography [30,31]. The highest binding densities were localized in the SFO and AP, with considerable aggregation also found in the OVLT and median eminence. CVOs express a deficient blood brain barrier and have a high capillary density, fenestrated capillaries, large perivascular spaces, and specialized ependymal cells (tanycytes), enabling them to detect circulating substances and tranduce the information to neural messages [32]. Circulating Ang II, like other peptides, cannot cross the blood brain barrier and, not surprisingly, localizes specifically to CVOs [33]. This may explain very low amounts of demonstrable angiotensinogen in CVOs, as the CVOs that express very high Ang II binding sites, SFO and OVLT, have been implicated in the action of blood-borne and CSF-borne Ang II.

The amygdaloid complex is another area in which there is a mismatch of the components of the RAS. The bed nucleus of the stria terminalis and the central

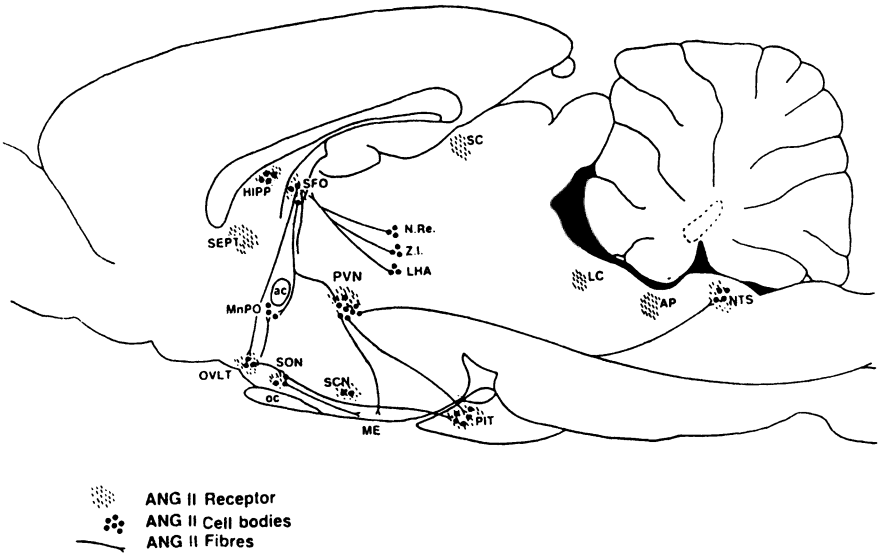


**Figure 1.** Schematic diagram of a longitudinal section of the rat brain showing Ang II-containing cell bodies (black circles), Ang II receptors (shaded areas), and angiotensinergic pathways (lines). (Adapted from Steckelings et al [3].)

nucleus of the amygdala lack demonstrable Ang II receptors, and the medial amygdaloid nucleus lacks detectable Ang II immunoreactivity but contains other components of the RAS [21].

While other areas contain all other components of the RAS, they lack demonstrable Ang II receptors. They include the nuclei of the band of Broca, medial septal nuclei, and anterior and lateral hypothalamic areas [21].

## ANGIOTENSINERGIC PATHWAYS

Steckelings et al. [3] summarized the major localization of Ang II neuronal perikarya, receptors, and fibers (figure 1). To demonstrate angiotensinergic pathways, anatomical tracing in combination with immunohistochemical staining for angiotensins has been used. Lind et al. [34] demonstrated two major angiotensinergic efferent pathways from the SFO, one projecting to the median preoptic nucleus (MnPO) and the other to the PVN. The SFO also has an afferent projection arising in perifornical parts of the lateral hypothalamic area [34]. In addition, Jhamandas et al. [35] demonstrated that 46% of SFO neurons retrogradely labelled from the SON were also labelled for angiotensin. Fewer cells demonstrating both markers were demonstrable in the MnPO and OVLT. The presence of these pathways is supported by electrophysiological and microinjection studies [35,36]. Lind et al. [37] suggested that angiotensinergic projections may include those from

the PVN to presympathetic neurons in the intermediolateral cell column of the spinal cord as well as from the PVN to the posterior pituitary.

## CENTRAL ACTIONS OF ANGIOTENSIN II

Central cardiovascular and body fluid regulating actions of angiotensins may be achieved by either actions of brain angiotensins or actions of blood-borne Ang II on CVOs. In this review we focus on central actions of Ang II generated by the brain.

Ang II is the principle bioactive peptide of the brain RAS. Whereas a role for Ang III and IV in mediating cardiovascular actions of the brain RAS is gradually emerging [38,39], this will not be discussed.

Acute intracerebroventricular (icv) administration of Ang II into the third or lateral ventricles induce cardiovascular and behavioral responses, including pressor responses in a number of species, including rats [40–43]. Pressor responses to centrally administered Ang II may be attributed to an increase in sympathetic neuronal activity as well as release of arginine vasopressin (AVP). In rats with peripheral blockade of AVP, icv Ang II increases renal sympathetic nerve activity and blood pressure (figure 2) [44]. Peripheral sympathectomy results in a prolonged latency for the pressor response to icv Ang II. This implies the presence of a fast component in the pressor response that is induced by sympathetic neural activation [45]. Inhibition of AVP's actions by peripheral $V_1$ receptor blockade, vasopressin antibodies, or hypophysectomy attenuates the pressor response to icv Ang II [46,47]. Furthermore, in rats with diabetes insipidus that lack vasopressin, pressor responses to central Ang II are depressed [48]. Combined peripheral α-adrenoceptor and $V_1$ receptor blockade completely prevents the pressor response to central Ang II [46]. This confirms a role for both sympathetic activation and vasopressin release in the pressor response to icv Ang II.

In addition to increasing sympathetic activity and AVP release, centrally injected Ang II may induce the release of a humoral inhibitor of the $Na^+K^+$ ATPase, as indicated by a decrease in $^{86}Rb$-uptake when rat arteries were incubated with plasma supernate of dogs treated with icv Ang II [49,50]. The release of this humoral inhibitor was blocked by central saralasin pretreatment, which is consistent with the involvement of central Ang II receptors in its release [50].

Microinjection of Ang II into the ventrolateral medulla (VLM) of rats elicits cardiovascular changes that parallel, but are of smaller magnitude than, those produced by microinjection of the excitatory amino acid, L-Glu. Ang II injected into the caudal VLM produces depressor and bradycardic effects that are opposite to the pressor and tachycardic effects elicited by injection into the rostral ventrolateral medulla (RVLM) [51]. Ang II responsive sites in the RVLM were localized to the subretrofacial nucleus, which contains neurons that project to sympathetic preganglionic neurons of the intermediolateral cell column [52,53]. Chan et al. [54] have shown that 30% of RVLM neurons with spinal projections are excited by iontophoretic application of Ang II. Sasaki and Dampney demonstrated that microinjection of Ang II into the RVLM increases renal sympathetic nerve activity [55].

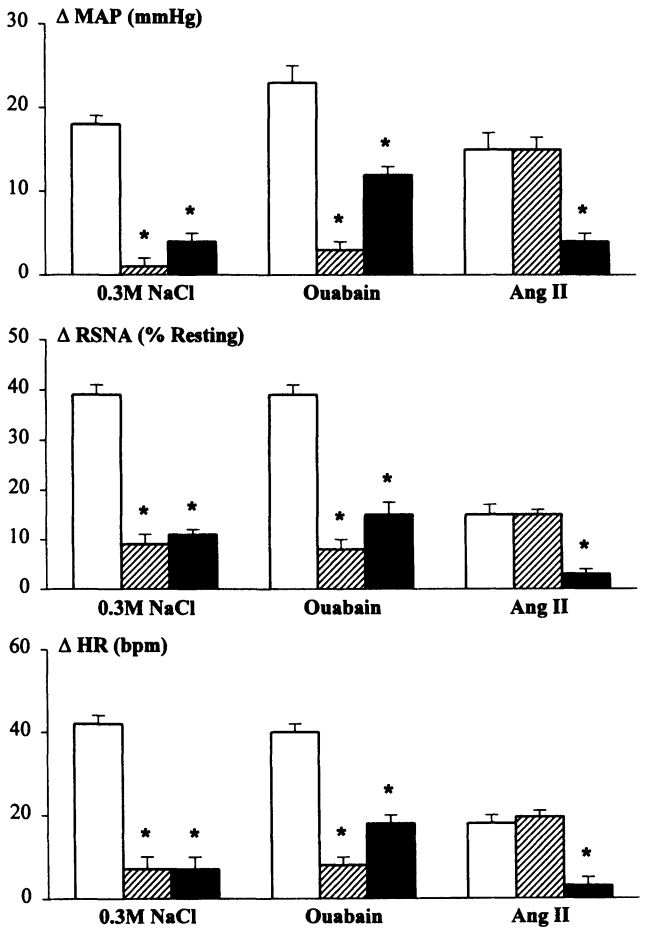


**Figure 2.** Peak increases in MAP, RSNA, and HR in response to icv administration of 0.3M NaCl (3.8μl/min for 10min), ouabain (0.6μg), and Ang II (30ng) following pretreatment with icv aCSF (white bars), Fab fragments (66μg, hatched bars), or losartan (10μg, black bars). Arginine vasopressin antagonist (30μg/kg iv) was administered prior to each treatment. Values are mean ± SEM. n = 7–8 *$p < 0.05$ vs aCSF. (Adapted from Huang and Leenen [44].)

In cats and rabbits, topical application or microinjection of Ang II antagonists into the VLM produced blood pressure and heart rate changes opposite to those evoked by Ang II [55,56]. Bilateral injection of an Ang II antagonist into the RVLM in normotensive rats reduced blood pressure to virtually spinal levels [57]. These studies all support the concept for a role of the brain RAS in modulation of tonic sympathetic drive generated by vasomotor neurons of the VLM.

Ang II action in the VLM also appears to be involved in modulation of phasic cardiovascular function. In anesthetized rats, microinjection of Ang II into the

RVLM suppressed the baroreflex response. This effect could be prevented by coadministration of either the nonpeptide $AT_1$ or $AT_2$ receptor antagonist, losartan, or PD 123319, respectively [58]. In the same study, microinjection of PD 123319 to block effects of endogenous Ang II elicited an enhancement of baroreflex responses, whereas losartan had no significant effect, indicating a role for $AT_2$ receptors. In anesthetized rabbits, saralasin, a peptide Ang II receptor antagonist, facilitated sympathetic baroreflex function and decreased resting renal sympathetic nerve activity when microinfused into the RVLM and inhibited baroreflex response when microinfused into the caudal VLM [59]. The presence of Ang II receptors associated with vagal efferents within the nucleus tractus solitarius (NTS), the first relay station of the baroreflex pathway, is also consistent with a modulatory role of the brain RAS in baroreflex function [60]. Microinjection of Ang II into the NTS of anaesthetized rats resulted in depressor responses at low doses (1 ng) or a pressor response at higher doses (10 ng) accompanied by bradycardia [61,62]. Bilateral microinjection of the Ang II antagonist, ($Sar^1$,$Thr^8$) Ang II, into the NTS enhanced baroreflex-mediated bradycardia [63]. In conscious rats microinjection of saralasin had a similar effect [64]. Overall these studies suggest that Ang II decreases the gain of the baroreflex. While pressor responses to Ang II in the NTS appear to be mediated by sympathetic activation, the attenuation of reflex bradycardia has been attributed to a decrease in parasympathetic activity.

## CIRCULATING ANG II

Circulating Ang II, as previously mentioned, binds to receptors in CVOs and causes centrally mediated effects. Microinjection of 0.1 ng or less of Ang II into the SFO, the third ventricle close to the OVLT, or the AP induces pressor responses [65–67]. It is likely that the pressor effects of circulating Ang II are mainly mediated by the SFO and area postrema, whereas the OVLT and MnPO are more involved in the pressor responses to CSF Ang II [4].

## THE BRAIN RAS AND SALT-SENSITIVE HYPERTENSION

The brain RAS contributes to the development and maintenance of certain forms of hypertension, particularly salt-sensitive hypertension. The spontaneously hypertensive rat (SHR) and the Dahl salt-sensitive (Dahl S) rate are genetic models of salt-sensitive hypertension in which a hyperactive brain RAS has been implicated. In these models high salt intake exacerbates the development of hypertension, and the latter appears to depend on brain Ang II, leading to an increase in sympathetic outflow.

The extent of the involvement of a tonically active brain RAS in the development of hypertension in SHR on a regular salt diet is still unresolved. On the one hand, studies on biochemical aspects of the brain RAS are consistent with increased activity of the brain RAS in SHR. Angiotensinogen content is greater in young (four weeks old) SHR than in age matched normotensive control Wistar Kyoto (WKY) rats in the preoptic area, SFO and OVLT [68]. Angiotensinogen content in

the septum, preoptic nuclei, and PVN [68,69] is increased in adult SHR when compared to WKY rats. Renin activity is higher in ten-week-old SHR in the pituitary but markedly lower in several central regions, including the SFO and the periaqueductal central grey [69]. Ang II immunoreactivity in adult SHR is twice as much that of WKY rats in the SON and PVN [70]. However, Ang I and Ang II content was lower in the hypothalamus in adult stroke-prone SHR when compared to WKY rats [71]. SHR also exhibit increases in Ang II receptors. Autoradiography demonstrates the number of Ang II binding sites in the SFO is greater in young and adult SHR than in age-matched WKY rats [72]. On the other hand, studies evaluating the hemodynamic consequences of blockade of the brain RAS have provided conflicting results. Central antisense oligodeoxynucleotide inhibition of angiotensinogen mRNA in adult SHR decreases angiotensinogen in the brain stem and hypothalamus and lowers blood pressure to normotensive level up to 24 h after administration [73]. Peripheral administration of the antisense oligodeoxynucleotide at the same dose does not change blood pressure significantly consistent with a central action. Central injection of recombinant adeno-associated virus-antisense to $AT_1$ mRNA decreased blood pressure by ~20 mmHg in adult SHR for up to 9 weeks and slowed the development of hypertension in young SHR [74]. Icv administration of the ACE inhibitor, captopril, attenuated the development of hypertension in SHR [75]. However, centrally administered ACE inhibitors may also increase brain bradykinin or neuropeptides [76,77]. Adult SHR also exhibit a greater decrease in blood pressure following acute injections of losartan, an $AT_1$ receptor antagonist, into the anterior hypothalamus or following acute injections of an Ang II peptide antagonist, ($Sar^1$,$Ile^8$) Ang II, into the RVLM when compared with blood pressure in age-matched WKY rats [78,79]. In contrast, in adult SHR, neither icv $AT_1$ receptor blockade using losartan nor combined $AT_1$ and $AT_2$ receptor blockade using saralasin and ($Sar^1$,$Thr^8$) Ang II caused a significant decrease in blood pressure [80,81]. Chronic icv administration of losartan (1 mg/kg/d) did not affect either the development or maintenance of hypertension or sympathetic hyperactivity in SHR on a regular salt diet (figure 3) [82,83]. At a higher dose (10 mg/kg/d), icv or sc administration caused a similar decrease in resting blood pressure, indicating that the depressor effect was due to leakage of losartan out of the central nervous system and blockade of peripheral $AT_1$ receptors [82]. The evidence so far indicates that the activity of the brain RAS is higher in SHR than in WKY rats and appears to be involved in acute blood pressure regulation. However, chronic central blockade of $AT_1$ receptors appears not to affect the development or maintenance of hypertension in SHR on a regular salt diet.

Our studies have focused on the role of brain endogenous ouabain-like compound(s) ("ouabain") and its interaction with the brain RAS in the development of salt-sensitive hypertension.

In salt-sensitive Dahl S rats and SHR, centrally released "ouabain" plays a critical role in the development or exacerbation of hypertension when these rats are fed a high salt diet. The increase in blood pressure by high sodium is associated with decreases in sympathoinhibition and increases in sympathoexcitation, resulting in an

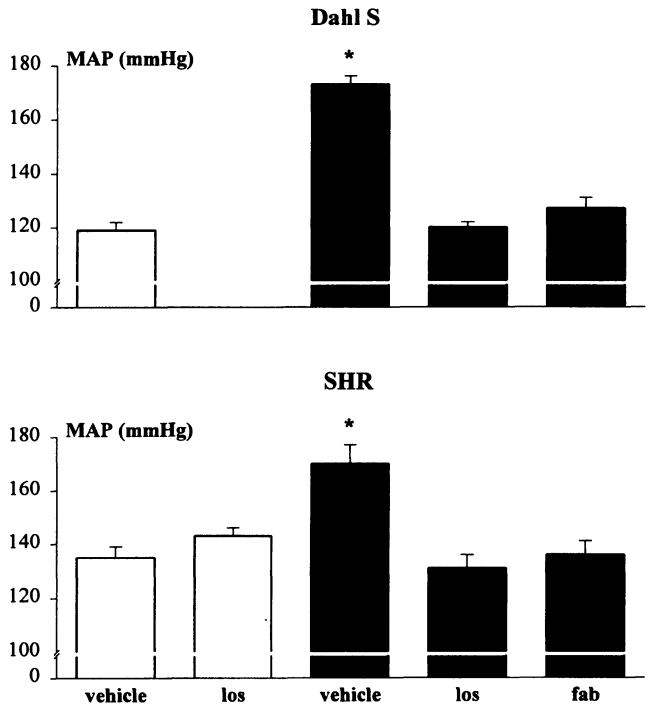


**Figure 3.** Resting MAP at 9 weeks of age in Dahl S rats (upper panel) and SHR (lower panel) on regular (0.4% NaCl, white bars) or high (8.0% NaCl, black bars) salt diet for 4 weeks treated chronically with icv vehicle, losartan (1 mg/kg/d, los), or Fab fragments (200 μg/d, fab). Values are mean ± SEM. n = 7–8. *$p < 0.05$ vs other groups. (Adapted from Huang and Leenen [83,94].)

increase in sympathetic neuronal activity. In these studies sympathetic activity was estimated by assessment of renal sympathetic nerve activity and cardiovascular responses to air jet stress and to icv injection of guanabenz, an $\alpha_2$-adrenoceptor agonist. SHR on a high salt diet exhibit an enhanced response to guanabenz which possibly reflects an upregulation and/or decreased $\alpha_2$-receptor occupancy in the anterior hypothalamus as a result of decreased norepinephrine release from sympathoinhibitory neurons [84,85]. Dahl S rats and SHR on high dietary salt intake exhibit increases in hypothalamic, pituitary, and pons "ouabain" content, compared to their normotensive controls [86,87]. Concomitant chronic icv administration of Fab fragments, which bind ouabain and related steroids with high affinity [88,89] prevents sodium-induced sympathoexcitation and increase in blood pressure [90,91]. In similar doses, Fab fragments administered iv are ineffective in preventing sodium-induced increases in blood pressure, indicating that the actions of "ouabain" are central. Although there is a small increase in brain "ouabain" content in normotensive rats on a high salt diet, there is no attendant increase in sympathetic

activity or blood pressure, and icv Fab fragments do not change blood pressure. It is possible that the "ouabain" concentrations in WKY/Dahl R rats on a high salt diet do not reach the threshold required for an increase in blood pressure. Alternatively, salt-sensitive rats may also have an increased sensitivity to "ouabain" and, therefore, may respond at lower central "ouabain" concentrations.

The brain RAS appears to play a critical role in salt-induced hypertension in both SHR and Dahl S rats. SHR on a high-salt diet exhibit an enhanced depressor response to icv injection of captopril compared to SHR fed a regular salt diet [92]. Microinjection of losartan into the anterior hypothalamus elicits a larger depressor response in SHR on a high-salt diet vs. a regular salt diet [93]. Recent studies in our laboratory demonstrated that chronic blockade of central $AT_1$ receptors by icv losartan prevents exacerbation of hypertension in both SHR and Dahl S rats on a high-salt diet (figure 3) [83,94]. Chronic central infusion of CV-11974, a nonpeptide $AT_1$ receptor antagonist, prevented the development of hypertension in Dahl-Iwai salt-sensitive rats on a high salt diet [95]. These studies suggest that hyperactivity of the brain RAS contributes to the exacerbation of hypertension in SHR, Dahl S, and Dahl-Iwai salt-sensitive rats on a high-salt diet.

Chronic blockade of either brain "ouabain" by icv administration of Fab fragments or brain Ang II receptors by icv administration of losartan affects sodium induced changes in sympathetic activity and blood pressure in SHR and Dahl S to a similar extent [83,94]. SHR on a high salt diet treated with chronic icv infusion of Fab fragments and/or losartan do not exhibited an enhanced decrease in renal sympathetic nerve activity to icv guanabenz. Responses were comparable to that seen in SHR with control icv infusion on a regular salt diet. In addition, both chronic icv Fab fragments and losartan treatment normalize dietary salt-induced enhanced sympathetic responses to air jet stress. This suggests that the dietary salt-induced increase in sympathoinhibition and increase in sympathoexcitation is mediated by central "ouabain" and Ang II. SHR on a high salt diet treated chronically with icv Fab fragments exhibited larger sympathoexcitatory and pressor responses to acute icv injection of Ang II than responses of rats receiving control infusion (figure 4). This is consistent with the concept that blockade of brain "ouabain" decreases the activity of the brain RAS, which leads to a decreased occupancy and/or upregulation of Ang II receptors, resulting in enhanced responses to exogenous Ang II [83].

Consistent with findings in SHRs, in Dahl S rats on a high salt diet, chronic blockade of either brain "ouabain" or $AT_1$ receptors for 4 weeks prevents sodium-induced exacerbation of hypertension to a similar extent [94]. In Dahl S rats on a high salt diet, chronic icv infusion of Fab fragments or losartan normalized the sodium-induced enhancement of mean arterial pressure (MAP) and renal sympathetic nerve activity responses to icv guanabenz and air jet stress [94]. This finding is also consistent with the concept of an interaction of brain "ouabain" and brain Ang II in mediating sodium-induced sympathoexcitation and hypertension.

We hypothesized that a high dietary salt intake in salt-sensitive rats may alter central control of blood pressure by transiently or intermittently increasing CSF

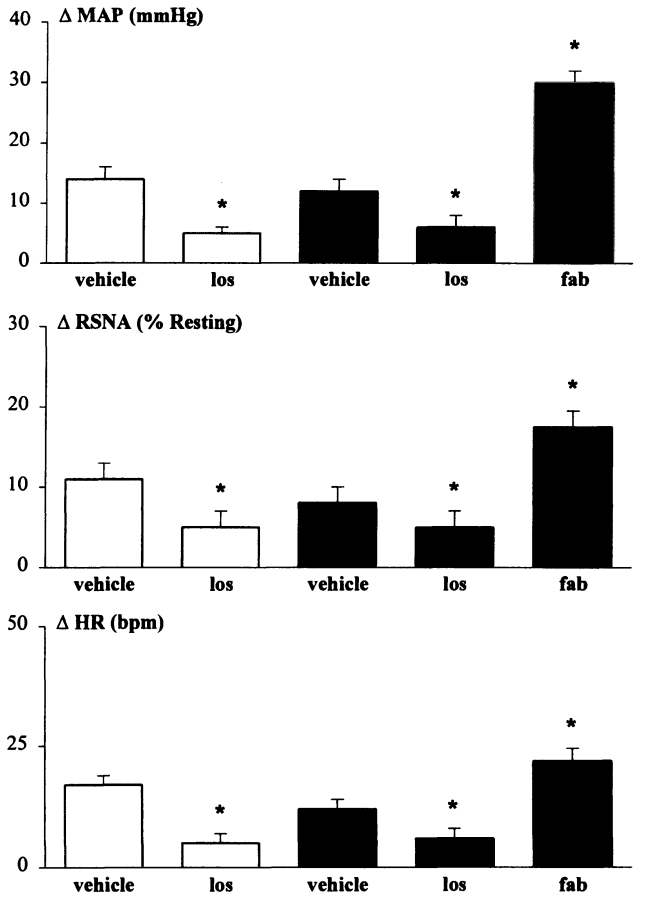


**Figure 4.** Peak increases in MAP, renal sympathetic nerve activity (RSNA), and heart rate (HR) in response to icv Ang II (30 ng) in SHR on regular (0.5% NaCl, white bars) or high (8.0% NaCl, black bars) salt diet treated chronically with icv vehicle, losartan (1 mg/kg/d, los), or Fab fragments (200 μg/d, fab), Values are mean ± SEM. n = 7–8. *$p < 0.05$ vs icv vehicle on same diet. (Adapted from Huang and Leenen [83].)

sodium concentration, causing an increase in central "ouabain" and sympathetic outflow [90]. However, to what extent such an increase in central sodium indeed occurs in still controversial [96,97]. In normotensive rats, acute icv administration of hypertonic saline, ouabain and, Ang II cause similar sympathoexcitatory and pressor responses which are all abolished by icv pretreatment with the $AT_1$ receptor blocker, losartan [44]. Fab fragments, on the other hand, only block responses to hypertonic saline and ouabain (figure 2) [44]. This would suggest that an increase in central sodium concentration increases brain "ouabain", which exerts its

sympathoexcitatory and pressor effects mostly via brain Ang II. Chronic increases in CSF sodium in normotensive, salt-resistant Wistar rats cause an increase in both central "ouabain" sympathoexcitation and blood pressure, mimicking the effects of high dietary sodium in salt-sensitive rats [98]. Concomitant icv infusion of either Fab fragments or losartan abolished the sympathoexcitation and the increase blood pressure, supporting the role of both brain "ouabain" and the brain RAS in mediating the effects of acute or chronic increase in central sodium [98]. Thus, if changes in central sodium concentrations do indeed occur on high sodium intake, they cause a similar pattern of central changes as those caused by high sodium intake in SHR and Dahl S and may therefore play a major role. High sodium intake may cause increases of CSF sodium which are larger in salt-sensitive rats than in resistant rats. In addition salt-sensitive rats may show larger responses to similar increases in CSF sodium.

The central pathways that mediate sympathoexcitation and hypertension induced by either central sodium administration or a high sodium diet have yet to be identified. An area that has been implicated in these effects is the anteroventral third ventricle (AV3V) region. An interaction of brain "ouabain" and brain Ang II in this region is supported by histological findings. Ouabain immunoreactive neurons have been demonstrated in the paraventricular and supraoptic nuclei of the hypothalamus [99]. Dense immunoreativity to ouabain in nerve terminals is observed in the AV3V, including the OVLT, the SFO, and median eminence [100]. In these areas Ang II receptors and other components of the brain RAS are also densely distributed [21,101]. Studies that utilized electrolytic lesions of the AV3V showed that this region mediates the pressor effects of acute icv injections of hypertonic saline, Ang II, and ouabain [102–104]. This region is also crucial for the development of salt-sensitive hypertension [105,106]. Excitotoxic lesions of the ventral AV3V, including the OVLT, did not affect pressor responses to acute icv injection of Ang II but attenuated responses to acute icv hypertonic saline or ouabain administration [107]. Ventral AV3V lesions prevent hypertension induced by chronic icv administration of hypertonic saline [108]. Ang II binding in the OVLT is increased in SHR when compared to WKY rats [109]. The firing rate of MnPO neurons that are Ang II responsive is higher and the threshold current required to evoke SFO stimulation-induced excitation is lower in SHR [110]. In SHR on a regular salt diet, losartan microinjected into the MnPO did not change resting blood pressure but attenuated pressor responses to acute icv injection of hypertonic saline and ouabain [111,112]. However, microinjection of either Fab fragments or losartan into the MnPO of SHR reversed sodium-induced exacerbation of hypertension [111,112], indicating that ouabain release and activation of $AT_1$ receptors within the MnPO mediates salt-sensitive hypertension.

Another area which has been implicated in mediating salt-sensitive hypertension is the anterior hypothalamic area (AHA). SHR on a high salt diet exhibit increases in the number of $\alpha_2$-adrenoceptors and decreased stores and turnover of noradrenaline in the AHA compared to SHR on a regular salt diet [113–115]. A decrease in noradrenaline release in the AHA, which consequently decreases

sympathoinhibition, may contribute to salt-sensitive hypertension. As the AHA has reciprocal connections to the OVLT in the ventral AV3V [116], mechanisms in these areas may act along the same pathway to mediate salt-sensitive hypertension.

Local osmotic stimulation of the PVN increases the release of Ang II assessed by microdialysis in this nucleus [117]. Hypertension in Dahl S rats on a high salt diet is attenuated by lesions of the PVN [118].

Thus, several nuclei/areas have been identified to be involved in the sympathoexcitatory and hypertensive responses to central sodium and high salt intake in salt-sensitive rat strains. However, at present it is still quite unclear what the actual stimulus is and what the actual pathways are leading to sympathoexcitation.

## CLINICAL RELEVANCE

It is evident from the findings presented in this review that the brain RAS contributes substantially to the development and maintenance of salt-sensitive hypertension in rats. To what extent this also applies to humans has not yet been evaluated. Further studies in both animals and humans are needed to establish the dose-response relationships for chronic oral treatment with blockers of the RAS varying in penetration into the brain. When the brain RAS contributes significantly, one may expect better responses to blockade of both peripheral and brain RAS as compared to only peripheral blockade.

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# $AT_1$ ANGIOTENSIN RECEPTOR BLOCKADE AND ANGIOTENSIN CONVERTING ENZYME INHIBITION: EFFECTS ON VASCULAR REMODELING AND ENDOTHELIAL DYSFUNCTION IN SHR

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***Summary.*** Small arteries of different vascular beds exhibit structural and functional remodeling in spontaneously hypertensive rats (SHR) compared to those of Wistar-Kyoto control rats (WKY). These differences may be reduced by treatment with angiotensin I-converting enzyme inhibitors (ACEI). It is unclear whether this beneficial effect is the result of inhibition of the generation of angiotensin (Ang) II by ACEI or the result of increased bradykinin accumulation or other mechanisms. To evaluate the role of Ang II, SHR were treated for twelve weeks with the $AT_1$ angiotensin receptor antagonist losartan or with the angiotensin-converting enzyme inhibitor enalapril once blood pressure had been elevated for some time, at ten weeks of age. Losartan induced a dose-dependent blood pressure reduction, which was associated with a blunting of cardiac and aortic hypertrophy, similar to that elicited by enalapril. Small arteries from the coronary, renal, mesenteric, and femoral circulations exhibited a dose-dependent blunting of remodeling under losartan treatment, accompanied by abolition of the impairment of endothelium-dependent relaxation, as was also found in rats treated with enalapril. Treatment with $AT_1$ selective Ang II receptor antagonists is able to induce regression of cardiovascular hypertrophy and endothelial dysfunction in genetic hypertension in the rat similar to that induced by ACEI. This suggests that part of the mechanism whereby ACEI exert their beneficial effects is via inhibition of Ang II generation.

Elevated peripheral resistance is the hallmark of high blood pressure, and it is in large measure the result of energy dissipation at the level of so-called resistance arteries, which include small arteries (vessels with lumen diameters of 100–300 μm) and even smaller arterioles [1]. Small arteries present significant changes in structure

*N.S. Dhalla, P. Zahradka, I. Dixon and R. Beamish (eds.). Angiotensin II Receptor Blockade: Physiological and Clinical Implications.* 

and function in hypertension [2,3]. Structurally they exhibit what is called "eutrophic remodeling" [4], that is a reduction in the lumen and external diameter with normal media cross-section (or volume of the media per unit length), combined with some degree of growth. There is controversy on whether vessels have the same number of smooth muscle cells, whether there is cell hyperplasia or cell hypertrophy [5,6]. The more frequent change found in small arteries is that slightly larger and more numerous cells are restructured around the lumen of the blood vessel resulting in a smaller lumen and outer diameter, with a predominant eutrophic remodeling component and a limited component of growth [7]. How this re-arrangement occurs is unknown, but may result from changes in cell adhesion molecules or intercellular matrix deposition or spatial arrangement of fibrillar material. These changes have been well described in spontaneously hypertensive rats (SHR) [6–11]. These changes may be involved in the pathogenesis of elevated blood pressure, although this is still unclear [2,12–14], and may also participate in mechanisms underlying some of the complications of hypertension, particularly at the level of the coronary microcirculation. SHR exhibit these alterations in the structure and function of small arteries in many vascular beds, including such pathophysiologically critical ones as the heart and kidney [15–17], and in the brain [15,18]. Associated with eutrophic or hypertrophic remodeling, these vessels present impaired endothelium-dependent relaxation as a result of acetylcholine-induced contractions through production of endothelium-derived contracting factor (EDCF) [19,20].

Factors implicated in the induction of structural changes in small arteries in hypertension include hyperplasia or cell hypertrophy in response to Ang II [21], other vasoactive peptides, or other agents such as catecholamines may play a role. Thus for some time it has been thought that treatment with agents that interrupt the renin-angiotensin system could result in regression of vascular remodeling in hypertension. The mechanisms involved in the production of functional changes in small arteries remain unclear. Endothelial vasorelaxant dysfunction may involve reduced nitric oxide production or enhanced degradation of nitric oxide because of the effect of superoxide anions [20] or may involve excess production of vasoconstrictor endoperoxides (EDCF)[19]. Whether treatment that interrupts the RAS would improve these abnormalities has not been definitively established. Therefore, there has been interest in determining whether some antihypertensive drugs that block the RAS may improve the structure and function of small arteries in vascular beds, such as the renal or the coronary circulations, which may be involved in major long-term complications of hypertension. Correction of structural alterations of small arteries in SHR has been demonstrated in previous studies using angiotensin-converting enzyme inhibitors (ACEI) [13,14,15,17,22,23]. The favorable effect of ACEI may be due to inhibition of Ang II generation or kinin degradation or may be due to other potential effects of these agents. Some studies performed using the Ang II antagonist losartan [24] and more recent studies with the Ang II receptor antagonist D8731 [25] have suggested that improvement of small artery structure may be induced by prolonged treatment with specific Ang II antagonists. The latter study [25] documented prevention of structural vascular

changes in several important vascular beds with this Ang II receptor antagonist that was similar to prevention of the same with the angiotensin-converting enzyme inhibitor lisinopril. In these studies prevention of the development of vascular damage in SHR was the objective, and treatment was initiated at four weeks of age. There have, however, been few attempts to learn whether regression of vascular changes in critical vascular beds could be achieved with Ang II receptor antagonists once hypertension, with its attendant adaptive vascular alterations, had already developed. Treatment of SHR with calcium channel antagonists [17,26], ACEI [17,26,27], or Ang II receptor antagonists [28] has resulted in correction of abnormal endothelial function along with the regression of vascular remodeling.

We have examined the effects of the Ang II antagonist losartan on small artery structure at the level of four vascular beds. Coronary, renal, mesenteric and femoral, were evaluated in SHR which had already become hypertensive, and which were treated from weeks of age for a period of 12 weeks [28], in comparison to effects of the ACEI enalapril given for a similar period of time. Of the vascular beds investigated, the coronary and renal small arteries may be critical in relation to hypertensive complications, whereas mesenteric and femoral small arteries may contribute to elevated peripheral resistance. These resistance-sized arteries were investigated after they were mounted on an isometric wire-myograph, as performed in most studies in the past [6,8,9,11,13–17,22,24,25,27]. In addition, mesenteric arteries were investigated isobarically in a pressurized chamber, which may represent a more physiological approach for the study of these vessels [7,19,26,29], in order to compare results with those obtained isometrically.

## METHODS

SHR were housed individually and treated from 10 weeks of age for 12 weeks with losartan or enalapril administered in the drinking water, with the concentration of drug adjusted daily to ensure a dose of 20 or 50 mg/kg per day of losartan or 10 mg/kg per day of enalapril. Blood pressure of treated and untreated SHR and of age-matched Wistar-Kyoto (WKY) control rats was measured every two weeks with the tail-cuff method after warming the rats, which were slightly restrained in a plexiglass cage. Rats were killed by decapitation, and tissues were immediately dissected, blotted dry, and weighed. Coronary, renal arcuate, and femoral arteries were obtained as described previously [16,17]. They were mounted on an isometric wire-myograph and measurements performed as described already in detail [11, 16,17,27]. The vessels were contracted with 10 μmol/l norepinephrine and relaxed with a single maximal concentration of 10 μmol/l acetylcholine to evaluate endothelium-dependent contraction. Mesenteric small arteries obtained from the same rats were mounted as pressurized preparations, at a intravascular pressure of 45 mmHg [7]. The function of the vascular endothelium was evaluated by extraluminal application of acetylcholine (10 μmol/L) in PSS containing 10 μmol/L norepinephrine. Measurements were performed on the pressurized vessels after they were deactivated with 10 μmmol/L ethyleneglycol-bis-(β-aminoethyl ether)N,N,N′,N′-tetraacetic acid (EGTA) for 10 minutes.

## RESULTS

### Effect of treatment on blood pressure, and on heart and aorta weight

Systolic blood pressure measured with the tail-cuff method in rats treated with losartan or enalapril rose significantly less ($p < 0.01$) than that of untreated SHR. At 22 weeks of age, blood pressure of SHR treated with a low dose of losartan (20 mg/kg per day in the drinking water) rose to 181 ± 1 mmHg, that of SHR treated with a high dose of losartan (50 mg/kg per day in the drinking water) rose to 156 ± 4 mmHg. That of SHR reated with enalapril (10 mg/kg per day in the drinking water) rose to 148 ± 4 mmHg. whereas the blood pressure of untreated SHR rose to 210 ± 2 mmHg. Blood pressure of WKY rats was unaffected by treatment. The weight of the heart, which was significantly elevated in SHR relative to body weight (4.41 ± 0.08 mg/g body weight), was dose-dependently reduced under treatment with losartan (to 3.47 ± 0.06 mg/g at the high dose, $p < 0.01$) and significantly reduced to a similar degree by enalapril (3.67 ± 0.06 mg/g), $p < 0.01$). The weight of 2 cm segments of thoracic aorta of SHR (12.7 ± 0.2 mg/100 g body weight) were significantly reduced as well by treatment in a dose-dependent manner (to 9.5 ± 0.1 mg/100 g by the higher dose of losartan, and to 10.3 ± 0.3 mg/100 g by enalapril, both $p < 0.01$ vs. untreated SHR), whereas there were no changes in WKY when corrected for weight.

### Effect of losartan or enalapril on structure of small arteries

Small arteries from the coronary, renal (arcuate arteries), mesenteric, and femoral circulations, whether studied on the wire-myograph or as pressurized preparations, exhibited the expected combination of eutrophic and mild hypertrophic remodeling [4,7,8,11]. Lowering of blood pressure with losartan or enalapril did not significantly affect lumen diameter, but a significant dose-dependent reduction in media width to lumen diameter ratio was found in the four vascular beds examined on the wire myograph from SHR treated with losartan (figure 1) and in the mesenteric arteries studied as pressurized preparations (not shown). Similarly, enalapril treatment resulted in a significant decrease in media to lumen ratio in the four vascular beds, without significant change in lumen diameter (figure 1).

### Effect of treatment on endothelial function of small arteries

Wire-mounted mesenteric small arteries of WKY contracted with a submaximal concentration of methoxamine were completely relaxed by 10 μmol/L acetylcholine. In contrast, and as expected [19,30], arteries from untreated SHR exhibited contractions when stimulated with this dose of acetylcholine. Small arteries from losartan or enalapril-treated SHR showed an abolition of these contractions elicited by elevated concentrations of 10 μmol/L acetylcholine, whereas responses to the endothelium-independent vasodilator nitroprusside were similar in treated or untreated SHR. Pressurized, precontracted mesenteric small arteries from WKY were almost completely relaxed by 10 μmol/l acetylcholine (>95%), whereas those from untreated SHR relaxed significantly less (<80%). Pressurized mesenteric small arter-

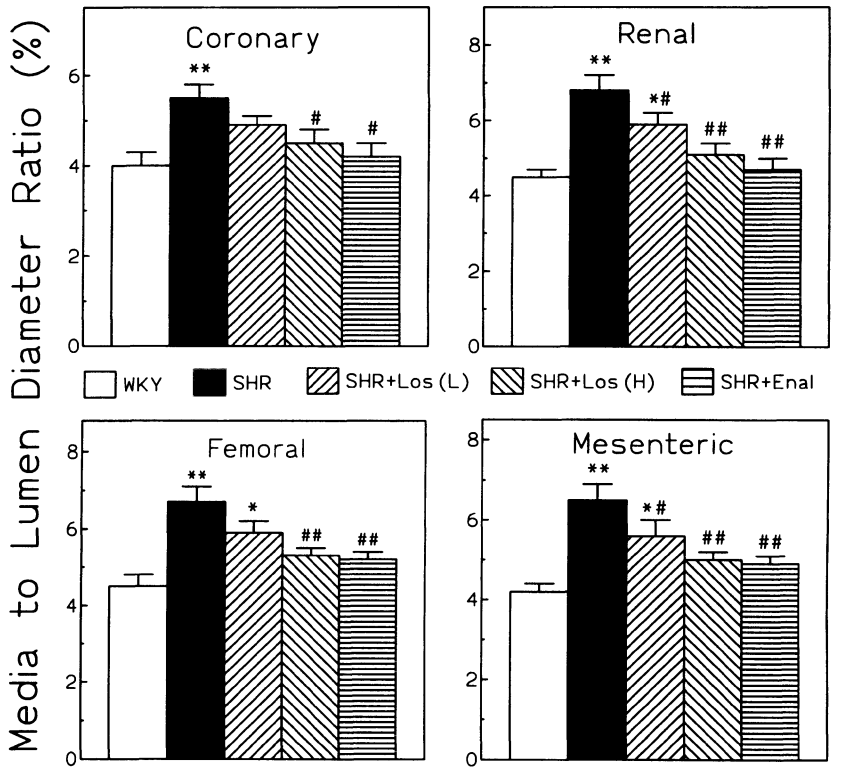


**Figure 1.** Bar graphs show the media width to lumen diameter ratio of small arteries dissected from the coronary, renal cortical, mesenteric, and femoral vasculature from Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR), the latter treated or not with a low dose of losartan (Los (L), 20 mg/kg per day in the drinking water), a high dose of losartan (Los (H), 50 mg/kg per day), or enalapril (Enal, 10 mg/kg per day) and studied on an isometric wire-myograph. *$p < 0.05$, **$p < 0.01$ vs. WKY; # $p < 0.05$, ## $p < 0.01$ vs. SHR. (Drawn in part using data from Li J-S et al. [28].)

ies from losartan or enalapril-treated SHR showed a normalization of relaxation in response to acetylcholine (>90%).

## DISCUSSION

Chronic administration of the ACEI enalapril or the orally active, nonpeptidic selective Ang II $AT_1$ receptor antagonist losartan to SHR results in a dose-dependent regression of hypertrophy. However, correction of endothelium-dependent dysfunction in small arteries occurs already at the lower dose of losartan, which is associated with only a moderate degree of correction of small artery structure. This may indicate that normalization of the impairment of endothelial function may

precede correction of structure, as already suggested by other studies in which short-term treatment with a calcium channel blocker normalized endothelium–dependent relaxation at a time when no correction of vascular structure was detectable [31]. It is difficult to know whether blood pressure lowering is a necessary condition for regression of hypertensive cardiovascular changes. In other studies using the ACEI perindopril, regression of vascular hypertrophy was dose-dependent and parallel to blood pressure lowering [15], as with losartan in the study reported here. This would support the view that structural regression is determined in large measure by blood pressure lowering. With losartan there may, however, slightly greater reversal of hypertrophy than with the ACEI cilazapril and the calcium channel antagonist mibefradil [17] or even with enalapril in this study. Other studies have shown similar effects of ACEIs and other $AT_1$ receptor antagonists [25].

Losartan appears to exert its effect on vascular growth [32] by blockade of the $AT_1$ angiotensin receptor and thus by interruption of the RAS, which may explain the similarity of its results with ACEI. Bradykinin accumulation may contribute to the effects of ACEI, but these results suggest that in relation to structural regression, bradykinin, which does not accumulate under the angiotensin antagonist, plays at most a minor role.

The improvement in endothelial dysfunction (production of endothelium-derived contracting factor or EDCF) [19,30] after treatment with ACEI has sometimes been attributed to the beneficial effects of kinin accumulation. This would be expected with ACE inhibition but not with angiotensin receptor blockade. The improvement found in SHR treated with losartan may be a consequence of blood pressure lowering or of some other phenomenon. Angiotensin $AT_1$ subtype receptors are present in the endothelium of arteries [33], and their blockade could result in the improvement of endothelial function reported in this study. Endothelial effects of $AT_1$ blockade could also result from the unopposed action of angiotensin peptides on $AT_2$ receptors, which are not blocked by losartan. In the kidney, $AT_2$ receptor stimulation via generation of cGMP antagonizes $AT_1$ receptor stimulation of $PGE_2$ production [34]. The vasorelaxant and antigrowth effects of cGMP could contribute to the results of treatment with losartan, resulting in effects similar to or even greater than those induced by ACEIs.

In conclusion, both the orally active, nonpeptidic selective angiotensin $AT_1$ receptor antagonist losartan and the ACEI enalapril induced a regression of cardiovascular hypertrophy in the heart and small arteries of pathophysiologically critical vascular beds, such as the coronary and renal circulations, and simultaneously improved endothelial dysfunction. Neither the present nor previous studies conclusively demonstrate whether the effects of ACEI or $AT_1$ receptor antagonism are superior. On the other hand, if beneficial effects such as those mentioned occur in hypertensive humans treated with these drugs, as has already been demonstrated with ACEIs [35–38], this class of agents could have a significant beneficial effect on blood vessels of hypertensive humans, and could improve outcome, which remains to be demonstrated.

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# CELLULAR PHYSIOLOGY OF ANGIOTENSIN II RECEPTORS IN VASCULAR SMOOTH MUSCLE CELLS

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***Summary.*** Angiotensin (Ang) II operates both as a systemic hormone modulating blood pressure and as a paracrine factor that is synthesized in response to stress. Regardless of its origin, the response of cardiovascular tissues to Ang II is mediated by specific cell surface receptors. In vascular tissues, two angiotensin receptors have been characterized according to their sensitivity to the specific antagonists losartan and PD123319 [1]. The $AT_1$ receptor (losartan-sensitive) accounts for the majority of Ang II binding activity in adult tissue. It is significant, however, that upon injury and during fetal development, the proportion of $AT_2$ receptors (PD123319-sensitive) increases [2]. Until recently the $AT_1$ receptor has been regarded as the principal mediator of the responses evoked by Ang II. As a consequence the $AT_2$ receptor has been studied less intensively and fewer details of its biological functions have been defined. The developmental regulation of $AT_2$ receptor expression, however, suggests that it may be important in ontogeny. Furthermore, the generation of an $AT_2$ receptor knockout mouse has revealed that this receptor, although its absence is not lethal, governs both the drinking response and motility [3]. In our laboratory, which has made use of porcine tissue for its studies, the application of antagonists specific to either the $AT_1$ or the $AT_2$ receptor has revealed that both receptors are independently required for VSMC growth. This review will, therefore, summarize recent advances in our understanding of Ang II-mediated SMC growth with respect to the role of individual angiotensin receptor subtypes and their associated signaling systems. Since both receptors have also been found essential for the vascular response to injury, an emphasis has been placed on defining the relationship between SMC growth and Ang II as it applies to the development of coronary artery disease and restenosis.

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## THE RENIN-ANGIOTENSIN SYSTEM IN RESTENOSIS

Perhaps one of the most profound advances in the clinical treatment of occlusive coronary artery disease has been the advent of coronary angioplasty [4]. While the initial intervention is highly successful (>90%), there is a significant problem with late (4 to 10 months) failure which may reach as high as 40% [5,6]. This regression is caused primarily by the formation of a secondary occlusion or restenosis that differs significantly from the original atherosclerotic lesion. Restenosis is a proliferative disease of the arterial wall that occurs in response to vascular trauma and is characterized by migration and rapid luminal proliferation of cells originally in the medial smooth muscle layer, deposition of extracellular matrix, and infiltration of neutrophils and macrophages [7,8].

Cellular stress (vascular damage) has been linked to activation of the renin-angiotensin system [9]; therefore, it seemed appropriate to examine the role of angiotensin (Ang) II in the formation of the restenotic lesion. A porcine coronary artery culture model (modified from Koo and Gotlieb [10]) was used to examine the effect of balloon angioplasty on the contribution of Ang II to the formation and proliferation of a neointima. Using this model of restenosis, we were able to demonstrate that neointimal proliferation is suppressed in a concentration-dependent manner by losartan ($AT_1$ antagonist), reaching a maximum inhibition of 70% at 10 μM [11]. A similar effect was observed with PD123319 ($AT_2$ antagonist), with a 76% reduction at 10 μM. A combination of both losartan and PD123319, on the other hand, showed no additional improvement. Interestingly, while it has been shown that ACE inhibitors successfully reduce stenosis in rodent models [12,13], they have proven ineffective in our porcine model. In fact, captopril produced a slight increase in the magnitude of the neointima following balloon angioplasty [11].

The importance of species-specific responses should be considered carefully in light of the MARCATOR study, which demonstrated that the clinical use of ACE inhibitors was largely ineffective in reducing restenosis postangioplasty [14]. The observed species-specificity for ACE inhibitors may be explained by the presence of alternative pathways (figure 1) for the synthesis of Ang II [15]. The importance of a chymase-dependent system has recently been recognized in humans [16–18]. It may, therefore, be predicted that receptor antagonists will be more effective in preventing restenosis since they block the cell response directly. It may be prudent, however, to consider a recent report by Huckle et al. [19] that compared the utility of $AT_1$, $AT_2$ and, dual $AT_1/AT_2$ receptor blockade in both the rat and the pig. These investigators showed the $AT_1$ antagonist (L-158,809) produced a significant reduction in neointimal area in the rat (37%) while having a minimal effect in the pig (12%). Although the latter was not statistically significant, a 12% reduction in neointimal thickness could theoretically increase flow by approximately 50%. While little effect was observed by these authors with $AT_2$ or $AT_1/AT_2$ antagonists, evidence that $AT_2$ receptor blockade will effectively prevent neointimal formation has, nevertheless, been reported [20]. It is, therefore, tempting to speculate that

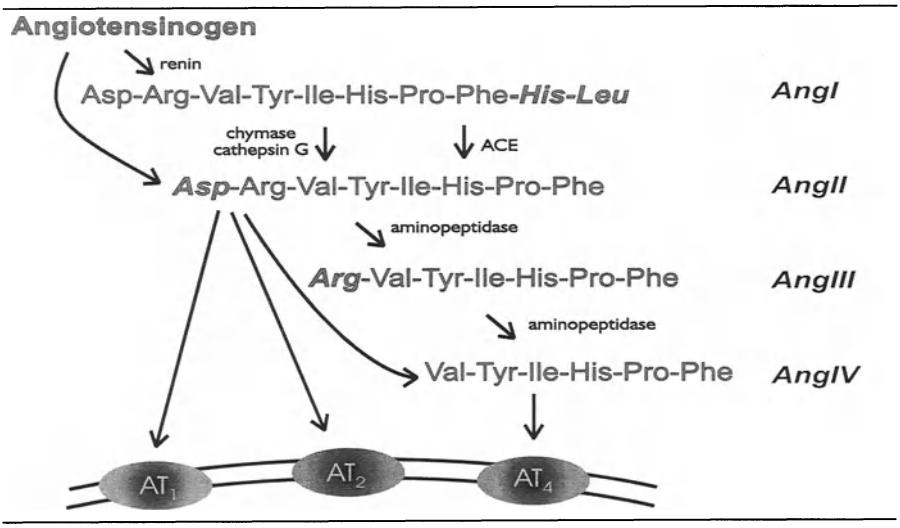


**Figure 1.** An overview of angiotensin II metabolism.

restricting the interaction of Ang II with its receptors might limit the development of restenosis post-angioplasty, regardless of whether the vascular source of Ang II is ACE-dependent or not.

## THE SMOOTH MUSCLE RESPONSE TO ANGIOTENSIN II

Vascular smooth muscle cells (VSMC) express both $AT_1$ and $AT_2$ receptor subtypes as demonstrated through ligand binding and Northern blotting experiments [21,22]. In adult vascular tissues, the $AT_1$ receptor clearly predominates, as indicated by the insensitivity of the Ang II binding sites to PD123319 [23]. This disparity in receptor number has led to increased interest in the $AT_1$ receptor as a target for therapeutic intervention and interest in using specific receptor antagonists, such as losartan. Numerous studies have demonstrated that the $AT_1$ receptor is involved in both the contractile and the proliferative responses to Ang II [24,25]. Based on these reports, it has been assumed that the $AT_1$ receptor almost exclusively mediates the biological responses associated with Ang II. This interpretation has provoked little debate because information concerning $AT_2$ receptor function is lacking. More recently, however, we have observed that both receptor subtypes may be required for a complete smooth muscle cell (SMC) response to injury [11]. Similar results have been reported by Levy et al. [26] who found that vascular hypertrophy and fibrosis could be reduced by $AT_2$ receptor blockade as well as by $AT_1$ receptor antagonists. Since the studies reported to date have shown the $AT_1$ and $AT_2$ receptors do not stimulate the same intracellular responses, we propose that these receptors mediate distinct signaling pathways and that activation of both receptors may be necessary to evoke cell proliferation.

Methods to assess the alteration in cell growth state can be readily applied to studies at the tissue or animal level, but a comprehensive and detailed characterization of the molecular mechanisms that regulate these processes is more effectively achieved with cells in culture. Two cell culture systems have, therefore, been employed in the course of our studies. Our initial studies used rat aortic A10 SMCs to monitor the stimulation of growth by Ang II. The results supported the premise that the $AT_2$ receptor is an important factor in angiotensin-dependent growth [27]. Additional studies with A10 SMCs have established that $AT_2$ receptor density varies with cell-growth state [28]. This latter point was corroborated by Kambayashi et al. [29] who observed that $AT_2$ receptor expression was induced by placing rat aortic SMCs into serum-reduced conditions. Experimental conditions designed to achieve quiescence, as described by Saward and Zahradka [28] and Kambayashi et al. [29], were not previously employed by other investigators; consequently, a contribution of $AT_2$ receptors to SMC growth may not have been detectable. Regardless, our results [28] and those of Kambayashi et al. [29] clearly indicate that establishment of a quiescent state, which requires a period of 3 to 7 days in reduced serum, should be considered as an essential protocol for studies designed to survey $AT_2$ receptor function.

Our second culture system employed porcine coronary artery SMCs [30] which proliferate in response to Ang II, as determined by assays for protein, RNA and, DNA synthesis [Saward and Zahradka, submitted]. Evidence that SMCs traverse the cell cycle and proceed through mitosis has also been obtained. Transition through the cell cycle was confirmed by experiments (PCNA immunostaining, thymidine uptake) which established that S phase is not reached sooner than 36 hours, while an increase in cell number is not observed before 96 hours after addition of the Ang II. These data demonstrated that SMC proliferation is activated by Ang II, although passage through the cell cycle is relatively slow.

The experiments with porcine artery SMCs were subsequently extended to characterize the receptor contribution to angiotensin-mediated SMC proliferation. As was observed with the A10 cell and organ culture systems, the proliferation of coronary artery SMCs was inhibited by PD123319, supporting the evidence that $AT_2$ receptor activation is important for cell growth [31]. Interestingly, losartan was also observed to inhibit SMC growth, exhibiting an efficacy that is comparable to, but not additive with, that of PD123319. Since these antagonists are highly selective for their respective receptors, a new paradigm must be developed to justify the involvement of both angiotensin receptor subtypes in the control of cell proliferation. To account for these observations, we have postulated that the $AT_1$ and $AT_2$ receptors operate through independent intracellular signaling systems, each activating distinct processes that are obligatory for cell cycle progression. Thus, the redundancy in receptor function that is perceived when an endpoint such as cell growth is used to monitor the response to Ang II may not be evident when short-term responses are considered. As part of our effort to define the contribution of the $AT_1$ and $AT_2$ receptors to SMC growth and proliferation, two unrelated systems,

prostaglandins and phosphatidylinositol 3-kinase (P13K), were investigated for their potential to operate as intracellular mediators for Ang II.

## PROSTAGLANDINS AS MEDIATORS OF SMC GROWTH

Prostaglandins operate as paracrine factors for endothelial smooth muscle communication and also function as intracellular mediators for specific growth factors [32]. Previously, we reported that prostaglandin synthesis was required for bradykinin-dependent inhibition of A10 SMC growth [33]. Prostaglandins also serve as signaling intermediates following treatment with Ang II since indomethacin inhibits growth stimulation of both A10 and porcine coronary artery SMCs by Ang II [27, Saward, Yau and Zahradka, unpublished]. To verify that indomethacin interferes specifically with prostaglandin synthesis, Ang II was tested for its ability to provoke the release of arachidonic acid by coronary artery SMCs. In this experiment, it was observed that Ang II caused an increase in arachidonic acid release that was blocked in the presence of an $AT_2$ receptor antagonist [Saward, Yau, and Zahradka, unpublished]. This indirect assessment of prostaglandin production indicates that Ang II may stimulate phospholipase activity via the $AT_2$ receptor. A comprehensive evaluation of the various prostanoids produced in response to Ang II is ongoing.

Although the prostaglandin(s) produced in response to Ang II remains to be identified, we have investigated whether the direct addition of a specific prostaglandin in vitro could influence SMC growth. Prostacyclin ($PGI_2$) had no detectable effect on cell growth. On the other hand, the ability of $PGE_2$ to induce c-fos proto-oncogene expression, trigger MAP kinase phosphorylation, stimulate DNA synthesis, and increase cell number suggests that $PGE_2$ may indeed activate cell cycle progression [Yau and Zahradka, unpublished]. These data clearly establish that the synthesis of specific prostaglandins in response to Ang II has the potential to promote SMC growth.

## PHOSPHATIDYLINOSITOL 3-KINASE: A UNIQUE MEDIATOR OF THE VASCULAR RESPONSE TO ANGIOTENSIN II

Evidence that receptor antagonists block the angiotensin-mediated proliferative response of SMCs in both primary culture and an organ culture model suggests that excess Ang II may promote the development of certain vascular pathologies. Alternatively, either circulating or local Ang II could intensify the response originating from other hormonal factors. For this reason, reliance upon a single therapeutic approach may not be tenable. Treatment strategies may be improved if common intracellular mediators could be identified and their activity suppressed. We have, therefore, been interested in defining common signal transduction pathways that are activated in response to both Ang II and other hormonal agents.

PI3K is a fundamental intracellular signaling component that is associated with tyrosine kinase (TK) receptors [34]. Phosphorylation of the tyrosine moiety within the SH2 domain of a TK receptor or a coupling protein, such as IRS-1, produces a docking site for the p85 subunit of PI3K which is followed by activation of the

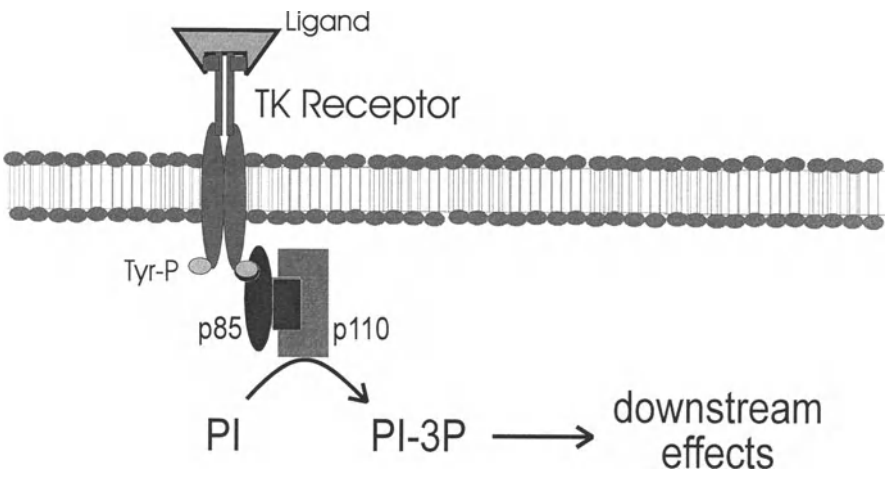


**Figure 2.** Phosphatidylinositol 3-kinase is activated by tyrosine kinase (TK) receptors.

catalytic p110 subunit (figure 2). In contrast, G protein-coupled receptors operate by stimulating GTP/GDP exchange, causing the release of the Gα subunit, which leads to increases in second messengers, such as cAMP and $Ca^{2+}$. Recent evidence that TK and G protein receptors do not operate through mutually exclusive systems is supported by reports that tyrosine phosphorylation also occurs when G protein receptors are activated [35,36]. The identification and characterization of a PI3K isoform (p110γ) that is activated by $G_{\beta\gamma}$ subunits independently of p85 [37] provides a candidate enzyme for the coupling of TK and G protein receptor-mediated signaling.

The potential for a connection between PI3K and G protein receptors raises the possibility that PI3K activation may influence SMC growth in response to Ang II. This prospect was tested and confirmed by experiments that showed that wortmannin and LY294002, both potent inhibitors of PI3K, blocked Ang II-stimulated hyperplasia [38]. An analysis of phosphatidylinositol 3-phosphate (PI-3P) production 15 minutes after treatment of SMCs with Ang II revealed that PI3K activity was significantly increased. This increase in PI-3P could be prevented by treatment with an $AT_1$ receptor antagonist (losartan). Participation of the p85/p110 PI3K in the cellular response to Ang II was indicated by a transient increase in p85 tyrosine phosphorylation which peaked at 15 minutes and returned to basal levels by 30 minutes. Immunocytochemistry localized the p85 subunit to the perinuclear region in quiescent cells. After 15 minutes of Ang II stimulation, however, p85 was uniformly distributed throughout the cell. Concurrent with the decrease in p85 phosphorylation at 30 minutes, p85 relocalized to the perinuclear region. These data were confirmed by a subcellular fractionation that showed p85 increased in the membrane fraction of Ang II-treated SMC concomitant with a decrease in

the cytoskeletal fraction. To confirm that the changes in p85 generated by Ang II are coupled to PI3K activity, PI3K activity was measured in vitro after immunoprecipitation of p85. The increased synthesis of PI-3P that was observed with the immunoprecipitate from an Ang II-treated cell lysate clearly establishes that the early response of PI3K to Ang II is mediated by p85, and not $G_{\beta\gamma}$, subunits.

The available data strongly support the existence of a direct relationship between PI3K and G protein activation. To explore this possibility, the coupling of PI3K to prostaglandin receptors, which are also G protein-coupled, was examined. Not only did addition of $PGE_2$ to quiescent SMC stimulate the production of PI-3P, but also it produced a translocation of the p85 subunit [Yau and Zahradka, unpublished]. Furthermore, the PI3K inhibitors wortmannin and LY294002 prevented prostaglandin-mediated SMC growth. Thus, it appears likely that activation of PI3K is indispensable for certain aspects of G protein receptor function.

The data described above suggest that G protein receptors can stimulate the activity, phosphorylation, and migration of the p85/p110 isoform of PI3K and that this pathway is vital for both Ang II and prostaglandin-mediated SMC growth. Although these results establish that PI3K activation is an early event, we have also noted that PI3K inhibitors effectively prevent cell proliferation if they are added to cells more than 60 minutes following stimulation with Ang II [Saward and Zahradka, unpublished]. These data support the possibility that either p85/p110 PI3K activity is continuous over extended periods after Ang II stimulation or PI3K (either p85/p110 or p110γ) is sequentially activated by autocrine factors, such as prostaglandins, which are produced as cells progress through the cell cycle. A comprehensive analysis of p85/p110 and p110γ over a broad time course will be necessary to define the function of the individual PI3K isoforms with respect to G protein-coupled receptors.

## SYNOPSIS

Ang II is a vasoactive hormone that is synthesized in response to specific disease states or a chronic reduction in blood pressure brought about by physical trauma. This process represents a mechanism that may have evolved to augment the autonomic systems that modulate acute changes in blood pressure. While Ang II exists as a blood-borne hormone that affects all tissues, a local renin-angiotensin system has been identified in many tissues. The synthesis (or release) of Ang II at the tissue level occurs in response to numerous stimuli.

This review has described a number of observations that indicate angiotensin receptors and their associated intracellular signaling systems play an important role in the growth stimulation of smooth muscle, which may be involved in hypertension and restenosis. First, we have indicated that either the $AT_1$ or the $AT_2$ receptor may serve as a useful target for interventions designed to reduce the neointimal proliferation that occurs subsequent to balloon angioplasty. Second, we have established that (1) a relationship exists between changes in $AT_2$ receptor expression and SMC

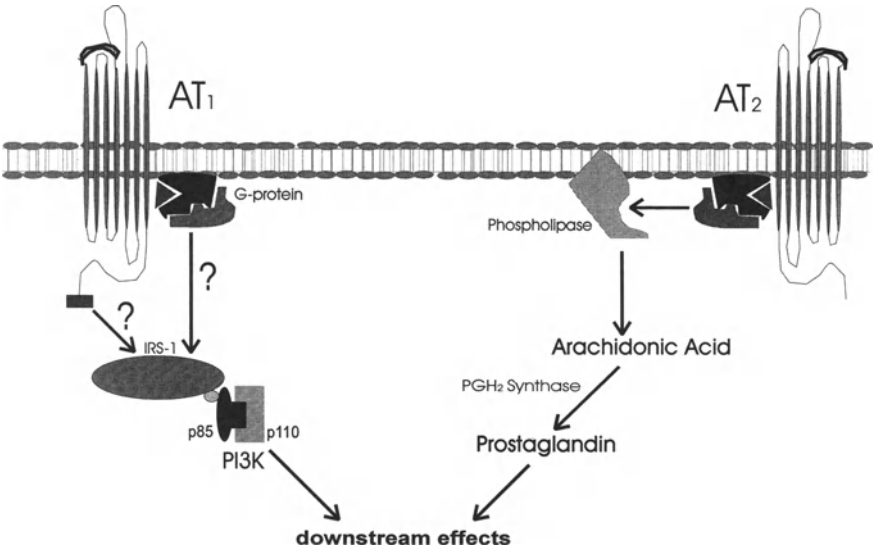


**Figure 3.** A model for activation of distinct signaling pathways by $AT_1$ and $AT_2$ receptors.

growth state, and (2) both $AT_1$ and $AT_2$ receptor subtypes must be activated for Ang II-dependent stimulation of SMC proliferation. We have also confirmed that several intracellular events that are influenced by Ang II are mediated by specific receptor subtypes. Based on this information, a model that links both PI3K activation with the $AT_1$ receptor and prostaglandin synthesis with the $AT_2$ receptor has been developed (figure 3). Experimental data indicating Ang II stimulates tyrosine phosphorylation of IRS-1 (Saward and Zahradka, in preparation), which agree with recent reports by Saad et al. [39] and Du et al. [40], have been incorporated into this model, since this observation provides a possible mechanism for coupling p85/p110 PI3K to the $AT_1$ receptor. Finally, we have found that inhibitors of specific signal transduction processes also have the capacity to interfere with SMC growth. In particular, inhibition of PI3K, which functions as a key signal transduction molecule for both tyrosine kinase and G protein-coupled receptors, can prevent cell growth in the presence of multiple growth factors. This feature of PI3K has made it valuable to research groups attempting to control tumor growth. It also makes it a candidate for the therapeutic intervention of cardiovascular conditions linked to hypertrophy or hyperplasia.

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# ANGIOTENSIN II ENHANCED THE EXPRESSION OF INHIBITORY GUANINE NUCLEOTIDE REGULATORY PROTEIN IN VASCULAR SMOOTH MUSCLE CELLS: BLOCKADE BY $AT_1$ ANTAGONIST

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***Summary.*** In the present studies, we have investigated the effect of angiotensin (Ang) II on guanine nucleotide regulatory protein (G protein) expression and functions in A-10 vascular smooth muscle cells (VSMCs). Ang II treatment of VSMC enhanced the levels of inhibitory guanine nucleotide regulatory protein (Gi) as well as Gi mRNA in a concentration-dependent manner as determined by immunoblot and Northern blot analysis, respectively. However, the GTPγS-mediated inhibition of forskolin (FSK)-stimulated adenylyl cyclase activity and the receptor-mediated inhibition of adenylyl cyclase by Ang II and C-$ANP_{4-23}$ [des($Gln^{18}$, $Ser^{19}$, $Gln^{20}$, $Leu^{21}$, $Gly^{22}$) $ANF_{4-23}$-NH2] (C-$ANP_{4-23}$) were attenuated in Ang II-treated cells. On the other hand, Gs protein expression and functions were not altered by Ang II treatment. Losartan treatment of the cells was able to partially restore the Ang II induced enhanced expression of Gi protein as well as the attenuated responsiveness of adenylyl cyclase to Ang II and C-$ANP_{4-23}$ inhibition. The results suggest the implication of $AT_1$ receptor in Ang II-induced increases of Giα protein expression in vascular smooth muscle cells.

## INTRODUCTION

Ang II, a vasoactive peptide and a key component of renin-angiotensin system, elicits a wide variety of biological responses, including vasoconstriction, stimulation of aldosterone secretion, and renal sodium reabsorption [1]. In addition, Ang II is a growth promoting factor for several cell types, such as fibroblasts, adrenocortical cells, cardiac myocytes, and vascular smooth muscle cells [2,3]. Ang II induces cell hypertrophy in cultured aortic smooth muscle cells as a result of increased protein synthesis [4,5] which is associated with increased expression of the growth associated

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nuclear proto-oncogene, c-fos, c-jun and c-myc [6,7]. Ang II also stimulates tyrosine phosphorylation of multiple substrates [8], including mitogen-activated protein kinase (MAPK) [8–10].

Ang II elicits its physiological effects by interacting with two distinct receptor subtypes, designated as $AT_1$ and $AT_2$ [11], based on their interaction with nonpeptide antagonists, losartan and PD123177, respectively [12]. The presence of $AT_1$ receptor subtype has been shown in rat vascular tissues. However, a small proportion of $AT_2$ receptors is also present in rat aorta [13,14]. Most of the physiological effects of Ang II are mediated by $AT_1$ receptors. $AT_1$ receptors are coupled to several second messenger systems, such as stimulation of phospholipase C (PLC) [15], D (PLD) [16], and $A_2$ ($PLA_2$) [17] and inhibition of adenylyl cyclase/cAMP [18–20] and plasma membrane calcium channels [21]. The activation of PLC results in the formation of two second messengers, inositol triphoshate ($IP_3$) and diacylglycerol (DAG). DAG activates protein kinase C (PKC), shown to phosphorylate various proteins, including inhibitory G-binding proteins, Gi [22,23]. The phosphorylation of Gi regulatory protein uncouples the inhibitory hormone receptor from adenylyl cyclase and thereby attenuates the hormone-mediated inhibition of adenylyl cyclase.

Adenylyl cyclase/cAMP system is composed of three components: receptor, catalytic subunit, and guanine nucleotide regulatory proteins (G proteins). The G proteins act as transducers and, in the presence of guanine nucleotides, transmit the signal from the hormone-occupied receptor to the catalytic subunit. The hormonal stimulation and inhibition of adenylyl cyclase are mediated through the stimulatory (Gs) and inhibitory (Gi) guanine nucleotide protein, respectively [24,25], resulting in the increased or decreased formation of cAMP, respectively. G proteins are heterotrimeric, consisting of $\alpha$, $\beta$, and $\gamma$ subunit. The $\alpha$ subunit binds and hydrolyzes GTP and confers specificity in receptor and effector interactions [25]. Four different isoforms of Gs have been identified which appear to be products of alternate splicing of a common precursor [26,27]. On the other hand, three distinct forms of Gi$\alpha$, namely, Gi$\alpha$-1, Gi$\alpha$-2, and Gi$\alpha$-3, have been identified and shown to be products of three different genes [28,29].

Genetic linkage between the Ang II gene and hypertension has been established [30]. Levels of Ang II have been reported to be elevated in hypertensive human beings [31]. Transgenic mice overexpressing Ang II have also been shown to have elevated blood pressure [32,33]. In addition Ang II has been shown to be differentially regulated in tissues involved in blood pressure regulation [34]. We have recently demonstrated increased expression of Gi$\alpha$ protein and Gi$\alpha$ mRNA and associated functions in spontaneously hypertensive (SHR) and deoxycorticosterone acetate (DOCA)-salt hypertensive rats as compared to their control rats [35,36]. The enhanced expression of Gi$\alpha$ and associated functions in SHR were restored towards control level by angiotensin-converting enzyme (ACE) inhibitor [37], which inhibits the conversion of Ang I to Ang II and thereby decreases the levels of Ang II. Taken together, it is possible that the enhanced levels of Ang II reported in

hypertension [31] may be responsible for the observed enhanced expression of Gi proteins in hypertensive rats [35,36]. To examine this possibility, the present studies were undertaken to examine the effect of Ang II treatment, in the absence or presence of losartan, on the expression of G proteins and adenylyl cyclase activity in vascular smooth muscle cells (VSMC, A-10).

## MATERIALS AND METHODS

### Cell culture and incubation

Pure VSMC (A-10) from embryonic thoracic aorta of rat was obtained from American Type Culture Collection, Rockville, MA, USA. The cells were plated in 7.5 $cm^2$ flasks and incubated at 37°C in 95% air and 5% $CO_2$ humidified atmosphere in Dulbecco's modified Eagle's medium (DMEM) (with glucose, L-glutamine, and sodium bicarbonate) that contained antibiotics and 10% heat-inactivated fetal calf serum (FCS). The cells were passaged, upon reaching confluence, with 0.5% trypsin containing 0.2% EDTA and utilized between passages 5 and 15. Confluent cell cultures were starved by incubation for 3 h in DMEM without FCS at 37°C. These cells were then incubated with different concentrations of Ang II for 24 h at 37°C, as described previously [38]. The treatment with different concentrations of losartan was done in the presence of $10^{-7}$ M Ang II. After incubation, cells were washed twice with ice-cold homogenization buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). The VSMC were scraped into ice-cold homogenization buffer using a rubber policeman and collected by centrifugation at 4°C for 10 min at 600 × g. The cells were then homogenized in a Dounce homogenizer (10 strokes), and the homogenate was used for adenylyl cyclase assay and immunoblotting.

### Adenylyl cyclase activity determination

Adenylyl cyclase activity was determined by measuring [$^{32}$P]-cAMP formation from [$\alpha$-$^{32}$P]ATP, as described previously [35,36]. Briefly, the assay medium contained 50 mM glycylglycine, pH 7.5; 0.5 mM MgATP, [$\alpha$-$^{32}$P]ATP (1.5 × $10^6$ cpm); 5 mM $MgCl_2$ (in excess of the ATP concentration); 100 mM NaCl, 0.5 mM cAMP; 1 mM 3-isobutyl-1-methyl xanthine; 0.1 mM EGTA; 10 $\mu$M GTP$\gamma$S and an ATP-regenerating system consisting of 2 mM phosphocreatine, 0.1 mg of creatine kinase/ml, and 0.1 mg of myokinase/ml in a final volume of 200 $\mu$l. Incubations were initiated by the addition of the membrane preparations (20–30 $\mu$g) to the reaction mixture, which had been thermally equilibrated for 2 min at 37°C. The reactions conducted in triplicate for 10 min at 37°C, were terminated by the addition of 0.6 ml of 120 mM zinc acetate. cAMP was purified by co-precipitation of other nucleotides with $ZnCO_3$, by addition of 0.5 ml of 144 mM $Na_2CO_3$, and subsequent chromatography by the double-column system, as described by Salomon et al. [39]. Under the assay conditions used, adenylyl cyclase activity was linear with respect to protein concentrations and time of incubation. Protein was determined essentially as described by Lowry et al. [40] with bovine serum albumin as standard.

### Immunoblotting

Immunoblotting was performed as described previously [36,41]. After SDS-PAGE, the separated proteins were electrophoretically transferred to nitrocellulose paper (Schleicher and Schuell) with a semi-dry transblot apparatus (Bio Rad) at 15 V for 45 min. After transfer, the membranes were washed twice in phosphate-buffered saline (PBS) and incubated in PBS containing 8% dehydrated milk at room temperature for 2 h. The blots were then incubated with antisera against G proteins in PBS containing 3% dehydrated milk and 0.1% Tween-20 at room temperature for 2 h. The antibody-antigen complexes were detected by incubating the blots with goat anti-rabbit IgG (Bio-Rad) conjugated with horseradish peroxidase for 2 h at room temperature. The blots were washed three times with PBS before reaction with enhanced-chemiluminescence (ECL) Western-blotting detection reagents from Amersham. Quantitative analysis of the G proteins was performed by densitometric scanning of the autoradiographs employing the enhanced laser densitometer, LKB Ultroscan XL, and quantified using the gel scan XL evaluation software (version 2.1) from Pharmacia (Quebec, Canada).

### Total RNA extraction

Total RNA was extracted from VSMC, as described earlier [41,42].

### Radiolabelling of the probes

cDNA inserts encoding for Giα2, Giα3 and Gsα were radiollabeled with [α-$^{32}$P]dCTP by random priming, essentially as described by Feinberg et al. [43]. Specific activities of the labelled probes ranged from 1 to 3 × $10^8$ cpm/μg of DNA. The 32-mer oligonucleotide recognizing the 28S rRNA was end-labeled with [γ-$^{32}$P]ATP using T4 polynucleotide kinase, as described by Sambrook et al. [41].

### Northern analysis

DMSO/glyoxal-treated total RNA was resolved on 1% agarose gels and transferred to nylon membrane, as described previously [41,42]. Filters, after prehybridization at 65°C for 6 h in hybridization solution (600 mM NaCl, 8 mM EDTA, 120 mM Tris at pH 7.4, 0.1% sodium pyrophosphate, 0.2% SDS, heparin 500 U/ml), were then hybridized overnight in hybridization solution containing dextran sulphate (10% w/v) and the cDNA probe at 1 to 3 × $10^6$ cpm/ml, as described previously [41,42]. Filters were then rinsed at 65°C for 2 × 30 min in 300 mM NaCl, 4 mM EDTA, 60 mM Tris at pH 7.4, and 0.2% SDS, and 1 × 30 min in 150 mM NaCl, 2 mM EDTA, 30 mM Tris at pH 7.4, and 0.1% SDS. Autoradiography was performed with X-ray films at −70°C. In order to assess the possibility of any variations in the amounts of total RNA in individual samples applied to the gel, each filter was hybridized with the $^{32}$P end-labelled oligonucleotide, which recognizes a highly conserved region of 28S ribosomal RNA. The blots that had been probed with the G protein cDNA were de-hybridized by washing for 1 h at 65°C in 50% formamide, 300 mM NaCl, 4 mM EDTA and 60 mM Tris at pH 7.4, and

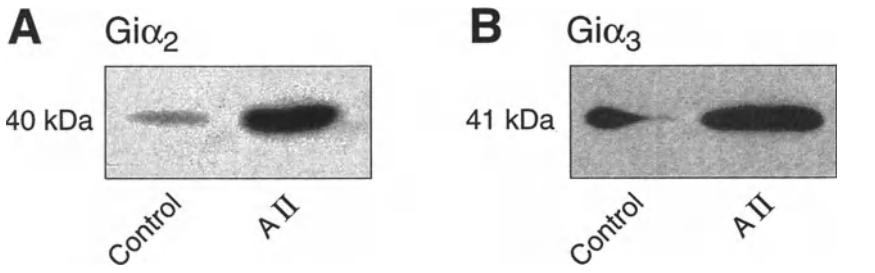


**Figure 1.** Determination of the levels of Giα-2 (**A**) and Giα-3 (**B**) proteins in membranes from control and angiotensin (Ang) II-treated vascular smooth muscle cells by immunoblotting. Vascular smooth muscle cells (VSMCs) were incubated in the absence (lane 1) or presence of $10^{-7}$M Ang II for 24h as described under Methods. Membranes were prepared as described under Methods and were used for immunoblotting. The membrane proteins were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred to nitrocellulose that was then immunoblotted using AS/7 antibody for Giα-1 and Giα-2 or EC/1 antibody for Giα-3 and were detected by using ECL Western blotting, as described under Methods. The autoradiograms are representative of three separate experiments. Reproduced from [38] with permission.

rehybridized overnight at room temperature with the oligonucleotide. Quantitative analysis of the hybridization of bound probes was performed by densitometric scanning of the autoradiographs by employing the enhanced laser densitometer, LKB Ultroscan XL, and quantified using the gel scan XL evaluation software (version 2.1) from Pharmacia (Quebec, Canada).

## MATERIALS

ATP, cAMP, and other chemicals necessary for total RNA extraction, and Northern blot analysis were obtained from Sigma Chemical Co. (St. Louis, MO., USA). Creatine kinase (EC.2.7.3.2), myokinase (EC.2.7.4.3), GTP and GTPγs were purchased from Boehringer-Manheim (Canada). 3-Isobutyl-1-methyl-xanthine (IBMX) was purchased from Aldrich Chemicial Corporation (Milwaukee, Wisconsin). [α-32P]ATP, [α-$^{32}$P]dCTP, and carrier-free [$^{32}$P]orthophosphate were purchased from Amersham Corp. (Oakville, Ontario, Canada). Ang II and C-ANP$_{4-23}$ were from Peninsula Laboratories Inc. (CA., USA).

## RESULTS

### Effect of Ang II treatment on G protein expression

We have recently shown an augmentation of Giα-2 and Giα-3 protein levels in hearts and aorta from SHR and DOCA-salt HR [35,36]. The enhanced expression of Gi protein was restored towards control levels by captopril treatment [37] indicating that Ang II may be responsible for the observed increases in Gi protein expression. In order to investigate this possibility, the effect of Ang II on Gi protein levels was investigated by immunoblotting using specific antibodies against different isoforms of Gi proteins. As shown in figure 1A, AS/7 antibodies, which react with

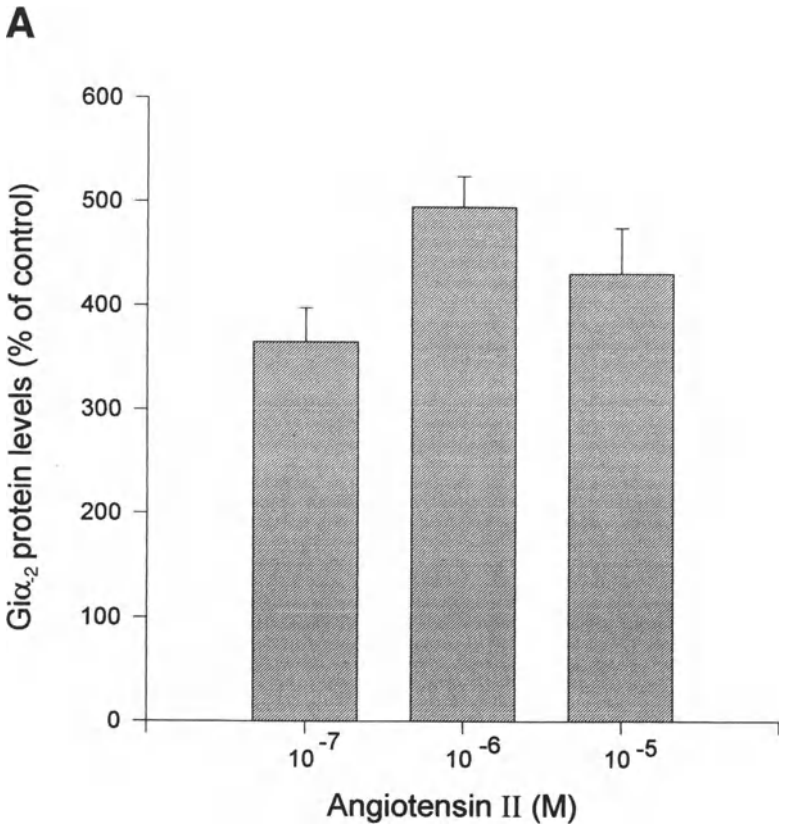


**Figure 2.** Effect of various concentrations of Ang II treatment on the levels of Giα-2 and Giα-3 in membranes from vascular smooth muscle cells (VSMCs). VSMCs were incubated in the absence or presence of various concentratons of Ang II for 24hr as described under Methods. The levels of Giα-2 (**A**) and Giα-3 (**B**) proteins were determined by immunoblotting using antibodies AS/7 and EC/1, respectively, as described under Methods. Quantification of G proteins was performed by densitometric scanning using an enhanced laser densitometer (LKB). The values are mean ± SEM from three separate experiments.

both Giα-1 and Giα-2, recognized a single protein of approximately 40KDa, referred to as Giα-2 (Giα-1 is absent in VSMC) [44], while antibodies EC/2 detected a single protein of 41KDa, referred to as Giα-3, on immunoblots of both VSMC from control and Ang II treated cells (figure 1B). However, the relative amounts of immunodetectable Giα-2 and Giα-3 were significantly increased in a concentration dependent manner in cells pretreated with Ang II, as determined by densitometric scanning (figure 2A and B).

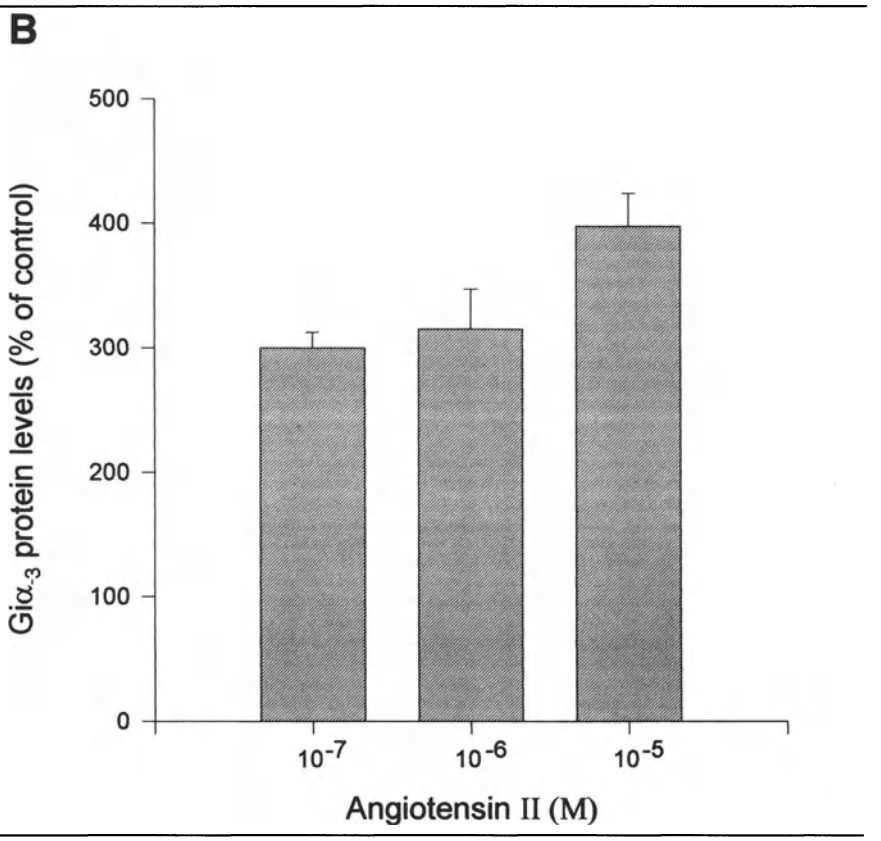


**Figure 2 (continued)**

**Effect of losartan on Ang II induced enhanced expression of G protein**

To examine if the enhanced expression of Gi protein by Ang II is mediated through $AT_1$ receptor, the effect of losartan, an $AT_1$ receptor antagonist was investigated. The results are shown in figure 3. Losartan reduced the Ang II-induced enhanced levels of Giα-2 (figure 3A) and Giα-3 (figure 3B) in a concentration dependent manner. At $10^{-4}$M, losartan decreased the Giα-2 and Giα-3 protein levels by about 30%.

We have also determined the mRNA levels of Giα-2 and Giα-3 in control and Ang II-treated cells by using cDNA probes of Giα-2 and Giα-3. Ang II at $10^{-7}$M was also able to enhance the mRNA levels of Giα-2 and Giα-3 by about 25–30%, and losartan was also able to reduce the enhanced mRNA levels of Giα-2 and Giα-3 in a concentration dependent manner. At $10^{-4}$M, losartan decreased the expression of enhanced Giα-2 and Giα-3 mRNA by about 25–30% (figure 4A, B).

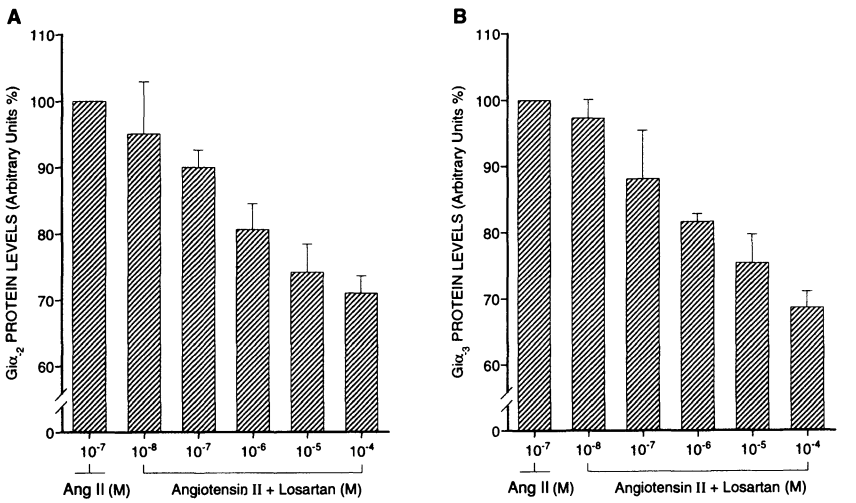


**Figure 3.** Effect of losartan on Ang II-induced enhanced expression of Giα-2 and Giα3 proteins in vascular smooth muscle cells (VSMCs). VSMCs were incubated in the absence or presence of $10^{-7}$M Ang II alone or in combination with various concentrations of losartan for 24hr as described under Methods. The levels of Giα-2 (**A**) and Giα-3 (**B**) proteins were determined by immunoblotting using AS/7 and EC/1 antibodies as described under Methods. Quantification of G proteins was performed by densitometric scanning using an enhanced laser densitometer (LKB). Values are means ± SEM of three separate experiments.

On the other hand, Ang II treatment of the cells did not alter the levels of Gs protein or Gsα mRNA (data not shown).

### Effect of Ang II treatment on Gi functions

In order to investigate if the augmentation of Giα proteins by Ang II treatment is also reflected in Gi functions, the effect of Ang II and C-ANP$_{4-23}$, which inhibit adenylyl cyclase activity through Gi regulatory protein [18,20,45,46], was examined in control and Ang II-treated cells. The results are shown in figure 5. Ang II and C-ANP$_{4-23}$ inhibited adenylyl cyclase activity by about 30 and 35% in control cells, respectively, which was completely attenuated by Ang II treatment. Losartan at $10^{-6}$M was able to restore the inhibition by about 40–45% when the inhibition of adenylyl cyclase by C-ANP$_{4-23}$ or Ang II was taken as 100%. In addition the receptor-independent Gi functions, as determined by examining the effect of low concentration of GTPγS on forskolin-stimulated adenylyl cyclase, were also attenuated by Ang II treatment (data not shown).

## DISCUSSION

The present studies demonstrate that Ang II treatment of the vascular smooth cells for 24h enhanced the expression of Giα-2 and Giα-3 proteins, whereas the

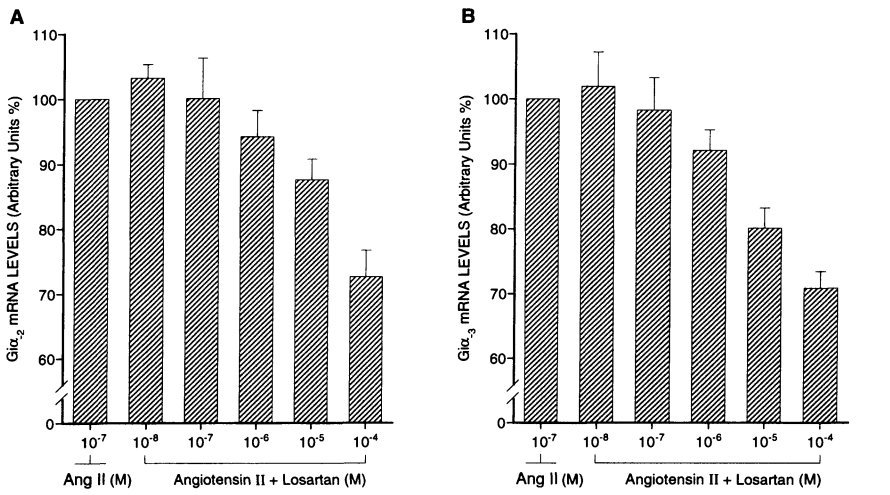


**Figure 4.** Effect of losartan on Ang II-induced enhanced expression of Giα-2 and Giα-3 mRNA in vascular smooth muscle cells (VSMCs). VSMCs were incubated in the absence or presence of $10^{-7}$M Ang II alone or in combination with various concentrations of losartan for 24 hours as described under Methods. The mRNA levels of Giα-2 (**A**) and Giα-3 (**B**) were determined by Northern blotting using cDNA probes of Giα-2 and Giα-3 as described under Methods. Quantification of Gi mRNA was performed by densitometric scanning using an enhanced laser densitometer (LKB). Values are means ± SEM of three separate experiments.

expression of Gsα was unchanged. These results are in agreement with the data reported earlier [47], where systemic infusion of Ang II resulted in the augmented levels of Giα-2 and Giα-3 proteins in glomerular and mesenteric vascular smooth muscle membranes. The levels of Giα-2 and Giα-3 mRNA were also increased by Ang II treatment. This may not be due to variation in the amounts of total RNA loaded in individual samples applied to the gels because hybridization with an oligonucleotide that recognizes a highly conserved region of the 28S RNA showed a similar amount of 28S RNA loaded from control and Ang II-treated cells (data not shown). These results suggest that the genes for Giα-2 and Giα-3 enhanced by Ang II treatment may be responsible for the observed increase in protein levels. The reduction in the Ang II-induced levels of Giα protein by losartan suggest the involvement of $AT_1$ receptor in Gi protein synthesis. The $AT_1$ receptor-mediated increase of protein synthesis has been shown previously [45,48]. The mechanism(s) responsible for Ang II-mediated Gi protein synthesis is not known and remains to be explored. However, one possible mechanism may be PKC, because staurosporin, a PKC inhibitor, was able to inhibit Ang II-induced enhanced expression of Giα protein in VSMC (Anand-Srivastava et al., unpublished observations). Our results on G protein expression are in agreement with those of Sims et al. [47], who have shown that Ang II infusion in rats resulted in enhanced protein levels of Giα-2 and

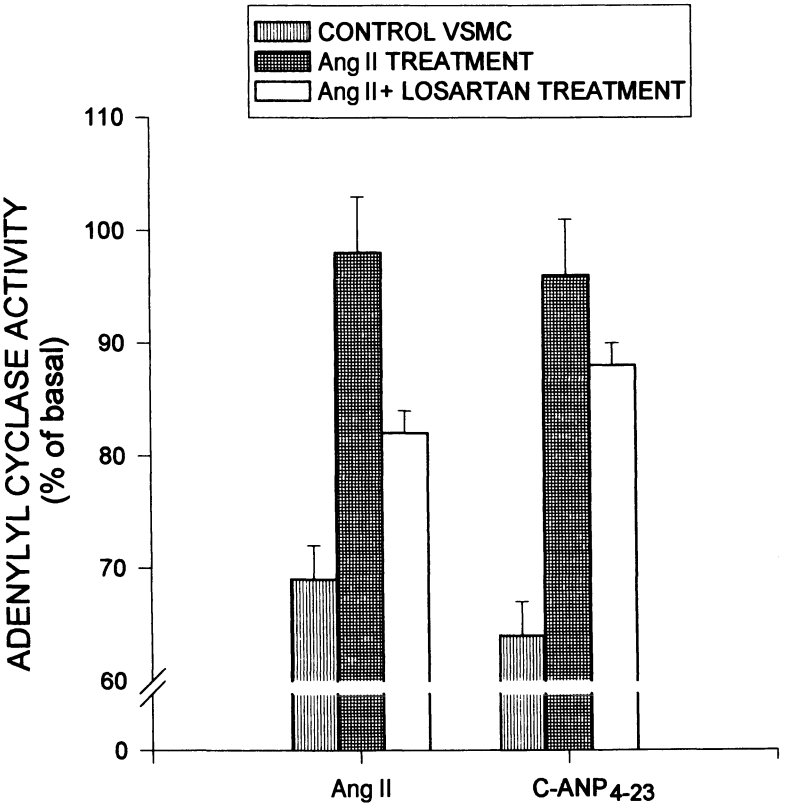


**Figure 5.** Effect of losartan on Ang II-induced attenuation of C-ANP$_{4-23}$ and Ang II-mediated inhibition of adenylyl cyclase in vascular smooth muscle cells (VSMCs). VSMCs were incubated in the absence (control, ▥) or presence of $10^{-7}$M Ang II alone (▩) or in combination with $10^{-6}$M losartan (□) for 24hr as described under Methods. Adenylyl cyclase activity was determined in the absence or presence of $10^{-5}$M Ang II or $10^{-7}$M C-ANP$_{4-23}$ in the membranes prepared from these cells as described under Methods. Basal adenylyl cyclase activity was taken as 100%. Values are means ± SEM. of three separate experiments.

Giα-3 in systemic and renal vasculature. The enhanced expression of Giα proteins in cultured adrenal cells in response to Ang II has also been demonstrated [49].

A relationship between the levels of G protein and functions has been reported by several investigators [35,50]. Increased level of Giα proteins and increased responsiveness of adenylyl cyclase to ANP, oxotremorine, and Ang II inhibition in aorta and heart from SHR and DOCA-salt hypertensive rats has recently been shown [35,36]. Similarly decreased levels of Giα-2 and complete attenuation of ANP receptor-mediated inhibition of adenylyl cyclase in platelets from SHR have also been reported [50]. However, the increased levels of Giα-2 and Giα-3 from Ang II treatment were not reflected in increases in Gi functions. On the other hand, the C-

$ANP_{4-23}$ and Ang II receptor-mediated inhibitions of adenylyl cyclase were completely attenuated by Ang II treatment. Several mechanisms may be involved in the observed attenuation of adenylyl cyclase inhibition by C-$ANP_{4-23}$ and Ang II. One of the possibilities may be that Ang II treatment has resulted in the down regulation of ANP as well as Ang II receptors in the vascular smooth muscle cells [51,52]. Alternatively the Gi protein levels, although enhanced by Ang II treatment, may have been phosphorylated by Ang II mediated activation of PKC, another signaling pathway of Ang II action. The phosphorylation of Gi protein may, therefore, result in the uncoupling of receptors from adenylyl cyclase. The phosphorylation of Giα proteins and the uncoupling of inhibitory hormone receptors from adenylyl cyclase by PKC has been reported [23]. Taken together, it may be possible that the observed attenuation of ANP-C and Ang II receptor-mediated inhibition of adenylyl cyclase caused by Ang II treatment may be attributed to be downregulation of the receptors or/and to the phosphorylation of Giα protein, which may be responsible for the uncoupling of these receptors from adenylyl cyclase.

The partial reversal of the ANP-C and Ang II receptor-mediated adenylyl cyclase inhibition by losartan may be due to its competitive binding, thereby preventing Ang II-mediated desensitization. However, the inability of losartan to completely restore the attenuated responsiveness of adenylyl cyclase to C-$ANP_{4-23}$ may be due to the low concentrations of losartan used in these studies, which may not have been enough to saturate all the $AT_1$ receptor subtype, Alternatively it may be because some effects of Ang II are being produced via a non-losartan sensitive $AT_2$ receptor subtype. Or perhaps it is by some other mechanism.

In conclusion we have shown that treatment of vascular smooth muscle cells with Ang II enhanced the levels of Giα-2 and Giα-3 proteins and mRNA but not levels of Gsα. The enhanced levels of Giα proteins were not associated with increased Gi functions. The Ang II-induced enhanced levels of Gi and attenuated inhibition of adenylyl cyclase by C-$ANP_{4-23}$ and Ang II were restored partially towards control levels by losartan, suggesting the implication of $AT_1$ receptor subtype in the observed effects of Ang II.

## NOTES

1. This work was supported by grants from the Medical Research Council of Canada and Quebec Heart Foundation.
2. Dr. Anand-Srivastava was a recipient of the Medical Research Council Scientist Award from the Medical Research Council of Canada during the course of these studies.

**Abbreviations:** C-$ANP_{4-23}$, a ring deleted analog of atrial natriuretic factor C-$ANP_{4-23}$ [des($Glu^{18}$, $Ser^{19}$, $Glu^{20}$, $Leu^{21}$, $Gly^{22}$)$ANP_{4-23}$-$NH_2$]. Gi, inhibitory guanine nucleotide regulatory protein, Gs, stimulatory guanine nucleotide regulatory protein, AngII, angiotensin II. GTPγS, guanosine 5′-0-(3-thiotriphosphate) FSK, forskolin, DOCA, deoxycorticosterone acetate.

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# COMPARISON OF THE INHIBITORY ACTIONS OF ANGIOTENSIN $AT_1$ RECEPTOR ANTAGONISTS IN THE PERIPHERAL VASCULAR BED

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***Summary.*** The effects of nonpeptide angiotensin $AT_1$ and $AT_2$ receptor antagonists on pressor responses to the angiotensin peptides were investigated in the cat and the rat. Under constant flow conditions, injections of angiotensin (Ang) I, Ang II, Ang III, Ang I-(3–10), ($Pro^{11}$,D-$Ala^{12}$) Ang I and Ang IV into the hindlimb perfusion circuit caused dose-dependent increases in perfusion pressure, while Ang I-(4–8) was without effect. The order of potency was Ang I = Ang II = Ang III > ($Pro^{11}$,D-$Ala^{12}$) Ang I > Ang I-(3–10) = Ang IV. Losartan, EXP 3174, and candesartan decreased vasoconstrictor responses to Ang II in a selective manner. EXP 3174 (1 mg/kg iv) and candesartan (1 mg/kg iv) shifted the dose-response curve to Ang II to the right in a nonparallel manner, whereas losartan shifted the curve to the right in a parallel manner. The $AT_2$ receptor antagonist PD 123,319 had no significant effect on vasoconstrictor responses to the angiotensin peptides in the regional vascular bed of the cat. In the rat, candesartan decreased pressor responses to Ang II, whereas PD 123,319 had no effect on the response to the peptide. These results indicate that vasoconstrictor responses to Ang peptides in the regional vascular bed of the cat and pressor responses to Ang II in the systemic vascular bed of the rat are mediated by the activation of $AT_1$ receptors, whereas $AT_2$ receptors play little, if any, role in the mediation or modulation of responses to Ang II in the cat or the rat.

## INTRODUCTION

Angiotensin (Ang) II has potent vasoconstrictor activity in a number of regional vascular beds in a variety of species [1–3]. Ang II is formed from Ang I by the angiotensin-converting enzyme (ACE) located on the surface of pulmonary capillary endothelial cells [1–6]. Recent studies, however, have suggested that in addition to

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the pulmonary capillary, bed, there is significant ACE activity in the upstream resistance vessel elements within the pulmonary, hindlimb, and mesenteric vascular beds of the cat [7–9]. Binding studies have revealed the existence of at least two different Ang II receptor subtypes, $AT_1$ and $AT_2$ [10,11]. $AT_1$ receptors have been found in many tissues, including vascular smooth muscle, liver, and kidney [12–17]. $AT_2$ receptors have been found in bovine cerebellum and uterus and in rat adrenal medulla [10,11,13,18]. The function of the $AT_2$ receptor in vivo is uncertain, although recent evidence suggests that this receptor subtype may play a role in fetal growth and development [19] and has been reported to mediate a vasodepressor response to Ang II and Ang III in the rat [20]. The $AT_1$ receptor, however, is believed to be responsible for most, if not all, cardiovascular responses to Ang II [7–9,21–24].

ACE inhibitors and Ang receptor antagonists have been used to counteract the hypertensive, cardiac, and vascular hypertrophic effects of Ang II [25]. The development of Ang receptor blocking agents was aided by the discovery of imadazole analogs, which were nonpeptide Ang II receptor antagonists [10,26]. DuP 753 (losartan), a nonpeptide Ang II receptor antagonist, was developed from these studies and is used in the treatment of hypertension [10,26]. Candesartan is a recently synthesized nonpeptide Ang II receptor antagonist displaying high affinity for the $AT_1$ subtype [27–31]. Candesartan is the metabolite of the orally active compound TCV-116 and is 30–100 fold more potent than the parent compound [29,32,33]. Candesartan inhibits Ang II-induced aldosterone secretion in rats [33] and decreases arterial pressure in renal hypertensive rats [31]. Candesartan has been reported to shift the dose-response curve for Ang II to the right in a nonparallel fashion and reduce maximal contractile responses to Ang II in rabbit aortic strips [28]. The present study was undertaken to investigate and compare the effects of $AT_1$ and $AT_2$ receptor blocking agents on responses to Ang peptides in the cat and the rat.

## MATERIALS AND METHODS

### Regional vascular bed experiments

For experiments in the regional vascular bed of the cat, adult cats of either sex weighing 2.0 to 5.4 kg were sedated with ketamine hydrochloride (10–15 mg/kg im) and were anesthetized with pentobarbital sodium (30 mg/kg iv). Supplemental doses of pentobarbital were given during the course of the experiment to ensure a uniform level of anesthesia. The trachea was cannulated, and the animals were ventilated with a Harvard model 607 ventilator at a volume of 40–60 ml at 15–22 breaths/m. The animals were maintained at 37°C with a heating blanket. An external jugular vein was catheterized for the intravenous (iv) administration of drugs, and a carotid artery was catheterized for the measurement of systemic arterial (aortic) pressure. For constant-flow perfusion of the hindquarters vascular bed, a 3- to 4-cm segment of distal aorta was exposed through a ventral midline incision and was cleared of surrounding connective tissue by blunt dissection. After administration of heparin sodium (1000 U/kg iv), the abdominal aorta was ligated, and

catheters were inserted into the aorta proximal and distal to the ligature. Branches of the aorta distal to the origin of the external iliac arteries were ligated to restrict blood to the hindlimbs. Blood was withdrawn from the proximal catheter and pumped at a constant flow rate with a Sigmamotor model T-8 pump into the distal aortic catheter. Perfusion pressure was monitored from a lateral tap in the perfusion circuit located between the pump and the distal aortic catheter. Hindlimb perfusion pressure and systemic (aortic) pressures were measured with Statham P23 transducers and were recorded on a Grass model 7 polygraph. Mean pressures were derived by electronic averaging, and the flow rate was set so that hindquarters perfusion pressure approximated systemic arterial pressure and was not changed during an experiment. The flow rate was determined by timed collection and ranged from 24 to 30 ml/m. Agonists were injected directly into the hindlimb perfusion circuit distal to the pump in small volumes (30 and 100 μl) in a random sequence, and the hindlimb vascular bed was denervated by ligating and cutting the lumbar sympathetic chain ganglia between L3 and L4. These procedures have been described previously [9,23,24].

**Mesenteric vascular bed experiments**

For experiments in the mesenteric vascular bed, the cats were anesthetized and instrumented in a manner similar to that described for the hindlimb experiments. For constant-flow perfusion of the mesenteric vascular bed, the superior mesenteric artery was approached through a midline abdominal incision and carefully cleared of surrounding connective tissue. The mesenteric vascular bed was denervated by ligating and cutting the perivascular nerves to the small intestine as they course along the superior mesenteric artery. Following the administration of heparin sodium (1000 U/kg), the femoral artery was cannulated and connected to the inlet side of the perfusion circuit. The outlet side of the perfusion circuit was connected to a catheter that was inserted into the superior mesenteric artery. Blood flow to the small intestine was maintained constant with a Sigmanotor model T-8 perfusion pump. Superior mesenteric arterial perfusion pressure was measured by a lateral tap in the perfusion circuit that was located between the pump and the outlet side of the perfusion circuit. Superior mesenteric arterial perfusion pressure and systemic arterial pressure were measured with Statham P23 pressure transducers and were recorded on a Grass model 7 polygraph. Mean pressures were derived by electronic averaging, and the perfusion rate was set so that superior mesenteric arterial perfusion pressure approximated systemic arterial pressure and was not changed during the experiment. The flow rate was determined by timed collection and ranged from 26–36 ml/min. The agonists used in these experiments were injected, in small volumes (30 and 100 μl), directly into the superior mesenteric artery perfusion circuit distal to the pump. These procedures have been described previously [8].

**Systemic arterial pressure experiments**

For experiments on systemic arterial pressure in the rat. Sprague-Dawley rats of either sex weighing 340–540 g were anesthetized with pentobarbital sodium

(50 mg/kg ip). Supplemental doses of pentobarbital were given as needed to maintain a uniform level of anesthesia. The trachea was cannulated, and the rats breathed room air spontaneously or were ventilated with room air enriched with 95% $O_2$ and 5% $CO_2$ with a Harvard model 683 rodent ventilator at a tidal volume of 2.4–2.6 ml at a rate of 30–35 breaths/m. Catheters were inserted into the external jugular vein for the iv administration of drugs and into the carotid artery for the measurement of systemic arterial (aortic) pressure. Systemic arterial pressure was measured with a Viggo-Spectramed pressure transducer and was recorded on a Grass model 7 polygraph. Mean pressure was derived by electronic averaging.

### Materials

DuP 753 (losartan potassium: Dupont-Merck, Wilmington, DE) was dissolved in 0.9% NaCl, and EXP 3174 (Dupont-Merck) was dissolved in a 5% $NaHCO_3$/dextrose (50:50) solution. Candesartan (CV-11974) was dissolved in a 1N $Na_2CO_3$/0.9% NaCl solution (1:20). PD 123,319 (Research Biochemicals Inc., Natick, MA), acetylcholine chloride, Ang I, Ang II, Ang III, Ang IV, norepinephrine hydrochloride (Sigma Chemical Co., St. Louis, MO), ($Pro^{11}$,D-$Ala^{12}$) Ang I (generously provided by Dr. Leland Loose of Pfizer, Inc., Groton, CT), and endothelin-1 (Peptide Research Labs, Tulane University, New Orleans. LA) were dissolved in 0.9% NaCl. Captopril (Bristol Myers-Squibb, Princeton, NJ) was dissolved in 0.9% NaCl. U46619 (Upjohn, Kalamazoo, MI) was dissolved in 100% ethanol at a concentration of 10 mg/ml and was diluted in 0.9% NaCl. BAY K 8644 (Miles, New Haven, CT) was dissolved in a 1:4 solution of cremophor EL and tris(hydroxymethyl) aminomethane (Tris) and Tris·HCl (50 mM, pH 7.4). The resulting suspension was warmed, and polyethylene glycol and Tris (pH 7.4) were added to make a stock solution that was stored in a brown bottle in a freezer. Working solutions of all agonists were prepared on a frequent basis, stored in brown stoppered bottles, and kept on crushed ice during the course of an experiment.

### Statistical analysis

Responses were measured in absolute units (mmHg) as mean ± SE and were analyzed using a one-way analysis of variance and Scheffe's F test with a Bonferroni correction or a paired t-test [34]. A P value of less than 0.05 was used as the criterion for statistical significance.

## RESULTS

### Responses to Angiotensin peptides in the hindlimb vascular bed of the cat

Under constant flow conditions, injections of Ang I, Ang II, Ang III, Ang IV, Ang I-(3–10), and ($Pro^{11}$,D-$Ala^{12}$) Ang I into the hindlimb perfusion circuit caused dose-related increases in hindlimb perfusion pressure (figure 1). Injection of Ang I-(4–8) had no significant effect on hindlimb perfusion pressure (figure 1). When doses of the peptides are expressed on a nmol basis to take molecular weight into account, increases in hindlimb perfusion pressure in response to Ang I, Ang II, and

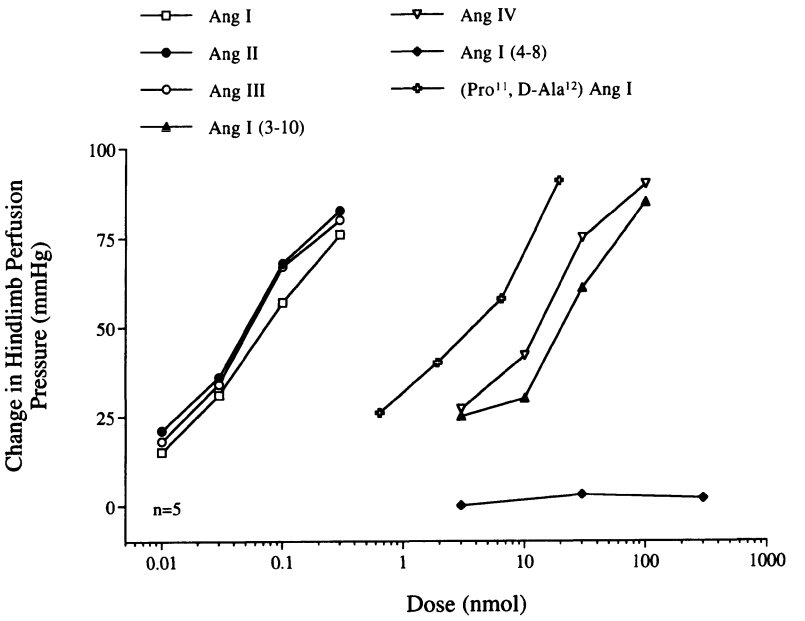



**Figure 1.** Dose-response curves comparing the increases in perfusion pressure in response to intraarterial injections of angiotensin I, II, III, I (3–10), I (4–8), IV, and ($Pro^{11}$, $D\text{-}Ala^{12}$) Ang I in the hindlimb vascular bed of the cat. Doses are expressed on a nmol basis to take molecular weight into account. n indicates number of animals.

Ang III were similar (figure 1). The dose-response curve for ($Pro^{11}$,$D\text{-}Ala^{12}$) Ang I was approximately 2 log units to the right of the dose-response curves for Ang I, Ang II, and Ang III. The dose-response curves for Ang I-(3–10) and Ang IV were similar and were three log units to the right of the dose-response curves for Ang I, Ang II, and Ang III (figure 1).

### Influence of losartan (DuP 753) and EXP 3174

The effects of the angiotensin $AT_1$ receptor antagonist, losartan (DuP 753), and its active metabolite, EXP 3174, on responses to Ang II were compared in the hindlimb vascular bed of the cat, and the results are shown in figure 2. Following administration of DuP 753 in a dose of 2.5 mg/kg iv and EXP 3174 in a dose of 1 mg/kg iv, responses to injection of Ang II into the hindlimb perfusion circuit were reduced significantly (figure 2). When the slopes of the dose-response curves for Ang II were compared before and after administration of DuP 753, (2.5 mg/kg iv) the curve was shifted to the right in a parallel manner which suggests that the blockade of responses to Ang II was competitive in nature (figure 2). When the slopes of the dose-response curves for Ang II were compared before and after

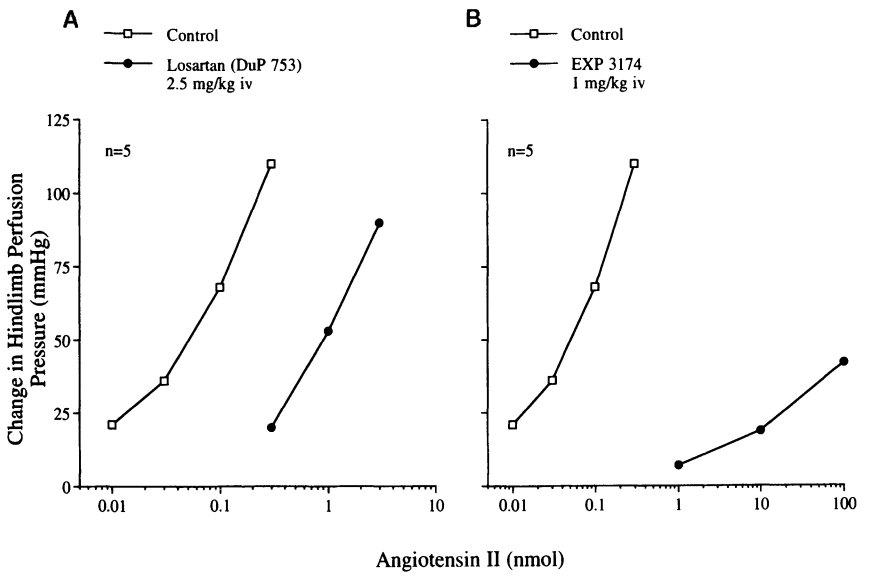



**Figure 2.** Influence of losartan (DuP 753; 2.5 mg/kg iv; *A*) and EXP 3174 (1 mg/kg iv, *B*) on responses to angiotensin II in the hindlimb vascular bed of the cat. The peptides were injected directly into the hindlimb perfusion circuit, and responses were determined before and 20 minutes after administration of the receptor antagonist. n indicates number of animals.

administration of EXP 3174, the curve for Ang II was shifted to the right in a nonparallel manner which suggests a non-competitive blockade. Responses to norepinephrine were not altered after administration of DuP 753 or EXP 3174 (data not shown).

### Influence of candesartan on responses to Angiotensin II

The effects of the newly developed nonpeptide $AT_1$ receptor antagonist candesartan (CV11974) on responses to Ang II were investigated in the hindlimb vascular bed of the cat. These results are shown in figure 3. Following administration of candesartan in doses of 3 µg/kg iv and 1 mg/kg iv, responses to injections of Ang II were reduced significantly (figure 3). When the slopes of the dose-response curves for Ang II were compared before and after administration of the low dose of candesartan, the curve was shifted to the right in a parallel manner suggesting that the blockade was competitive in nature (figure 3). When dose-response curves for Ang II were compared before and after administration of candesartan in a dose of 1 mg/kg iv, the curve for Ang II was shifted to the right in a nonparallel manner suggesting a noncompetitive blockade (figure 3). Responses to norepinephrine were not altered after administration of candesartan in doses of 3 µg/kg iv and 1 mg/kg iv (data not shown).

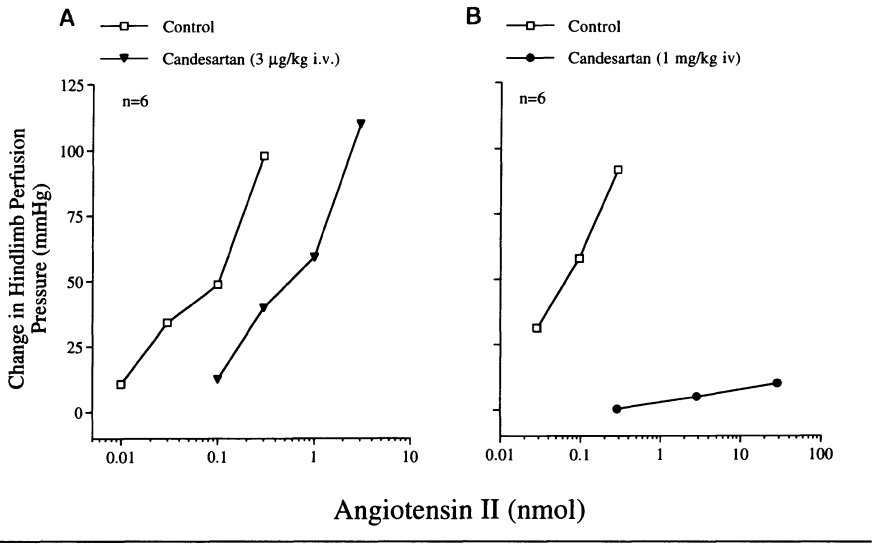



**Figure 3.** Influence of candesartan in doses of 3 μg/kg iv (*A*) and 1 mg/kg iv (*B*) on responses to angiotensin II in the hindquarters vascular bed. Responses to the peptide were determined before and 20 minutes after administration of the receptor antagonist. n indicates number of animals.

### Influence of captopril on responses to the Angiotensin peptides

The effects of the ACE inhibitor captopril on responses to Ang I and Ang II were studied in the hindlimb vascular bed of the cat. These results are shown in figure 4. Following administration of captopril in a dose of 4 mg/kg iv, responses to Ang I were reduced significantly at a time when responses to Ang II were not altered (figure 4). In a manner similar to that observed with Ang I, responses to Ang I-(3–10), the precursor for Ang IV, were reduced significantly after administration of captopril (data not shown). Responses to Ang IV and ($Pro^{11}$,D-$Ala^{12}$) Ang I were not altered after administration of captopril (data not shown).

### Duration of $AT_1$ receptor blockade

The duration of the inhibitory effects of DuP 753, EXP 3174, and candesartan on pressor responses to Ang II was assessed in the hindlimb vascular bed of the cat. The results of these experiments are shown in figure 5. Four hours after administration of DuP 753 in a dose of 2.5 mg/kg iv, EXP 3174 in a dose of 1 mg/kg iv, and candesartan in a dose of 1 mg/kg iv, responses to Ang II were reduced significantly at a time when responses to norepinephrine were not altered (figure 5).

### Influence of $AT_2$ receptor blockade

The effects of the $AT_2$ receptor antagonist PD 123,319 on responses to the angiotensin peptides were investigated in the hindlimb and mesenteric vascular beds

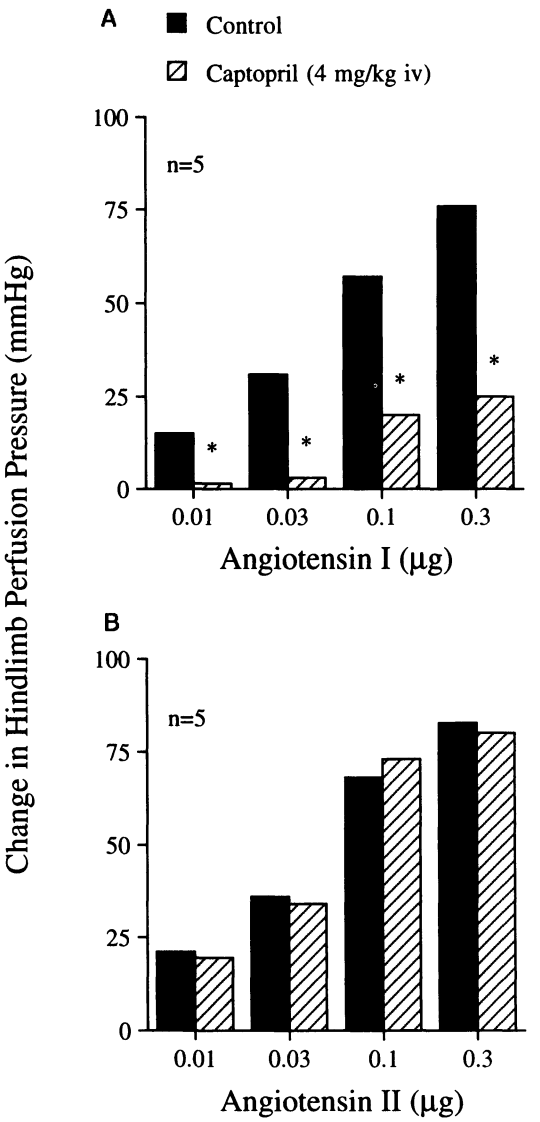



**Figure 4.** Influence of captopril (4 mg/kg iv) on responses to angiotensin I (*A*) and angiotensin II (*B*) in the hindlimb vascular bed of the cat. n indicates number of animals. * significantly different from control ($p < 0.05$).

of the cat. The results of these experiments are shown in figure 6. Following administration of PD 123,319 in a dose of 5 mg/kg iv, responses to Ang II, Ang III, or Ang IV were not changed significantly in the hindlimb vascular bed of the cat (figure 6). Following administration of PD 123,319 in doses of 10 and 20 mg/kg iv,

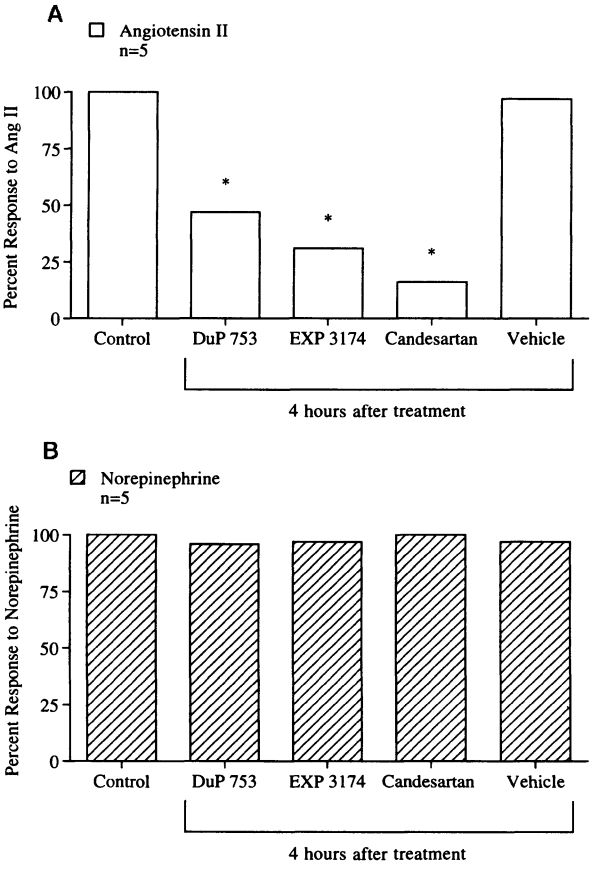



**Figure 5.** Influence of time on the inhibitory effects of DuP 753, EXP 3174, and candesartan on responses to angiotensin II (0.3 μg) and norepinephrine (1 μg) in the hindlimb vascular bed of the cat. n indicates number of animals. * response is significantly different from control ($p < 0.05$).

responses to Ang II, Ang III, and Ang IV were not altered in the mesenteric vascular bed of the cat (figure 6).

**Effects of candesartan and PD 123319 in the systemic vascular bed of the rat**

The effects of candesartan on changes in systemic arterial pressure in response to Ang II were investigated in the rat. These data are summarized in figure 7. Injections of Ang II in doses of 0.1–3 μg/kg iv caused dose-related increases in systemic arterial pressure. The increases in systemic arterial pressure in response to Ang II were reduced significantly following administration of candesartan in a dose of 1 mg/kg iv (figure 7). There was little tendency for the blockade to be sur-

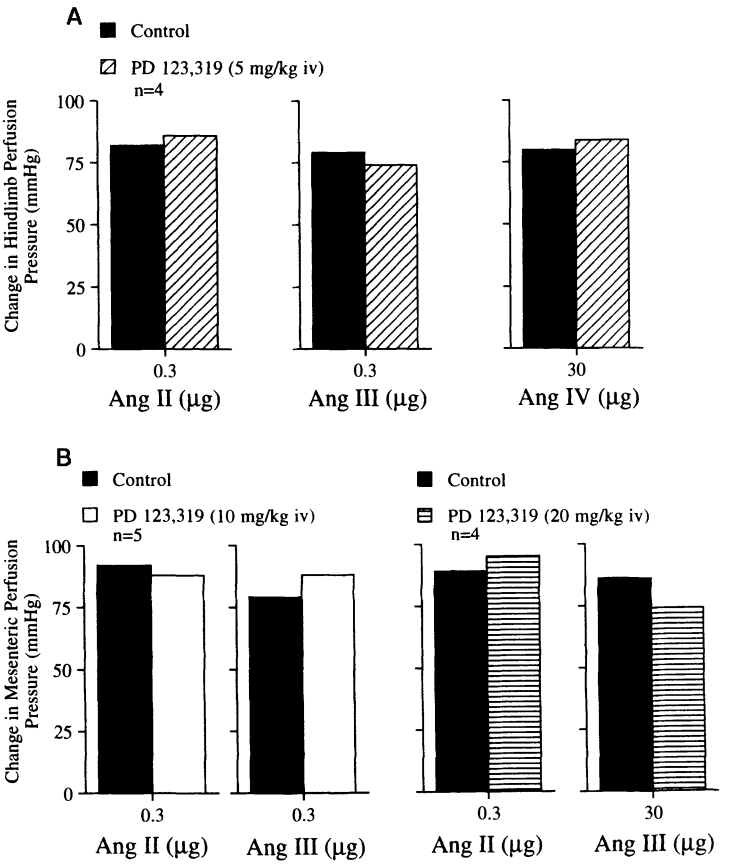



**Figure 6.** *A*, Influence of PD 123,319 on responses to angiotensin II, III, and IV in the hindlimb vascular bed of the cat. *B*, Influence of PD 123,319 in doses of 10 and 20 mg/kg iv on responses to angiotensin II and III in the mesenteric vascular bed of the cat. n indicates number of animals. * response is significantly different from control ($p < 0.05$).

mounted when larger doses of Ang II were injected after administration of the $AT_1$ receptor antagonist (data not shown). The $AT_1$ receptor blockade induced by candesartan was long in duration, and pressor responses to Ang II were inhibited at intervals up to 3 hours after administration of the $AT_1$ receptor antagonist (data not shown). Although pressor responses to Ang II were reduced for periods up to 3 hours after administration of candesartan, increases in systemic arterial pressure in response to iv injections of norepinephrine were not altered during this same time period (data not shown).

The effects of the $AT_2$ receptor antagonist PD 123319 on responses to Ang II were investigated in the rat. These data are summarized in figure 7. Increases in systemic arterial pressure in response to Ang II were not changed by the adminis-

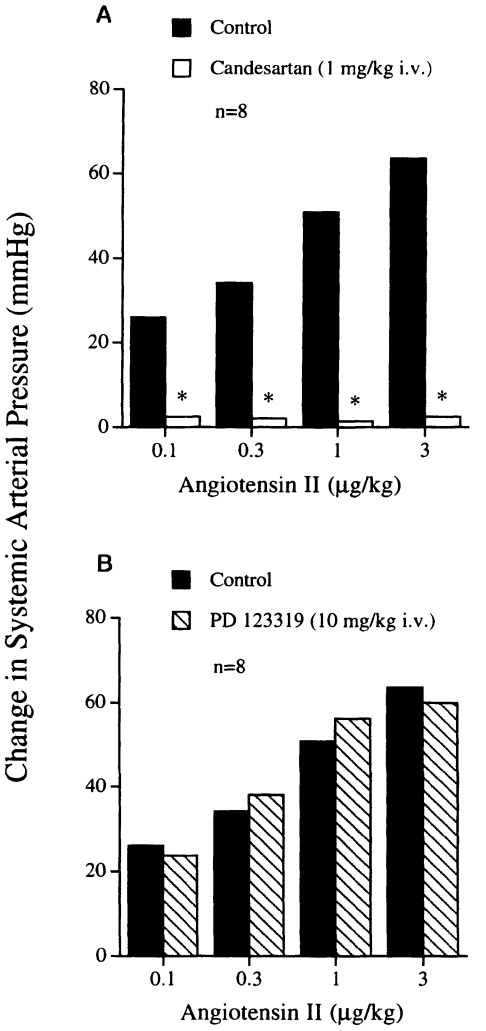
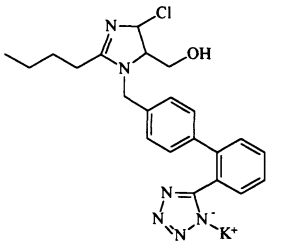
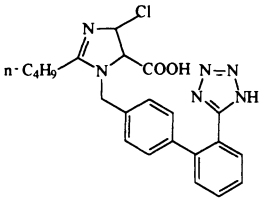



**Figure 7.** Influence of candesartan (*A*) and PD 123,319 (*B*) on responses to angiotensin II in the systemic vascular bed of the rat. n indicates number of animals. * response is significantly different from control ($p < 0.05$).

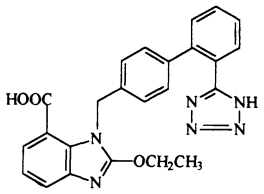
tration of PD 123319 in a dose of 10 mg/kg iv (figure 7). The subsequent administration of candesartan (1 mg/kg iv) significantly attenuated pressor responses to Ang II (data not shown). Increases in systemic arterial pressure in response to norepinephrine were not altered by candesartan or PD 123,319 (data not shown). The chemical structures for losartan, EXP 3174, candesartan, and PD 123,319 are shown




Losartan (DUP 753)




EXP 11374




Candesartan (CV-11974)

**Figure 8.** Chemical structures of the angiotensin $AT_1$ receptor antagonists losartan (DuP 753), EXP 3174, and candesartan (CV-11974).

in figure 8. The postulated sites of action of the angiotensin peptides and AT receptor antagonists are shown in figure 9.

## DISCUSSION

Results of the present investigation demonstrate that the nonpeptide angiotensin $AT_1$ receptor antagonists, DuP 753, EXP 3174, and candesartan have significant inhibitory effects on increases in hindquarters perfusion pressure in response to Ang II. Inasmuch as blood flow was maintained constant, the increases in perfusion pressure reflect increases in regional vascular resistance and show that vasoconstrictor responses to Ang II are antagonized by DuP 753, EXP 3174, which is an active metabolite of DuP 753, and candesartan. Vasoconstrictor responses to Ang II did not change over the period of time the experiments were carried out and were not altered by sodium meclofenamate or phentolamine, indicating that responses to the peptide were reproducible and were not modulated by the release of products in the

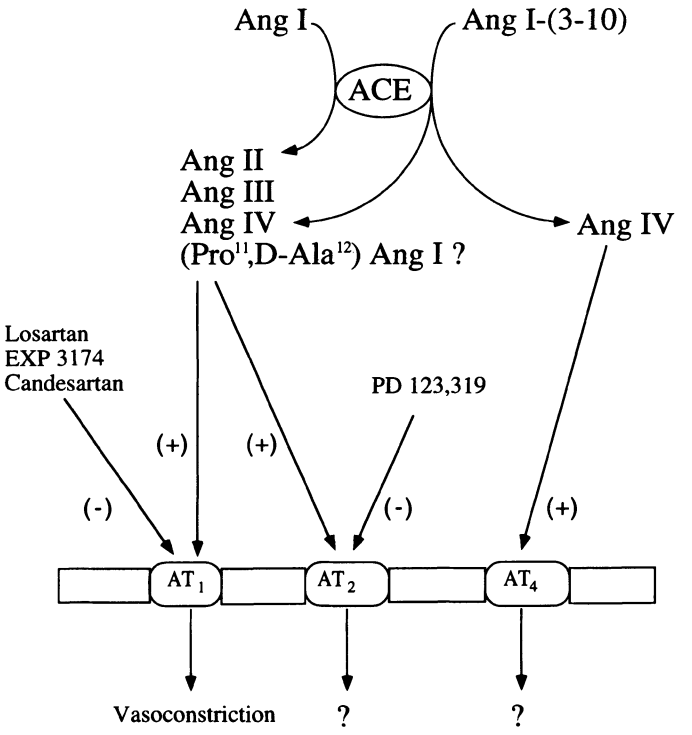



**Figure 9.** *A*, Diagram depicting the proposed sites of action of the angiotensin peptides in the cat and rat. Data from the present study suggest that Ang II, Ang III, and Ang IV cause vasoconstriction in the cat and rat by acting on the $AT_1$ receptor and that losartan (DuP 753), EXP 3174, and candesartan (CV-22974) are selective antagonists for the angiotensin $AT_1$ receptor. Data from the present study suggest that ($Pro^{11}$, D-$Ala^{12}$) Ang I causes vasoconstriction that is resistant to ACE inhibition, but blocked by $AT_1$ receptor antagonists. The role of the $AT_2$ and $AT_4$ receptors in mediating responses to the angiotensin peptides is uncertain. Bottom: Amino acid sequences for angiotensin (Ang) I, II, III, IV, and I-(4–8).

cyclooxygenase pathway or mediated in part by the release of norepinephrine from adrenergic terminals [7,9].

The inhibitory effect of DuP 753 on responses to Ang II was overcome when larger doses of the peptide were injected, and the shift to the right of the dose-response curve was parallel, suggesting that the DuP 753-induced blockade was competitive in nature. In addition to being a potent and competitive antagonist for Ang II, DuP 753 did not significantly effect vasoconstrictor responses to vasopressin, norepinephrine, neuropeptide Y, and U46619; biphasic responses to endothelin-1; or vasodilator responses to acetylcholine. These data indicate that the inhibitory effects of DuP 753 on responses to Ang II were highly selective, since responses to vasoactive agents, which act by a variety of receptor-mediated mechanisms, were

not altered. Moreover, DuP 753 had a long duration of action in that responses to Ang II in the hindquarters returned to only about 50% of control 4 hours after administration of the antagonist in a dose of 2.5 mg/kg iv. Although responses to Ang II returned gradually toward control value during the 4 hours of the experiment, responses to norepinephrine remained unchanged, indicating that the responsiveness of the vascular bed was not changed during the time that experiments were carried out. DuP 753 and other $AT_1$ receptor antagonists used in these studies had no significant effect on baseline pressures in the aorta and in the hindquarters vascular bed, suggesting that the nonpeptide $AT_1$ receptor antagonists had little, if any, agonistic activity in the cat and that Ang II did not play a major role in regulating vascular tone under baseline conditions in the cat. The results of the present studies in the hindquarters vascular bed of the cat are in agreement with studies in the pithed rat, a preparation in which the influence of autonomic reflexes has been removed and increases in diastolic pressure in response to Ang II are measured. In the pithed rat, DuP 753 shifted the Ang II dose-response curve to the right in a parallel manner without altering responses to norepinephrine or vasopressin. In a similar manner, DuP 753 shifted the Ang II dose-response curve in a parallel manner without altering contractile responses to norepinephrine or potassium chloride in the isolated rabbit aortic strip. The results of studies in the pithed rat, the rabbit aortic strip, and the cat hindquarters indicate that DuP 753 is a potent selective, competitive angiotensin $AT_1$ receptor antagonist which possesses little agonistic activity [23].

DuP 753 is metabolized to EXP 3174 in the rat. DuP 753 and EXP 3174 both inhibit responses to Ang II in the pithed rat and in isolated rabbit aortic strips. However, the properties of the blockade differ in that EXP 3174 is a noncompetitive antagonist. It has been reported that DuP 753 produced a biphasic inhibition of the pressor response to Ang II with a transient peak inhibition at 5 min followed by a gradual increase in blockade, suggesting the formation of an active metabolite in the rat [21,26,35]. Although it is not known if DuP 753 is metabolized to EXP 3174 in the cat, the inhibitory effects of the metabolite EXP 3174 on responses to Ang II were investigated in the hindquarters vascular bed [23]. Following administration of EXP 3174 in a dose of 1 mg/kg iv, responses to Ang II were reduced markedly, and there was little tendency for responses to the peptide to return toward control values during the 4 hours that responses were followed. Responses to norepinephrine remained unchanged over this same time. The EXP 3174-induced blockade was selective in that responses to U46619, endothelin-1, vasopressin, and norepinephrine were unchanged. The EXP 3174-induced blockade was not overcome until doses of Ang II that were much larger than those administered during the control period were injected. However, when high doses of the peptide were injected, the blockade was overcome. These data indicate that peptide doses that are required to overcome the EXP 3174 blockade are much larger than required DuP 753 doses. These data, consistent with results in the pithed rat and rabbit aortic strip, indicate that the EXP 3174 blockade is noncompetitive in nature [23].

However, when the dose of EXP 3174 was reduced to 0.1 mg/kg iv, a different pattern of effect on responses to Ang II was observed in the hindquarters vascular bed. Although responses to the peptide were reduced markedly, the blockade was readily overcome when larger doses of the peptide were injected, and the shift to the right of the Ang II dose-response curve was parallel, suggesting that the antagonism is competitive at the low dose studied. The reason for the difference in results regarding the nature of the blockade in the rat and the cat is uncertain, but may be attributed in part to the species or to the experimental preparation employed. Moreover, in studies in the rat, responses to Ang II were measured in terms of increases in arterial pressure, and since cardiac output was not measured, changes in systemic vascular resistance were not compared before and after administration of the nonpeptide antagonist. In the present study the inhibitory effects of the antagonists on responses to Ang II were investigated in the denervated hindquarters vascular bed in which blood flow was maintained constant with a pump. Although there may be differences in the nature of the blockade induced by EXP 3174 in different experimental preparations, studies in the literature indicate that EXP 3174 is approximately 20–40-fold more potent than DuP 753. This compares favorably with the present study in the hindquarters vascular bed where EXP 3174 is estimated to be approximately 10–30-fold more potent than DuP 753. Moreover, as observed with DuP 753, EXP 3174 had no significant effect on systemic arterial or hindquarters perfusion pressure, indicating that the antagonist had little, if any, agonistic effect. These data are in agreement with the studies in the rat [23].

EXP 3174 is formed from DuP 753 when the 5-hydroxymethyl group is oxidized to a 5-carboxylic acid group on the imidazole ring. Although DuP 753 is converted to EXP 753 in the rat, it is unknown if the compound is metabolized to EXP 3174 in the cat. Moreover, when the DuP 753-induced blockade was followed over time, there was no apparent biphasic pattern of inhibition with a gradual increase in the blockade developing after 5 min, as observed in experiments in the conscious rat. In the hindquarters vascular bed of the cat, the DuP 753-induced blockade decreased in intensity with time. These data may suggest that DuP 753 is not converted to a more active metabolite in the cat and may suggest that the metabolism of the nonpeptide antagonist may differ in the two species. Although some differences were observed with respect to the nature of the Ang II receptor blockade with EXP 3174 in the hindquarters of the cat and in several preparations in the rat, the present data are consistent with the results of Timmermans and coworkers and provide support for the conclusion that "their efforts have culminated in the discovery of DuP 753, an orally active, potent, nonpeptide angiotensin receptor antagonist" [21,26,35]. The present data suggest that nonpeptide antagonists, such as DuP 753, and related compounds may be useful in investigations into the role of Ang II in physiological and pathophysiological processes in the regional circulation in the cat. In terms of clinical effectiveness, it is now well established that losartan is an effective and well-tolerated drug for the treatment of essential hypertension [35].

Candesartan is a newly available $AT_1$ receptor antagonist which is long acting and very selective for the $AT_1$ receptor [27–33,36]. The present results show that

candesartan inhibits pressor responses to Ang II in the hindquarters vascular bed of the cat. The inhibitory effects of candesartan on responses to Ang II were selective in that responses to norepinephrine were not altered and are consistent with results of studies with DuP 753 and EXP 3174 [23]. The inhibitory effects of the lowest dose of candesartan (3 μg/kg iv) were overcome when larger doses of angiotensin were injected, and the shift to the right of the Ang II dose-response curve was parallel. When the dose of candesartan was increased to 1 mg/kg iv, the inhibitory effects of the $AT_1$ receptor antagonist were not overcome, the rightward shift of the Ang II dose-response curve was nonparallel, and the Ang II dose-response curve exhibited little, if any, positive slope.

The duration of the inhibitory effects of candesartan on the responses to Ang II was related to dose, and the recovery half-time ($T^1/_2$) of the inhibitory effect of the lowest dose studied (3 μg/kg iv) was approximately 90 min, whereas the half-time of the inhibitory effect of the 1 mg/kg iv dose was greater than 6 hr in duration. The inhibitory effects of candesartan on responses to Ang II were selective in that the vasoconstrictor responses to norepinephrine were not altered by the $AT_1$ receptor antagonist in all doses used in the present study. The selectivity of the inhibitory effects of candesartan was assessed in greater detail. Doses of candesartan, which markedly attenuated responses to Ang II, did not alter pressor responses to U46619 and BAY K8644, vasodilator responses to levcromakalim and acetylcholine, or biphasic responses to endothelin-1. These data indicate that candesartan is a highly selective, potent $AT_1$ receptor antagonist in the hindquarters vascular bed of the cat. Candesartan had no significant effect on baseline pressures in the aorta and hindquarters vascular bed, suggesting that the $AT_1$ receptor antagonist has little agonist activity and that Ang II plays little, if any, role in maintaining baseline pressures in the systemic vascular bed of the cat.

The characteristics of the inhibitory effect of Candesartan on responses to Ang II were dependent on the dose of the $AT_1$ receptor antagonist injected. At the lowest dose used, the shift to the right of the Ang II dose-response curve was parallel. However, at the high dose, the shift to the right of the angiotensin dose-response curve was nonparallel. These data suggest that Candesartan can act as a competitive antagonist at low doses, whereas at high doses, the $AT_1$ receptor blocking agent has the characteristics of a noncompetitive $AT_1$ receptor antagonist. The reason for the difference in results obtained with the lowest and higher doses of Candesartan is uncertain, but may be explained by the presence of "spare" angiotensin $AT_1$ receptors in the resistance vessel elements in the hindquarters vascular bed of the cat. Moreover, if "spare" $AT_1$ receptors were present, the activation of some fraction of the available $AT_1$ receptors would still be capable of eliciting a maximal response, and the Ang II dose-response curve would be shifted to the right in a parallel manner after treatment with a low dose of Candesartan [37–40]. Furthermore, when a greater fraction of the available $AT_1$ receptors are inactivated by the noncompetitive $AT_1$ receptor antagonist, decreasing the "$AT_1$ receptor reserve", the peptide can no longer elicit a maximal response and the slope of the Ang II dose-response curve would be decreased [39,40]. The results of the present study showing a parallel shift

to the right of the Ang II dose-response curve with the lowest dose of Candesartan studied, along with the data showing a nonparallel shift with the higher doses of the $AT_1$ receptor antagonist employed, provide support for the hypothesis that "spare" $AT_1$ receptors are present in the resistance vessel elements of the hindquarters vascular bed of the cat.

The results of experiments in the rat show that Ang II increases systemic arterial pressure in a dose-related manner. The increases in pressure in response to Ang II and Ang III were markedly inhibited by the administration of candesartan. The $AT_1$ receptor blockade was not surmounted when larger doses of Ang II were injected after administration of candesartan. The dose-response curve for Ang II was shifted to the right in a nonparallel manner. Although responses to Ang II and Ang III were markedly attenuated, candesartan had no significant effect on increases in systemic arterial pressure in response to norepinephrine. The inhibitory effects of candesartan on pressor responses to Ang II were long in duration and there was little, if any, tendency for responses to return toward control value 3 hours after administration of the $AT_1$ receptor antagonist in a dose of 1 mg/kg iv. Increases in systemic arterial pressure in response to norepinephrine did not change during the 3 hours after administration of the $AT_1$ receptor antagonist. The present data indicate that candesartan is a selective, potent, long-acting angiotensin $AT_1$ receptor antagonist and that the $AT_1$ receptor blockade is noncompetitive in nature in the rat.

Although increases in systemic arterial pressure in response to Ang II were markedly inhibited by candesartan, a hypotensive or vasodilator response to the angiotensin peptides was not observed after administration of the $AT_1$ receptor antagonist. These data suggest that the predominant actions of Ang II are mediated by the activation of $AT_1$ receptors in the systemic vascular bed of the rat and that noncompetitive blockade of the $AT_1$ receptor does not unmask a vasodilator response. Moreover, when the dose of candesartan was increased to 10 mg/kg iv in the rat, increases in systemic arterial pressure in response to Ang II were abolished and a vasodepressor response to the peptide was not observed.

It has been reported that Ang II and Ang III can produce biphasic changes in systemic arterial pressure in the anesthetized rat and that during $AT_1$ receptor blockade, pressure increases are eliminated, whereas the depressor responses were enhanced [20]. Moreover, it has also been shown that $AT_1$ receptor blockade eliminated responses to Ang III, whereas $AT_2$ receptor blockade enhanced pressor responses to Ang III [20]. In the present experiments, biphasic changes in systemic arterial pressure in response to the angiotensin peptides were rarely observed and were not statistically significant when data from all experiments were analyzed. Increases in systemic arterial pressure in response to Ang II were markedly attenuated, but were not reversed by candesartan, and increases in systemic arterial pressure in response to the angiotensin peptides in the rat were not modified by the $AT_2$ receptor antagonist, PD 123319. These data suggest that angiotensin $AT_2$ receptors do not mediate a depressor response or modulate responses to angiotensin peptides in the systemic vascular bed of the rat.

The reason for the difference in results in the present study and previous studies in the literature is uncertain, but may involve differences in anesthesia or experimental procedures employed. The results of the present study showing that candesartan inhibits Ang II-induced pressor in an insurmountable manner are consistent with results in isolated rabbit aorta. The present results are also consistent with results in the anesthetized rat showing that candesartan in a dose of 1 mg/kg iv can produce a 100% decrease in the pressor response to Ang II. The results of the present study extend previous work by showing that candesartan inhibits or abolishes vasopressor responses to angiotensin peptides in the rat in a highly selective manner.

The results of experiments with $AT_1$ and $AT_2$ receptor antagonists were similar in the rat and in the cat [7–9,24]. The studies in both species indicate that the major actions of Ang II are mediated by the activation of $AT_1$ receptors and that after administration of candesartan in a dose of 1 mg/kg iv or greater a nonequlibrium $AT_1$ receptor blockade is established and, for all practical purposes, vasoconstrictor responses to Ang II are abolished, Although vasoconstrictor responses to angiotensin peptides are abolished by higher doses of candesartan and the $AT_1$ receptor blockade is highly selective, a vasodilator response to angiotensin peptides is not observed in the regional vascular bed of the cat or in the rat [7–9,24]. A vasodilator response to angiotensin peptides blockade of $AT_1$ receptors was not observed and vasoconstrictor responses to the angiotensin peptides were not altered with the administration of the $AT_2$ receptor antagonist PD 123,319 in doses of 5, 10, or 20 mg/kg iv [7–9,24], angiotensin peptides [7–9,24]. The results of an extensive series of experiments with PD 123,319 have been interpreted to suggest that $AT_2$ receptors do not mediate or modulate responses to Ang II, III, or IV in the hindlimb, pulmonary, or mesenteric vascular beds in the cat or in the hindquarters and systemic vascular beds of the rat [7–9,24].

The mechanism by which angiotensin peptides induce vasoconstriction in the cat have been investigated. Although products of the cyclooxygenase pathway of arachidonic acid do not seem to play a prominent role, products of the lipoxygenase pathway appears to be important in $AT_1$ receptor-mediated vasoconstriction [41]. Results from experiments in which inhibitors of phospholipase C and protein kinase C reduce responses to angiotensin peptides suggest that the vasoconstrictor response to Ang II in the pulmonary vascular bed of the cat is mediated, in part, by $AT_1$ receptor activation of phospholipase C and protein kinase C [42,43].

Ang I and Ang I-(3–10) induce vasoconstrictor responses in the regional vascular bed of the cat that are markedly attenuated by ACE inhibitors [8,24]. The vasoconstrictor response to Ang I and Ang II are similar in magnitude and in time course and are not altered by the addition of a time-delay coil to the perfusion circuit in the cat [7–9,24]. These data suggest that angiotensin is readily and efficiently converted to an active peptide in small arteries near or at the site of action of the peptide upstream from the capillary bed [7–9,24]. In terms of relative vasoconstrictor activity in the hindlimb vascular bed of the cat, responses to Ang I, Ang II, and Ang III were very similar when doses are expressed on a nanomole basis to take in

account the molecular weight of the peptides [9,24]. Vasoconstrictor responses to Ang I-(3–10) and Ang IV were very similar, and although Ang I-(3–10) and Ang IV have full intrinsic activity, by our criteria, in the hindlimb vascular bed of the cat, dose-response curves for Ang I-(3–10) and Ang IV were 2 log units to the right of the Ang II dose-response curves [8,9,24,44]. The Ang I analog ($Pro^{11}$,D-$Ala^{12}$) Ang I was threefold more potent than Ang IV in the hindlimb vascular bed of the cat. Responses to ($Pro^{11}$,D-$Ala^{12}$) Ang I were not altered by ACE inhibitors but were attenuated by $AT_1$ receptor antagonists. The present studies with Ang I and Ang I-(3–10) suggest that ACE activity is high in the small arteries at or near the site of localization of $AT_1$ receptors in the precapillary bed [7–9,24]. These data suggest that this local ACE activity could play a role in the local regulation of vascular tone in the regional vascular bed of the cat [7–9,24]. The data showing similar responses to Ang II and Ang III suggest that the amino acid Arg at position 2 is not required for high affinity activation of $AT_1$ receptors in the regional vascular bed of the cat [7,9,24]. The Ang IV dose-response curve is parallel to the Ang II dose-response curve, but it lies two log units to the right of the Ang II curve. The data that supports these facts along with the observation that Ang I-(4–8) has no discernable pressor activity suggest that the amino acid Val in the number 3 position of the peptide is required for high-affinity binding to the $AT_1$ receptor and intrinsic activity in the cat [7–9,24]. The observation that vasoconstrictor responses to ($Pro^{11}$,D-$Ala^{12}$) Ang I are not altered by ACE inhibitors but are blocked by $AT_1$ receptor antagonists suggests either that other enzymatic pathways, such as the chymase pathway, are involved in the conversion of the analog to an active peptide or that this ACE-resistant analog itself possesses the ability to activate the $AT_1$ receptor.

In conclusion, the results of experiments with the angiotensin peptides described in this chapter are summarized in figure 9. The observation that Ang I, Ang II, Ang I-(3–10), and Ang IV have similar vasoconstrictor activity and that responses to Ang I and Ang I-(3–10) are inhibited by ACE inhibitors suggests that the precursors are efficiently converted to active peptides by ACE at or near the site of action in the hindquarters vascular bed. The active peptides, Ang II and Ang IV, are formed within the arterial segments and induce vasoconstriction by activating $AT_1$ receptors. Ang II and Ang III have similar vasoconstrictor activity, suggesting that the Asp residue in position 1 is not essential for the full expression of the vasoconstrictor response to the peptide. Ang IV has full intrinsic activity in its ability to induce vasoconstriction in the hindlimb vascular bed. However, the dose-response curve for Ang IV is 2 log units to the right of Ang II suggesting that it has low affinity for the $AT_1$ receptor and that the Arg residue in position 2 is needed for high-affinity binding to the $AT_1$ receptor. Since Ang I-(4–8) has no measurable activity, the Val residue in position 3 is essential for activity at the $AT_1$ receptor. The Ang I analog ($Pro^{11}$,D-$Ala^{12}$) Ang I is more potent than Ang IV and has full intrinsic activity. Vasoconstrictor responses to the Ang I analog are not blocked by ACE inhibitors, suggesting that the ACE-resistant substrate may be converted to an active peptide by a non-ACE pathway, such as the chymase pathway. It is also possible that

the analog may have the capacity to stimulate $AT_1$ receptors and induce vasoconstriction. The present results suggest that Ang II, Ang III, Ang IV, and ($Pro^{11}$,D-$Ala^{12}$) Ang I induce vasoconstriction by activating $AT_1$ receptors. The data with the $AT_2$ receptor antagonist PD 123,319 suggest that activation of the $AT_2$ receptor does not induce vasodilation and that $AT_2$ receptors do not mediate or modulate vasoconstrictor responses to angiotensin peptides. The role of the $AT_2$ receptor and the putative $AT_4$ receptor in the regulation of vascular tone in the regional vascular bed of the cat is yet unknown.

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# ANGIOTENSIN II RECEPTOR ANTAGONISTS IN PATIENTS WITH RENAL FAILURE AND ON HEMODIALYSIS

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***Summary.*** Limited experience with the use of the angiotensin II antagonist losartan demonstrated good antihypertensive efficacy in patients with renal impairment irrespective of the degree of renal function. In milder forms of renal impairment, there was no further impairment of renal function and no accumulation of the parent drug or the active metabolite. It has been suggested that this is due to losartan being excreted via the hepatic as well as the renal route and that the former possibly compensates for a reduced renal excretion of the drug. Even though losartan is not dialysable, this feature makes it possible to use the drug in patients on hemodialysis. Angiotensin II antagonists may be useful as an alternative treatment in patients with renal impairment, patients who are on hemodialysis and do not tolerate ACE inhibitors, or patients who have a contraindication to this class of drugs. However there is still insufficient data available on the use of losartan in more severe forms of renal impairment.

Angiotensin (Ang) II antagonists exert their pharmacological action by blockade of the $AT_1$ receptor [1]. Therefore, they do not demonstrate some of the adverse effects typical of angiotensin-converting enzyme (ACE) inhibitors, e.g., chronic nonproductive cough and angioedema [2], which are considered to be the result of accumulation of bradykinin, substance P, or other products consequent to the inhibition of the metabolism of these substances by ACE inhibitors [3]. Consequently, Ang II antagonists offer an alternative to ACE inhibitors in patients in whom interference with the renin-angiotensin system is desirable but has been precluded by adverse affects while they were on ACE inhibitors.

In patients with renal disease, there is often progressive deterioration of renal function. Although the detailed causes involved in this process are still largely

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unknown, they include the involvement of the intrarenal renin-angiotensin system. Postulated mechanisms are among others, glomerular hypertension [4] and glomerular hypertrophy [5] whereby Ang II is considered to contribute by inducing efferent arteriolar constriction and by Ang II exerting a growth promoting effect [6,7]. Indirect evidence for the involvement of the renin-angiotensin system with the previously mentioned mechanisms is provided by the favorable results obtained from treatment with ACE inhibitors [8]. The fact that a greater reduction in glomerular filtration rate at the time of initiation of ACE inhibitor therapy was associated with better protection against a later deterioration in glomerular filtration rate [9] underlies the importance of efferent vasoconstriction in the progression of renal impairment.

The presence of proteinuria is associated with an accelerated decline in renal function [10]. Although proteinuria represents a surrogate endpoint, it has been shown that greater reduction in proteinuria is associated with a slowing of the progress of the decline in renal function [11]. ACE inhibitors have also been shown to reduce proteinuria to a greater extent than other antihypertensive drugs [12].

ACE inhibitors have a renal protective effect in many human renal diseases [13]. Although it has been postulated that the benefit of ACE inhibition is in part due to the selective dilation of the efferent arteriole caused by bradykinin, renal blood flow, or renal plasma flow, studies are usually comparable between losartan vs. ACE inhibitors [14]. Ang II antagonists lack this particular mechanism, though their antiproteinuric effect is comparable to that of ACE inhibitors [15]. Therefore it would appear that Ang II receptor antagonists seem to affect surrogate endpoints, such as changes in blood pressure, changes in renal blood or plasma flow, and changes in proteinuria, identical to ACE inhibitors. Thus, it would be logical to expect Ang II receptor antagonists to be renal protective as well. Although to date there are no long-term human studies completed [16], the results obtained from animal studies are consistent with this hypothesis [17–19].

Losartan is the first of a series of Ang II antagonists that have become available for the treatment of hypertension. The long plasma half-life of the active metabolite E3174 provides good 24-hour blood pressure control and an excellent side effect profile [20]. Therefore this drug seems to be well tolerated by most patients. Compared to ACE inhibitors, Ang II antagonists provide a comparable blood pressure lowering effect [20].

There is still limited experience with Ang II antagonists in patients with severe impairment of renal function and in those on hemodialysis. The results of relatively small studies indicate good antihypertensive efficacy and safety of losartan irrespective of the degree of renal function or impairment [21] for a period up to 12 weeks [22]. There was no worsening of renal function, and neither the parent drug nor the active metabolite accumulated in the presence of impaired renal function [23], which may relate to the fact that losartan and its active metabolite, E3174, are excreted up to 35% by the kidney and up to 65% to the hepatic route. As the drug is not dialyzable [24], and since no significant change in the area under the curve

occurred with increasing impairment of renal function [23], one could assume that in the presence of reduced renal function, there is a compensatory increase in the hepatic excretion of the drug.

The following case report demonstrates the usefulness of the Ang II antagonist losartan in a patient on hemodialysis.

## CASE PRESENTATION

A 39-year-old female with End-Stage Renal Disease (ESRD) and hypertension was presented for discussion. She came to medical attention in 1979 when she developed pre-eclampsia. Unfortunately, shortly following an emergency cesarean section, the neonate succumbed to respiratory failure. A renal biopsy performed on that admission demonstrated IgA glomerulonephropathy. Following the delivery, she became normotensive off all antihypertensives. By 1981, her serum creatinine had risen to 220 umol/L, and it continued to worsen until she developed ESRD and commenced hemodialysis in 1987. In 1988, she underwent a cadaveric renal transplant, but had hyperacute rejection requiring a transplant nephrectomy. In 1991, she developed mild hypertension. She was treated initially with captopril with the dose titrated to 25 mg orally bid, but this had to be discontinued because of a persistent nagging cough. At this point is was possible to dialyze the patient with an AN69 dialyzer, which was more appropriate for this young patient. The use of the AN69 was precluded while the patient was on the ACE inhibitor because of the danger of an anaphylactoid reaction. Nifedipine XL was started, but despite escalating doses, her diastolic blood pressures remained at 100 mm Hg. Clonidine was added, but side effects of dry mouth, fatigue, and postural dizziness, were noted. Noncompliance was suspected because of marked variability in her frequently measured blood pressures. These drugs were discontinued, and she was switched to Verapamil in 1992. Following a parathyroidectomy for worsening of her renal osteodystrophy, she developed hypocalcemia leading to hypotension and congestive heart failure from complete AV nodal block. The verapamil was discontinued, and she was discharged off all antihypertensives while attempting nonpharmacological control. In 1994, she was attempted on amlodipine, but quit taking this because of ongoing headaches. In 1995, she was tried on atenolol, but failed this because of fatigue and insomnia, which made it difficult to manage her four children ranging in age from eight to seventeen. Finally, in 1996, she was placed on losartan and has tolerated this well.

Past medical history included several D&C's in 1987–88 with the histology showing a proliferative endometrium. In 1989, she had an abdominal hysterectomy. She has no known allergies. Physical examination revealed that her blood pressures were 136/84 on losartan. Pulse rate was 82. Respiratory rate was 16. Dry weight was 74.5 kg with 2.4 kg interdialytic weight gains. Fundal examination revealed slight arteriolar narrowing. Cardiovascular and respiratory examinations were normal. Her abdomen was soft with normal bowel sounds and no hepatosplenomegaly. No renal bruits were audible. Investigations included a chest

x-ray demonstrating a slight increase in the cardio-thoracic ratio and a normal EKG. An echocardiogram demonstrated borderline hypertrophy of left ventricular wall thickness.

## CASE DISCUSSION

This case illustrates several of the shortfalls of antihypertensive therapy and the usefulness of the angiotensin II receptor blockade class. Telemans et al. reported 5 anaphylactoid reactions occurring in 3 out of 13 patients receiving ACE inhibitors coincidentally dialyzed on Polyacrylonitrile AN69 dialyzers [25]. These serious reactions usually required treatment with intravenous fluids, hydrocortisone, epinephrine, and discontinuation of dialysis. The authors speculated that the negatively charged AN69 dialyzer interacted with the plasma resulting in activation of Hageman factor. Activated Hageman factor converts pre-kallikrein to kallikrein. Kallikrein then cleaves kininogen to bradykinin, which may mediate the anaphylactic symptoms [26]. Because the ACE inhibitors also lead to an increase in bradykinin, the interaction between the drugs and AN69 dialyzers may lead to the life-threatening reactions. Greater numbers of hemodialysis patients are being treated with high-flux dialyzers, so an alternative antihypertensive agent, such as the Ang II receptor antagonist, would be useful. Indeed, this patient was placed on an AN69 dialyzer after the captopril was discontinued because of cough. In our local experience, we have noted no adverse reactions with the combination of AN69 dialyzers and losartan. However careful observation is still warranted given two recent reports of anaphylactoid reactions with losartan [27], though they did not occur in dialysis patients. One other aspect to consider in this case is whether or not the patient may have benefited from Ang II receptor blockade at the time of diagnosis of her renal disease in 1979. Further clinical studies are pending.

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# THE PLACE OF ANGIOTENSIN II ANTAGONISTS IN RELATION TO THE CANADIAN HYPERTENSION SOCIETY GUIDELINES

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***Summary.*** Angiotensin (Ang) II antagonists are a novel class of drugs developed to block Ang II receptors. This approach is deemed more direct and more likely to be effective than the earlier pharmacological approach of inhibiting the enzyme (ACE inhibitor) responsible for converting Ang I to Ang II. Furthermore, it is expected that the approach will avoid problems associated with pathways that bypass ACE, e.g., tissue chymase.

Two major subtypes of receptor have been identified, $AT_1$ and $AT_2$. Blockade of the $AT_1$ receptor by a new class of agents typified by losartan lowers blood pressure in hypertensive subjects and has a theoretical role in blocking the action of Ang II in the pathophysiology of other disease states such as congestive heart failure (CHF) and left ventricular hypertrophy (LVH).

The Canadian Hypertension Society Consensus Reports on the drug treatment of hypertension were last updated in a series of publications in the Canadian Medical Association Journal in 1993. Specific reviews dealt with the pharmacotherapy of hypertension, the treatment of hypertension in the elderly, and the treatment of the diabetic hypertensive. The choice of antihypertensive therapy is driven by factors that include concurrent cardiovascular risk factors and the presence or absence of other disease states. The physician is challenged to find an appropriate medication for the individual patient that is not contraindicated, that is effective and affordable for the patient, and that does not cause adverse effects in managing this largely asymptomatic disorder.

Although no long-term studies of $AT_1$ blockers have been done in sufficiently large populations to determine the changes in clinical outcomes that are desirable in treating hypertension (e.g., reduction of myocardial infarction, stroke, and end-stage renal disease), the surrogate endpoint of blood pressure lowering indicates that $AT_1$ blockers will have an important place in the management of hypertension that is akin to that presently held by ACE

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inhibitors, alpha-1 blockers, and calcium channel blockers. Recent evaluation of losartan in the elderly (ELITE) indicates a potentially valuable role for losartan in the treatment of elderly hypertensive patients with impaired left ventricular function.

## INTRODUCTION

The angiotensin (Ang) II type 1 ($AT_1$) receptor blockers are a novel class of drugs with a relatively selective competitive antagonism at the $AT_1$ receptor subclass of Ang II receptors [1–4]. The index drug is losartan, but several analogs are under evaluation and some, e.g., valsartan, have been released in other countries.

Because of its pharmacological action as an $AT_1$ receptor blocker, losartan appears to offer advantages over the angiotensin-converting enzyme (ACE) inhibitors. The latter class of drugs is incapable of entirely preventing the production of Ang II from Ang I because of alternative synthetic pathways that include cardiac and vascular chymase. ACE inhibitors also lead to the accumulation of bradykinin and other kinins thought to be responsible in part for the troublesome cough that may affect as many as 5 to 10% of all ACE inhibitor recipients. However, potential benefits associated with the accumulation of bradykinin, such as a contribution to vasodilation, may be lost through the action of $AT_1$ blockade.

These known differences between the pharmacological actions of ACE inhibitors and $AT_1$ antagonists have led to considerable speculation on their potential advantages and disadvantages in the treatment of hypertension, congestive heart failure, and left ventricular hypertrophy.

Hypertension is a common problem, affecting approximately 10% of the North American population. The problem increases with aging, affecting 20 to 25% of seniors. Usual therapeutic interventions include nonpharmacological therapy of weight loss by dietary and regular aerobic exercise, moderation of salt intake, and moderation of alcohol intake, together with the management of other known cardiovascular risk factors such as smoking, diabetes, and dyslipidemia [5,6].

Two aspects of pharmacotherapy of hypertension have advanced substantially during the past two to three decades. First, there is now a substantial body of evidence that demonstrates the benefit of treating systolic hypertension above 160 mm Hg and diastolic hypertension above 100 mm Hg [7]. Second, there has been enormous growth in the availability of medications to treat hypertension, including novel classes, modified delivery forms, and drug combinations [8].

In the drug therapy of hypertension, there has been an increased emphasis on the importance of evidence rather than opinion in making recommendations on the simplification of treatment to once-or twice-daily monotherapy when possible, the avoidance of aggravation of other risk factors or concurrent disease states, and the rational management of comorbidity [9].

Given the high prevalence of hypertension, the usual lifelong duration of treatment, and the potential financial burden on the individual, the private third-party payor, or the provincial Pharmacare plan, the issue of cost deserves some consideration. There has been a tendency to emphasize the acquisition cost of medications

and ignore the direct and indirect costs associated with inadequate compliance leading to poor BP control, costs of additional visits to the primary care physician or the Emergency Room, laboratory tests used to monitor potential biochemical abnormalities, and the costs of investigation for secondary hypertension when BP control is inadequate.

Newer medications with fewer adverse effects, better tolerance with improved compliance, little or no need for additional laboratory investigations, and potentially greater benefits over lifetime management if compliance is enhanced may actually be more cost-effective and offer better value than less expensive medications. However, these are matters that require definitive health economic analyses and will not be discussed further.

The main emphasis of this review will be the potential place of $AT_1$ receptor antagonists in the context of the most recent Canadian Hypertension Society Consensus Conference reports [9–12]. There will be extensive cross-references to specific reports. The reader is advised to consult the original reports and their supporting references for a comprehensive understanding of background and evidence leading to specific recommendations. In addition, review of the fifth report of the Joint National Committee on Detection, Evaluation, and Treatment of High Blood Pressure (JNC V) [6], also published in 1993, is recommended.

The 1993 CHS reports introduced explicit evidence for recommendations that were based on a hierarchy of levels of evidence for rating studies of treatment, prevention, and quality assurance [9,11]. The highest level of evidence (Level I) is a randomized controlled trial (RCT) that demonstrates a statistically significant difference in at least one important outcome, e.g., survival or major illness. An RCT of adequate sample size to exclude a 25% difference in relative risk with 80% power is also acceptable as Level I evidence. Level II is defined as an RCT that does not meet the Level I criteria. Level III evidence is defined as a nonrandomized trial with contemporaneous controls selected by some systematic method (i.e., not selected by perceived suitability for one of the treatment options for individual patients) or appropriate subgroup analysis of a randomized trial. Lower levels of evidence describe before-after studies, case series, or case reports.

Grade A recommendations are based on one or more studies at Level I, Grade B recommendations are based on Level II evidence, Grade C recommendations on Level III evidence, and Grade D recommendations on evidence lower than Level III, including expert opinion.

This approach has been well accepted by most clinician-scientists but is not without dispute. Major counter-arguments include concerns about the lack of weight given to theoretical advantages of a drug and the denial of surrogate endpoints in Grade A evidence. While there is widespread sympathy for this concern because of the general belief that BP control is an adequate surrogate for long-term benefits of antihypertensive therapy, potential long-term effects of differing pharmacological classes may create as yet unknown long term advantages or disadvantages. There are troubling historical precedents in pharmacological treat-

ments that provide the desired surrogate endpoints without benefits of "hard" clinical outcomes (e.g., clofibrate reduced cardiac death, but overall mortality increased; several antiarrhythmics reduced the frequency of ventricular ectopy, but cardiac mortality increased; alpha-blockers improved hemodynamics in patients with heart failure, but did not improve cardiac mortality).

## METHODOLOGY

Relevant recommendations from the reports on pharmacological treatment of essential hypertension [10], hypertension in the elderly [11], and hypertension and diabetes [12] will be listed, and the potential value of $AT_1$ receptor blockers will be indicated.

## PHARMACOLOGICAL TREATMENT OF ESSENTIAL HYPERTENSION

Key decision making points in the management of essential hypertension are the level of blood pressure, the presence or absence of other risk factors such as abnormal lipid profile, raised blood glucose or insulin levels, or left ventricular hypertrophy and the presence or absence of other medical conditions such as ischemic heart disease, congestive heart failure, peripheral vascular disease, chronic obstructive pulmonary disease, and asthma. The presence of other risk factors or evidence of atherosclerosis should expedite therapy. One must recognize the presence of other disease states in the individual patient and avoid drugs that may aggravate any of these conditions when choosing a medication. Physicians are encouraged to select drugs that may benefit two or more coexistent disorders.

### The uncomplicated hypertensive

Recommendations 1 through 8 deal with the initial choice of monotherapy, alternatives in the face of a contraindication, an adverse response, or the lack of adequate response.

*Recommendation 1*

Initial therapy should be monotherapy with either a low-dose thiazide diuretic (e.g., hydrochlorothiazide, 25 mg daily) or a beta-blocker (grade A).

*Recommendation 2*

If the response is inadequate or if there are adverse effects, substitute the alternative drug (grade A).

*Recommendation 3*

If there is a partial response, consider a combination of a diuretic and a beta-blocker (grade A) or monotherapy with an alpha-blocker, ACE inhibitor, calcium entry blocker, or centrally acting drug (grade B).

On the basis of available evidence, it would seem entirely appropriate to include

within Recommendation 3 monotherapy with an angiotensin II antagonist ($AT_1$ blocker) at the same level of evidence as alpha-blockers, ACE inhibitors, calcium entry blockers, and centrally acting drugs (i.e., grade B).

*Recommendation 4*

If there is an adverse effect or a contraindication to diuretic and beta-blocker therapy, consider monotherapy with one of the other groups of drugs (grade B).

Likewise, it seems entirely reasonable to include an $AT_1$ receptor blocker as alternative monotherapy (grade B).

*Recommendation 5*

If the blood pressure is still not controlled, try combinations such as a low-dose diuretic with an ACE inhibitor, a calcium entry blocker, a centrally acting drug or an alpha-blocker (grade B).

$AT_1$ antagonists have been studied in combination with other classes of drugs, mainly diurectics [13–16], and offer advantages over monotherapy when monotherapy is not sufficiently effective (grade B).

*Recommendation 6*

Alternatively, a beta-blocker could be given with a vasodilator (such as a dihydropyridine calcium entry blocker), hydralazine, or an alpha-blocker (grade B).

Beta-blockers can be used in combination with $AT_1$ receptor antagonists (grade B).

*Recommendation 7*

Other combinations may be indicated (grade B/C).

It is entirely reasonable that such combinations may include $AT_1$ antagonists (grade B/C).

*Recommendation 8*

Resistant hypertension may require combinations of three or more drug groups (grade C). Consider possible reasons for the poor response to therapy, such as noncompliance or secondary causes of hypertension, including consumption of other drugs.

$AT_1$ antagonists can be used in combinations of three or more drugs (grade C).

**Congestive heart failure**

In the setting of congestive heart failure, several therapeutic regimens have been tested. Some are effective in lowering blood pressure but do not influence long term outcomes in congestive heart failure. Others have been shown to improve prognosis in congestive heart failure.

*Recommendation 14*

Diuretics and ACE inhibitors are first-line therapy (grade A).

An important new study on losartan has been published recently. The Evaluation of Losartan in the Elderly (ELITE) set out to compare the deterioration in renal function in elderly patients with congestive heart failure receiving either captopril 50 mg qid or losartan 100 mg daily [17]. There were no differences between treatments in renal function, but the losartan treatment reduced overall mortality and hospitalization. In particular, cardiac mortality resulting from sudden cardiac death was reduced by over 40%. On the basis of this evidence of reduced clinical events, it is reasonable to include losartan as an alternative to ACE inhibitors as first line therapy in patients with hypertension and congestive heart failure (grade B).

**Dyslipidemia**

*Recommendation 20*

In the setting of dyslipidemia alpha-blockers, ACE inhibitors, beta-blockers, calcium entry blockers, centrally acting agents, and low-dose thiazide diuretics may all be considered for monotherapy (grade D).

In the management of patients with dyslipidemia, $AT_1$ blockers offer an increased choice of lipid-neutral antihypertensive treatments (grade D).

**Left ventricular hypertrophy**

*Recommendation 26*

In the management of patients with hypertension and left ventricular hypertrophy, direct arteriolar vasodilators such as hydralazine or minoxidil should be avoided (grade B/C).

*Recommendation 25*

Left ventricular hypertrophy is a significant risk factor for cardiovascular complications (grade C). Its reversal has not been proven to reduce rates of cardiovascular events. There is insufficient evidence to base initial therapy on the reported effects of drugs on left ventricular hypertrophy.

Since angiotensin II is a powerful stimulus for myocardiocyte hypertrophy, there are theoretical reasons why $AT_1$ receptor blockers should be effective in this setting (grade D). However, definitive evidence is lacking.

**Chronic obstructive airways disease**

*Recommendation 29*

Patients with chronic obstructive pulmonary disease should avoid beta-blockers (grade C). ACE inhibitors can cause a chronic dry cough, which can be confused with chronic respiratory disorders. Discontinuation of therapy is usually required to eliminate the cough.

For those patients with diseases of the airways, $AT_1$ receptor antagonists offer a further choice of treatment. In particular, the chronic dry cough associated with ACE inhibitors is reduced substantially during antihypertensive therapy with $AT_1$ receptor blockers, with coughing frequency and severity similar to a placebo-treated group [18,19].

**Asthma**

*Recommendation 30*

For patients with reversible airway disease, all beta-blockers are contraindicated (grade C).

$AT_1$ receptor antagonists offer an alternative choice in the asthmatic patient (grade D).

**Gout**

*Recommendation 32*

In general, thiazide diuretics should be avoided (grade C).

$AT_1$ receptor antagonists offer an alternative in the management of the patient with a history of gout (grade D).

**Pregnancy**

*Recommendation 34*

Methyldopa, clonidine, hydralazine, and beta-blockers (e.g., atenolol, metoprolol, oxprenolol and labetalol) may be used alone or in combination (grade B/C).

Neither ACE inhibitors (grade C), calcium entry blockers (grade C) or $AT_1$ receptor blockers (grade C) antagonists should be prescribed for a woman capable of childbearing because of potential teratogenicity.

**Race**

In the management of black (African American) patients, there were two specific recommendations.

*Recommendation 37*

Thiazides in low doses are recommended over beta-blockers (grade B).

*Recommendation 38*

Calcium entry blockers are as effective as diuretics (grade B) and may be more effective than beta-blockers or ACE inhibitors (grade B).

$AT_1$ receptor antagonists provide another option for the management of hypertension in the black patient (grade D).

## HYPERTENSION IN THE ELDERLY

The drug of choice in the treatment of hypertension in the elderly is a low dose of a thiazide diuretic (grade A). Adequate control of hypertension in the elderly patient sometimes requires other medications.

**The elderly**

*Recommendation 15*

In general, the initial drug dose in elderly patients should be half of the usual recommended dose and should be increased gradually. Patients should be observed for changes in blood pressure and the development of side effects. (This recommendation is based on Level II evidence on diuretics and kinetic differences for calcium entry blockers.)

*Recommendation 16*

The number of tablets and different drugs should be kept to a minimum. During long-term treatment in the elderly, once-daily preparations are preferred for optimum compliance (grade B).

The usual starting dose of losartan in the elderly is 50 mg once daily. The once-daily treatment of losartan or losartan/hydrochlorothiazide combination offers another treatment choice for the elderly (grade D). Elderly patients with heart failure have improved outcomes during treatment with the $AT_1$ blocker losartan (grade B) [17].

When patients have a contraindication, demonstrated adverse effect, or lack of responsiveness to a thiazide diuretic, an alternative treatment is desirable.

*Recommendation 25*

Consideration should be given to agents other than thiazide diuretics in patients with clinically important pre-existing hyperlipidemia, hyponatremia, hypokalemia, or hypercalcemia (grade D). Mild hyperlipidemia or hyperuricemia is not a contraindication to low-dose diuretic therapy in elderly patients.

An $AT_1$ receptor blocker provides an alternative to other antihypertensive drugs in patients with these problems (grade D).

*Recommendation 33*

Caution because of the risk of acute renal failure or hyperkalemia, in elderly patients with severe hypertension or congestive heart failure, especially those with abdominal bruits, diabetic nephropathy, or decreased creatinine clearance, the serum creatinine and potassium levels should be measured before and after institution of therapy with ACE inhibitors (grade D). Acute mild increases in creatinine levels should not be considered a reason to discontinue ACE inhibitor treatment.

The pharmacological action of an $AT_1$ receptor blocker on the kidney is similar to that of an ACE inhibitor. Consequently, this recommendation to monitor serum creatinine and potassium should be observed after the introduction of $AT_1$ receptor-blocking therapy (grade D).

*Recommendation 34*

Centrally acting agents and peripheral alpha-blockers are effective for decreasing blood pressure (grade B). However, cognitive impairment resulting from therapy

with methyldopa, postural hypotension from peripheral alpha-blockers (e.g., prazosin, doxazosin and terazosin), drowsiness and rebound hypertension from clonidine, and depression from reserpine may limit the use of these otherwise effective antihypertensive drugs in older people.

$AT_1$ receptor antagonists appear well tolerated in the elderly and they offer advantages over other drugs in those with impaired left ventricular function. They offer a practical alternative to other drugs that influence cognitive function or produce postural hypotension (grade D).

## HYPERTENSION AND DIABETES

Stringent goals are set for the management of blood pressure in the setting of diabetes mellitus.

### Diabetes mellitus

*Recommendation 9*

The goal of treatment is a diastolic blood pressure of, at most, 90 mm Hg (grade B). In patients with microalbuminuria, it may be worth attempting to achieve approximately 80 mm Hg (grade D).

*Recommendation 10*

ACE inhibitors, calcium entry blockers (grade B), or alpha-blockers (grade C) should be the first-line antihypertensive agents.

*Recommendation 11*

Thiazides or beta-blockers should be second-line antihypertensive agents (grade C).

*Recommendation 12*

Centrally acting agents or vasodilators may be used if other agents are contraindicated or if there is difficulty in controlling the hypertension (grade C).

*Recommendation 13*

If first-line treatment is ineffective, contraindicated, or associated with side effects, the following should be tried: (1) change to or add another first-line agent (grade C), (2) add a cardioselective beta-blocker to a dihydropyridine calcium entry blocker (grade B), or (3) add a thiazide to an ACE inhibitor (grade B).

Because of their pharmacological actions and known beneficial effects on renal function and proteinuria, $AT_1$ receptor blockers provide an alternative to ACE inhibitors, calcium entry blockers, or alpha-blockers as first-line antihypertensive agents (grade D). $AT_1$ receptor antagonists offer an alternative to ACE inhibitors when the latter provoke cough in the diabetic hypertensive patient (grade C). This is an area that merits specific research studies that compare $AT_1$ receptor blockers with ACE inhibitors.

Diabetic patients commonly have associated morbidity such as autonomic dysfunction, impotence, peripheral vascular disease, and heart failure secondary to myocardial ischemia.

*Recommendation 17*

In the presence of autonomic neuropathy, alpha-blockers and centrally acting agents should be used with caution (grade C).

*Recommendation 18*

Beta-blockers should be used with caution in patients with heart failure and peripheral vascular disease (grade C). Beta-blockers, especially noncardioselective ones, may worsen hyperglycemia and in insulin-dependent diabetes, may prevent recognition of and delay recovery from hypoglycemia (grade B).

*Recommendation 19*

In the presence of impotence, centrally acting agents, thiazides, and beta blockers should be used with caution (grade C).

$AT_1$ receptor antagonists provide a reasonable alternative to alpha-blockers, ACE inhibitors, and calcium channel blockers in the setting of hypertension and diabetes complicated by these problems.

In summary, it must be considered entirely reasonable to include selective $AT_1$ receptor blockers such as losartan in the long list of alternative medications that are suitable for the treatment of the uncomplicated or complicated hypertensive patient when recommended first-line treatments are contraindicated, ineffective, or associated with intolerable adverse effects. It is predictable that this class of medication will be a suitable replacement in the majority of patients receiving an ACE inhibitor in whom a troublesome cough forces discontinuation of the ACE inhibitor. The results of ELITE [17] are particularly encouraging in those older hypertensives with impaired left ventricular function. It remains to be seen whether there will be any advantage of combining an ACE inhibitor with an $AT_1$ receptor blocker in the treatment of heart failure.

The ELITE trial [17] may have also helped with another conundrum of the $AT_1$ receptor blockers. Frequently questioned is the role of the $AT_2$ receptor and the possible implications of chronic stimulation of the $AT_2$ receptor by high levels of circulating and local angiontensin II that occur in left ventricular dysfunction and clinical congestive heart failure. The beneficial results of ELITE suggest that angiotensin II stimulation of unblocked $AT_2$ receptors is not hazardous and may even offer advantages. Further research is required to investigate the phenomenon further.

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# TRANSLATING CLINICAL GUIDELINES FOR MANAGEMENT OF HEART FAILURE AND HYPERTENSION INTO CLINICAL PRACTICE

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***Summary.*** Scientific data that are synthesized as practice guidelines appear to confront serious impediments in their translation and implementation into patient care. Considering the complexity of some guidelines, the time constraints of clinical practice, and the discrepancies between patients' characteristics and entry criteria for clinical trials, one can wonder whether practice guidelines and practicing medicine are compatible [1]. In the past, most of our efforts have gone into the creation of guidelines. It is time to turn our attention to their effective implementation.

## INTRODUCTION

Chart audit assessment of the quality of clinical care of patients with cardiovascular disease has shown significant discrepancies between clinical guidelines and actual clinical practice [2,3]. In heart failure, due to impaired left ventricular systolic performance, a distinguished panel of clinicians from the American Heart Association and the American College of Cardiology have recommended that therapy must include "ACE inhibitors for all patients . . . unless contraindicated" [4]. Furthermore "ACE inhibitors should be continued indefinitely" [4]. In practice, only 50% of patients with heart failure in tertiary and community hospitals are receiving ACE inhibitors [3]. It is unlikely that 50% of patients hospitalized with heart failure could have contraindications to ACE inhibitor therapy. Thus it is a reasonable assumption that a considerable proportion of patients with heart failure, who are candidates for ACE inhibitor therapy are not receiving the recommended therapy. The discrepancy between clinical recommendations and clinical practice is not restricted to heart failure management. Kosecoff et al. [5] found that consensus recommendations

*N.S. Dhalla, P. Zahradka, I. Dixon and R. Beamish (eds.). Angiotensin II Receptor Blockade: Physiological and Clinical Implications.* 

for the management of coronary artery disease from the US National Institutes of Health had no impact on case management in ten Washington State hospitals surveyed. The issue is also a serious concern for management of hypertension [5]. Indeed, only 17% of physicians surveyed in one US State used the US Joint National Committee's (JNC) report on detection, evaluation and treatment of high blood pressure in their practice [5]. These findings are also observed for acute illnesses such as myocardial infarction. Of patients eligible to receive thrombolytic therapy, only 73% received it while only 53% of eligible patients received beta blockers, and only 80% of eligible patients received aspirin [7].

This discrepancy between clinical recommendation and application in practice is not limited to cardiovascular disease. Lomas et al. [8] examined the effect of consensus guidelines for Cesarean section amongst obstetricians. Despite awareness of the guidelines and readiness of the hospital to change, there were no significant changes in the use of Cesarean section in sixteen community hospitals in the years after publication compared to before the publication of these recommendations [8].

The purpose of this chapter is to address why recommendations for practice guidelines by "expert groups" fail to be translated into clinical practice and to provide potential solutions to this ongoing problem. We can begin by examining the problem within the wider context of the determinants of the diffusion of new medical information and technology into practice [9]. A number of factors, each with their own intrinsic variability, are operative. These include variations in the performance characteristics of the physician who uses and interprets the new data; variations in the new technology (guidelines or drugs); biological characteristics of the patient with the disease; unpredictability of contentious responses to medical action (e.g., lawsuits); perplexity about ethical obligations of the clinician to the patient and society; and the clinician's view of the patient's own uncertainty (table 1) [9]. We can reframe these to the issue of clinical practice guidelines that can be considered within three areas specific to the physician, the technology, guidelines, or drugs and the patient (table 2). We begin by examining issues related to the physician.

## PHYSICIANS' AWARENESS OF PRACTICE GUIDELINES

A potential explanation for poor translation of clinical guidelines into practice is the lack of awareness of their existence. One full year after the U.S. 1984 JNC III recommendations were published, only 62% of physicians in the state of Maryland were aware of the report's recommendations [6]. These findings were similar to the Canadian obstetricians' awareness of guidelines for Cesarean Section. Lomas et al. [8] found only 67% of answers correct in responses from a Survey of obstetricians in the Canadian province of Ontario. Considering that the questionaire was multiple in nature and chance alone would have produced a 50% correct response, only a 17% improvement over chance was found. Only 3% of obstetricians were able to correctly identify all four of the recommended actions as well as the four actions that were not recommended.

**Table 1.** Determinants of the diffusion of new medical information and technology into practice

- variation in the performance characteristics of the experts who use and interpret the new technology
- variation in the performance characteristics of technology
- variation of biological response of the patient to disease
- unpredictability of contentious responses to medical action e.g., lawsuits
- perplexity about ethical obligations of clinician to patient and society
- clinician's view of patient's own uncertainty, needs and, expectations

Adapted from Reiser, Int J of Tech Assessment Health Care 2:7–12, 1986 [9].

**Table 2.** Some reasons for the limited implementation of clinical guidelines into clinical practice

*physician*
- lack of physician awareness of the guidelines
- time urgency in medical practice
- low prevalence of the clinical condition in a clinician's practice
- failure of learning new information. Problems of continuing medical education to communicate guidelines in a meaningful manner
- lack of ease of access to the proliferating number of guidelines

*guidelines*
- perceptions of the new guidelines
- ambiguities of practice guidelines or perception of inconsistency with other 'experts' or the literature
- conflicting messages about drugs therapy in recommendations and pharmaceutical advertising

*patient*
- patient attitudes to (new) drugs

There are a number of potential reasons for lack of awareness.

1. Medical practice has a time urgency. There is often insufficient time to read the literature, synthesize it, reason alone, and interact with colleagues to discuss the merit of new approaches (table 3).
2. There is no compensation for literature review or evaluation. Medical practitioners often have limited time to consider whether the entry criteria of the randomized clinical trials of the disease fit the specific patient that the physician is treating.
3. Difficulty of ready access to clinical practice guidelines impedes their implementation. Guidelines for all the various patient problems that a physician may encounter daily may not be readily accessible to the physician. Each specialty group or association publishes their guidelines in specialized journals. Practice guidelines may be in several different sources, not all of which are conveniently available.

Unfortunately, even the awareness of clinical guidelines is not necessarily a guarantee of their implementation. Awareness of JNC II report was not a significant determinant of practice behavior for management of hypertension [6].

**Table 3.** Time urgency and medical practice

| |
|---|
| • insufficient time to read the literature, synthesize the literature, interact with colleagues to reason together the new approaches |
| • no compensation for literature review or evaluation |
| • lack of time to consider whether or not the entry criteria of the RCT's on the disease fit the specific patient |
| • lack of ease of access to the proliferating number of guidelines |

## PERCEPTIONS OF NEW TECHNOLOGIES OR RECOMMENDATIONS

Some clinicians believe that technologies (including medications) that are efficacious are often slow in achieving an impact while technologies of questionable value diffuse rapidly into practice [10]. Similarly, there is a perception that many patients receive less than state-of-the-art medical care while expensive and/or inappropriate care is more prevalent than desired [10]. There is little data to support the contention that effective therapy slowly diffuses into clinical practice while technologies of questionable value diffuse rapidly. However, it is reasonable to suggest that physicians, who tend to be a conservative group in their approach, would be cautious about introducing new drugs into practice. Similarly, once physicians have become familiar with the dose ranges and side effects of certain drugs, they are less likely to stop using them in their practice. The latter is substantiated by the continued use of lidocaine in acute myocardial infarction despite recommendations to the contrary [7].

## PREVALENCE OF CONDITION IN THE CLINICIANS' PRACTICE

Physicians' pattern of practice vary considerably. While one family physician may see only young families, another family physician may see mainly an elderly population. The first physician may rarely see heart failure or cardiovascular disease and may not recall current recommendations.

The ease of diagnosis or degree of recognizability is another problem in prescribing. The diagnosis of heart failure requires a constellation of symptoms and findings on physical examination including elevated jugular venous pressure and an S3 gallop. The use of appropriate therapy is obviously conditional upon correct diagnosis.

Another issue with infrequently seen conditions is that the impact of treatment may not be as apparent. Is the difference in the scientific literature large enough to be clinically convincing? [11]. ACE inhibitors clearly reduce cardiovascular mortality in heart failure. In some studies, the number of patients needed to be treated with an ACE inhibitor to save one life (*NNT* or number needed to treat) is about 100 per year [12]. Some family practitioners may not see one hundred patients with heart failure per year. Yet, they may make as many as 20% of their patients cough when they prescribe an ACE inhibitor [13]. One might anticipate that a cardiologist with a large number of patients with heart failure should prescribe ACE inhibitors in a high proportion of patients to keep them out of the hospital for recurrent heart failure and to decrease the mortality in his patients.

**Table 4.** False assumptions and possible failure of traditional CME

- transmittal of rational information alone, independent of how it is presented, will consistently and predictably change (improve) clinical decisions
- once the message have been communicated (delivered), physicians will remember it forever
- once the message has been communicated (delivered), no one else will deliver a contradictory message
- the message will be remembered when the physician sees all patients with the condition
- based on assumption that transmittal of rational information alone, independent of how it is provided, will predictably improve clinical decisions

## FAILURE OF CONTINUING MEDICAL EDUCATION (CME) TO COMMUNICATE GUIDELINES

A physician's time to keep abreast of ongoing new medical information may be reduced as he practices through the years because of the pressures of a clinical practice as well as the demands of sick patients. Learning occurs through a process of continuing medical education that may be provided by the medical association, hospital, or medical school. Continuing medical education (CME) as a mandated function for continuing licensure is a "stick" rather than a "carrot" approach to medical education. Compulsory CME may not make the physician as receptive to change as CME born out of a physician's desire to improve the quality of his practice.

Traditional CME does not always communicate new medical knowledge in a manner that will ensure change in clinical practice. The problems of traditional CME include the presentation of the material with the belief that transmittal of rational information alone, independent of how it is presented, will consistently and predictably change (improve) clinical decisions (table 4). Furthermore, it is believed that once the message has been communicated, physicians will remember it. It is also believed that once the message has been communicated, no one else will deliver a contradictory message. Another assumption of traditional CME is that the message will be subject to instant recall when the physician sees patients.

## THE NATURE OF THE TECHNOLOGY OR DRUG RECOMMENDED

Some guidelines for management are unnecessarily complicated. The diagnosis, nonpharmacological management, and drug treatment of a condition may consist of 80 different recommendations that are phrased in paragraph form for scientific accuracy. Some guidelines may extrapolate too much from available data. For example, recommendations on management of lipid lowering drugs used goals for treatment that were not necessarily based on randomized trials. The intent was to compare different regimes with different LDL-C endpoints and compare clinical outcomes. They sought to test the hypothesis that lipid lowering drugs improved survival. Target or goal LDL cholesterol levels were extrapolated from the data. Physicians may have been reluctant to accept all the recommendations because a few have not been as rigorously tested. There is similar concern that recommendations on available evidence may not be optimal for all clinical decisions, especially when available evidence may not be of good quality [14].

**Table 5.** Potential reasons ACE inhibitors are not prescribed in heart failure

- concern about serious adverse effects e.g., renal failure, hyperkalemia, angioedema
- patients dislike of side effects: cough taste alterations
- need for monitoring e.g., renal function and potassium
- ACE inhibitors major benefit is prevention (death, recurrent hospitalization, etc.,) and there is always more reluctance to use preventive strategies for asymptomatic patients than treatment of symptoms

ACE inhibitors despite their beneficial effect on morbidity and mortality may produce side effects that are distressing for some physicians. While cough is the most frequent side effect occurring in as many as 20% of patients, 1% of patients risk angioedema [13]. Furthermore, the physician must monitor renal function and serum potassium to ensure the drug does not produce renal failure. These and other side effects are an impediment to the use this drug despite its beneficial effects in clinical trials (table 5).

## CONFLICTING MESSAGES ABOUT RECOMMENDED DRUGS

Conflicting messages and opinions can prevent action and clear decisions in the treatment of cardiovascular disease. A group of clinician experts could propose clinical practice guidelines that another specialist(s) might disagree with. If the disagreeing opinion is widely circulated, family physicians might not proceed quickly in implementing guidelines, which they conclude lack validity.

New drugs tend to receive more publicity in medical journals and in pharmaceutical advertising. This publicity may conflict with recommendations for older and more established drugs. There are several conflicting messages that are specific to ACE inhibitors which have clear advantages in the management of heart failure but do not have any morbidity and mortality data in the treatment of hypertension. The conflicting messages are briefly as follows. "ACE inhibitors will help your patients with congestive heart failure (CHF) because of the wealth of data that shows that they reduce CHF morbidity and mortality. Ang II antagonists will help your patient with CHF. The fact that there are no data that they reduce CHF morbidity and mortality should not concern you." Another conflicting set of messages is the following: "ACE inhibitors should be used to treat CHF because there are data that they reduce CHF morbidity and mortality. ACE inhibitors should be used to treat hypertension. The fact that there are no data to indicate whether or not they alter morbidity and mortality should not concern you."

## PATIENT ATTITUDES TOWARD DRUG TREATMENT

Patient concerns about drug therapy may limit the use of medically appropriate treatment of their condition (table 6). There are many patients that are fearful of

**Table 6.** Patient attitudes that may prevent accepting scientifically recognized drug treatment

- preference for nondrug treatment
- preference for alternate therapy e.g., herbal
- fear of drug treatment
- fear of life-long therapy
- dislike of consuming any synthetic substance
- concerns about the cost of drugs
- inability or limited ability to pay
- concerns about drug safety

traditional pharmaceutical remedies and prefer herbal remedies. Other patients fear the concept of lifelong therapy or are concerned about adverse effects. The high cost of some drugs is yet another consideration or deterrent for patients. While physicians are skilled in discussing with their patients the best options and therapy, there are patients who simply refuse to follow those recommendations.

## POTENTIAL SOLUTIONS FOR IMPLEMENTING CLINICAL PRACTICE GUIDELINES

There are various approaches to addressing each of the identified issues (table 7).

1. The lack of awareness of clinical guidelines may be addressed by providing improved continuing medical education to physicians.
2. Updated guidelines should be readily available either on computers or reference manuals so that physicians can use their time in the most effective manner. Computer programs specific for disease management can be developed and implemented.
3. Improved approaches to continuing medical education may be necessary. Active physician participation in educational interactions helps learning. The use of concise graphical educational material and highlighting and repeating essential messages coupled with the positive reinforcement of improved practices may be considered [15] (table 8).
4. The use of opinion leaders is another strategy. Changing physician prescribing patterns is a difficult undertaking. Schaffner et al. [16] studied the effect of several different strategies on the office practice of antibiotic prescribing and discovered that mailing brochures with recommended practice guidelines had no detectable effect. An office visit by a pharmacist/drug educator to discuss the guidelines had a modest effect. However, an office visit by another physician counsellor had a significant and indeed a marked impact on drug prescribing. Interestingly, physicians responded equally well to the recommendations of the physician counsellor to improve quality of care or to reduce drug cost [16]. Physicians respond to the influence of clinical leaders as well as concern for their patient's well being, patient's demands, perceived social good, and specific patient considerations [17].

**Table 7.** Potential solutions to problems of implementing clinical practice guidelines

- improved access to and availability of up to date clinical guidelines
- development of computer programs that will track patients with certain conditions to ensure implementation of recommended clinical care
- development and implementation of specific strategies to changing physician prescribing patterns
- development of specialized centres for the management of diseases such as heart failure

**Table 8.** Improving continuing medical education

- stimulate active physician participation in educational interactions
- use concise graphical educational material
- highlight and repeat essential messages
- provide positive reinforcement of improved practices in follow-up visit

Source: adapted from Soumerai and Avorn, JAMA 263:549–556, 1996 [15].

**Table 9.** Potential solutions: Academic detailing to improve clinical decision making

- conduct interviews to investigate baseline knowledge and motivation for current prescribing patterns
- focus programs on specific categories of physicians and their opinion leaders
- define clear educational and behavioural objectives
- ensure interaction of practicing physicians with a clinical (opinion) leader
- establish credibility through a respected organization, referencing authoritative and unbiased sources of information and present both sides of controversial issues

Source: adapted from Lomas et al., JAMA 265:2202–2207, 1991 [18].

Opinion leaders can have a significant impact on clinical practice [18]. The use of opinion leaders or academic detailing is an expensive but potentially useful way to implement change (table 9). The inherent danger of this approach is that a single or small group of physicians with a particular view point may take over the process in a local community. Thus, national consensus guidelines with local representation is the best approach.

5. Establishment of specialized centers is necessary for management of selected cardiovascular disease. There may be a need for highly specialized expertise in complex disease. The approach for the care of diabetes mellitus has been to establish specialty centers. One diabetologist has concluded that "a detailed understanding of intensive treatment programs (for diabetes mellitus) may be beyond the skill of the average primary care physician. . . ." "An experienced health care team should be involved with the patient's case. . . ." [19]. Perhaps specialized centers for the management of patients with cardiovascular risk factors in the absence of cardiovascular disease and after the diagnosis of myocardial infarction or heart failure might reduce morbidity and mortality from cardiovascular disease. As this is an expensive approach to medical care, its cost effectiveness would require careful scrutiny and justification.

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# COUGH INDUCED BY PHARMACOLOGICAL MODULATION OF THE RENIN-ANGIOTENSIN-ALDOSTERONE SYSTEM. ANGIOTENSIN CONVERTING ENZYME INHIBITORS AND ANGIOTENSIN II RECEPTOR ANTAGONISTS

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***Summary.*** Dry cough is now recognized as a common side effect of ACE inhibitor therapy. When cough becomes bothersome, no alternative is better than substituting a different class of antihypertensive agents for the offending ACE inhibitors. Losartan potassium represents a new class of antihypertensive agents with a different mechanism of action: it directly blocks the effects of angiotensin (Ang) II at the receptor level, without affecting kininase II-related factors, such as the bradykinin system. As shown in the Losartan Cough Study, the incidence of cough related to a treatment with an $AT_1$ type Ang II receptor antagonist, such as losartan, is significantly lower than that observed with lisinopril and is similar to that of HCTZ in patients with a rechallenged ACE-inhibitor cough. Ang II receptor antagonists represent a potential new treatment for hypertensive patients, which may be especially useful in patients in whom a pharmacological inhibition of RAAS is indicated, but who develop cough with ACE inhibitors.

It is well recognized that the renin-angiotensin aldosterone system (RAAS) plays a critical role in fluid/electrolyte homeostasis and blood pressure regulation. Pharmacological inhibition of the RAAS has been of particular interest in the management of pathophysiological states, such as congestive heart failure and hypertension, particularly since the introduction of angiotensin-converting enzyme (ACE) inhibitors in the early 1980s [1–4]. ACE inhibitors are generally well tolerated with a relatively low adverse-effect profile. However, their use can be limited by an annoying side effect, cough [5–29]. Recent research has focused on the development of new modulators of the RAAS: the angiotensin (Ang) II receptor antagonists. This lattest class of agents directly interfer with RAAS at the level of Ang II

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**Table 1.** Clinical characteristics of ACE inhibitors cough

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|---|
| Symptomatology |
| Dry (nonproductive), irritating, persistent |
| Sensation of "tickling" in the throat |
| May occur more frequently when reclining |
| Intermittent or continuous |
| May change the tone of voice |
| May cause vomiting |
| Onset |
| Generally within 2 months |
| May be triggered by viral respiratory tract infection in 36% of patients |
| Resolution |
| Generally within 1 week after ACE inhibitor discontinuation |
| Cross-reaction between ACE inhibitors |
| Resistant to conventional antitussive agents |
| Demographics |
| Females > Males |

Source: Based on reference 29, with permision.

receptors. They have been expected to be more specific and are less likely to induce cough as an adverse effect.

This review summarizes the available information on ACE inhibitor-related cough with respect to its characterization, frequency, mechanism, and treatment. Special attention has been paid to properly designed clinical trials to document the true incidence of ACE inhibitor-induced cough. Results are also presented from a prospective, randomized, double-blind, parallel-group, controlled trial, designed to compare the incidence and severity of cough associated with an ACE inhibitor, lisinopril; a type 1 ($AT_1$) Ang II receptor antagonist, losartan; and a thiazide diuretic, hydrochlorotiazide, which all modulate the RAAS [28].

## CLINICAL CHARACTERIZATION OF COUGH WITH ACE INHIBITORS

The general characteristics of the ACE inhibitor-related cough are listed in table 1. Description of ACE inhibitor-related cough from published case reports, abstracts, postmarketing surveillance studies, hospital series, and randomized controlled trials are generally consistent [30–50]. The cough is typically characterized as being nonproductive (i.e., dry cough), persistent, and irritating. Its onset is described as a tickling sensation in the back of the throat, occurring in most of the cases within two months of ACE inhibitor therapy. Delayed onsets of up to 15 months have been reported. A viral respiratory infection often contributes to the triggering of symptoms [50]. As it progresses, cough may become intermittent or continuous and exacerbate at night or when patient is reclining. Symptoms can interfer with speech, change the tone of the voice, or even cause vomiting. Usually these symptoms do not resolve with time unless the ACE inhibitor is withdrawn.

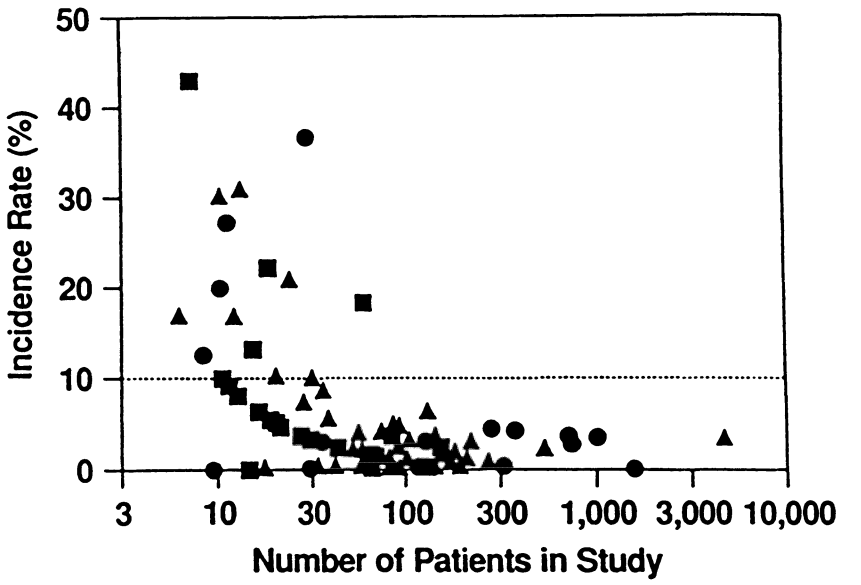


**Figure 1.** Incidence of dry cough with angiotensin-converting enzyme (ACE) inhibitors: ■, captopril; ▲, enalapril; ●, other ACE inhibitors. Based on reference 82, with permission.

## FREQUENCY OF COUGH

Cough is such a common symptom that it is often overlooked as a side effect of medication. In the medical literature, estimates on occurrence of ACE inhibitor-related cough vary extensively, reflecting different methodologies used to ascertain the presence of this side effect. Early postmarketing studies on captopril and enalapril have not been designed to measure the occurrence of cough. Estimates have mostly been based on spontaneous patient reports. Duration of treatment, complete follow-up, total population exposed, or interruption of ACE inhibitor for a reason other than cough have not always been taken into account. Consequently, this has led to an underestimation of the overall incidence of cough and probably better reflects severe cases of cough [51]. On the other hand, a considerably high incidence of cough has been reported in most studies with less than 50 patients [figure 1]. As a result the reported incidence of cough in the world literature ranges from 0% to over 30% [38,51–54].

Better documention regarding the overall occurrence comes from prospective trials specifically designed to assess the relationship between the development of cough and the use of ACE inhibitors (hospital clinic-based series and randomly allocated controlled trials). These studies relied on a more appropriate method of assessing side effects related to medication (double-blind challenge period, control group, patient-assessed standard side-effects questionnaires, life-table analysis) and

provided a more reliable evaluation of the overall incidence of ACE inhibitor-related cough, i.e., between 6 and 16% [31,50–52,55].

## PREDISPOSING FACTORS

Typical characterizations of adverse drug reactions include the identification of predisposing factors such as sex, age, race, type of agent involved, or any pertinent clinical situation. Because of the nature of most reports on ACE inhibitior-related cough, many of these data are not available. It is thus difficult to identify patients treated with ACE inhibitors who are at a higher risk of developing a persistent cough.

Some evidence suggested that the occurrence of ACE inhibitor-induced cough is related to gender. Indeed, the cough has been reported to occur more commonly in women than in men [31,35,50–57] with some studies reporting a threefold increase [58]. However, these data are difficult to interpret because of other contributing factors [55,59]. Moreover, females tend to spontaneously report cough more frequently than males regardless of the antihypertensive agent or placebo treatment [58].

Age, race and, smoking habit do not seem to be relevant prognostic factors. The cough is not likely to result from a pulmonary dysfunction; standard pulmonary tests are generally not affected, and asthmatic patients do not seem to be at increased risk of ACE inhibitor-related cough [31,32].

Cough is not likely to be dose-related. However, symptoms may improve with lowering the dose [35,60]. The cough appears to be nonspecific in terms of which ACE inhibitor is involved, the presence or absence of a sulfhydryl group in the molecule, or the duration of action [31,35,36]. The cross-reaction with any agent of the pharmacological class suggests that the cause of cough is related to the agent's mechanism of action [31,32,35,45,50,60,61]. Although recent reports suggest improvement in the severity and/or the frequency of dry cough after switching to a newer ACE inhibitor [62], no prospective studies are, at present, available to allow definite conclusions.

## MECHANISM OF COUGH

The underlying mechanism(s) by which ACE inhibitors as a class cause cough has yet to be elucidated. Moreover, it is also unclear why only a certain percentage of patients taking ACE inhibitors develop this side effect. Figure 2 summarizes proposed mechanisms that may contribute to the phenomenon.

ACE inhibitors produce their therapeutic effect by blockade of the RAAS, which prevents Ang II formation through a competitive inhibition of ACE. ACE is present in many tissues of the body, particularly in the vascular endothelium of the lungs. Since ACE (kininase II) is not specific to the RAAS, suspicion has fallen on substrates other than Ang I as the mediators of cough. It has been proposed that kinins (such as bradykinins), normally degraded in part by ACE, accumulate in the lung as a result of inhibition of ACE, thus promoting cough and bronchospasm [41].

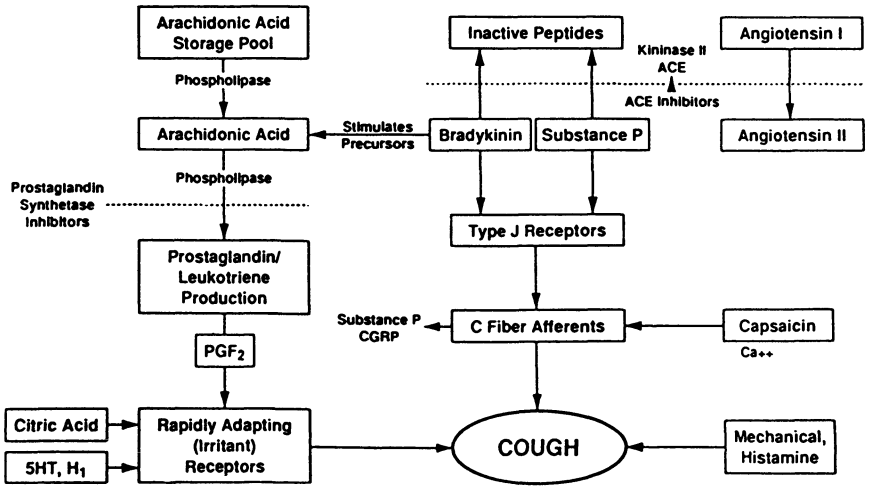


**Figure 2.** Mechanisms that may contribute to cough with angiotensin-converting enzyme (ACE) inhibitors: $PGF_2$, prostaglandin $F_2$; CGRP, calcitonin gene-related peptide; 5HT, 5-hydroxytryptamine; $H_1$, histamine. Based on reference 82, with permission.

This has been hypothesized from the following observations: the large amount of ACE in the lungs; the effects of bradykinin on C-fibers through type J receptors involved in the cough reflex; the bronchoconstrictive effect of bradykinin and the occurrence of cough after intradermal injection of bradykinin [49,63–67]. Accumulation of bradykinin could contribute to bronchial irritability and cough in susceptible persons by directly inducing smooth muscle contraction and local edema. Further, bradykinin could stimulate the formation of prostaglandins and leukotrienes, two bronchial inflammatory agents derived from arachidonic acid. In this respect, concurrent inhibition of prostaglandin formation with nonsteroidal anti-inflammatory drugs appears to attenuate the cough produced by ACE inhibitors [61,68].

It is difficult to accept that accumulation of kinins is the only mechanism of ACE inhibitor-induced cough, since the adverse affect appears to be variable in occurrence, unrelated to the dosage and with a female preponderance [34,35,37–41,69–71]. Several other mechanisms have been postulated as the explanation of ACE inhibitor-related cough [31,72–76]. These pertain to either bronchial hyperresponsiveness, irritative effect of various mediators, or enhanced pulmonary receptors. Many pulmonary tests (capsaisin, histamine, citric acid, methacholine) have been attempted in order to characterize one of the suggested mechanisms [31]. Substance P is another potent bronchoconstrictor which may be considered as a contributing factor to the phenomenon. Its biological effects are prolonged following inhibition of ACE. This could result in local accumulation in the upper respiratory tract, leading to cough through an interaction with type J receptors and

C-fiber afferents in susceptible persons [72,73]. A genetic predisposition has been suggested by Yeo and colleagues [77], since the proportion of the population homozygous for ACE (16%) corresponds to the incidence of cough. However, reports on the potential relation between the polymorphism of the ACE gene and ACE inhibition-cough are conflicting [78,79]. Turgeon and colleagues have suggested that persistent cough is rather an adverse reaction related to a genetic polymorphism of drug metabolism, since coughing patients carry mutant alleles of CYP2D6, a specific cytochrome P450 isoenzyme which catalyzes the oxidation of several cardiovascular drugs [80].

## MANAGEMENT OF ACE INHIBITOR-RELATED COUGH

The cough from ACE inhibitors does not respond well to standard antitussive agents, such as dextromethorphan or codeine. As mentioned, reduction of the dose may reduce the symptoms in some cases but can be limited by therapeutical issues. Trying to inhibit the cough by using NSAIDs is not recommend, since it exposes the patient to additional untoward effects and may represent a drug interaction interfering with the efficacy of the therapy. Thus far, the best alternative is the withdrawal of the ACE inhibitor. Cough generally disappears within 1 to 4 days, although disappearance after as long as 4 weeks has also been reported.

Before ordering extensive evaluation, diagnostic tests, or empiric treatments, clinicians should suspect ACE inhibitors as the cause of the cough although other conditions should be suspected including asthma, bronchitis, smoking, and heart failure.

## ANGIOTENSIN II RECEPTOR ANTAGONISTS AND COUGH

Losartan potassium (MK 954; DuP 753) is the first oral, nonpeptidic Ang II receptor antagonist to be marketed worldwide for the treatment of essential hypertension. It represents a significant addition to the cardiovascular armamentarium, and several other agents of this class are actually undergoing clinical trials [table 2]. By selectively and competitively antagonizing Ang II at its receptor level ($AT_1$), losartan represents a more direct approach than ACE inhibitors in blocking the RAAS. Consequently, through its lack of effect on the bradykinin system [81], it is not expected to be associated with dry cough.

To test the hypothesis that blockade of Ang II receptors does not cause cough to the same extent as cough seen during ACE inhibition, the Losartan Cough Study [28] was conducted in 135 patients with mild to moderate hypertension with a history of ACE inhibitor dry cough. This study was the first prospective, double-blind, controlled study specifically designed to evaluate as the primary endpoint the side effect of "dry cough" in patients treated with three different RAAS-modulating agents: losartan (50 mg once-daily), an $AT_1$ type Ang II receptor antagonist; lisinopril (20 mg once-daily), an ACE inhibitor; and hydrochlorothiazide (HCTZ) (25 mg once-daily), a diuretic. In lieu of placebo, HCTZ was used as a positive control for antihypertensive efficacy as well as a negative control for the develop-

**Table 2.** Angiotensin II ($AT_1$ type) receptor antagonists

| Name | Pharmaceutical industries |
|---|---|
| A-81988 | Abbott |
| Candesartan | Takeda Industries |
| D-8731 | Zeneca |
| GR-138950-C | Glaxo Research |
| Irbesartan | Sanofi/Bristol-Myers-Squibb |
| L-158809 | Merck Frosst |
| Losartan | Dupont-Merck Frosst |
| SC-52458 | GD Searle |
| SKF-108566 | SmithKline Beecham |
| Tasosartan | Wyeth Ayerst Research |
| Telmisartan | Boehringer Ingelheim |
| UP-296-6 | Laboratories UPSA |
| Valsartan | Ciba-Geigy |

ment of dry cough [82]. Assessment for the presence of cough was performed using a self-administered questionnaire throughout three consecutive study periods: a 6-week, single-blind positive challenge period with lisinopril; a 6-week, single-blind dechallenge placebo washout period; and an 8-week, double-blind rechallenge period with either losartan, lisinopril, or HCTZ. The questionnaire was similar to one previously used that was able to discriminate an ACE inhibitor from another drug on the basis of cough [83]. To de-emphasize the importance of cough as the primary endpoint, dry cough was one of nine symptoms assessed. The questionnaire was completed at each visit before the patients saw the investigator.

The results of the double-blind period showed that 72% of patients developed cough within 8 weeks when rechallenged for a third time with an ACE inhibitor compared to 29% and 34% with losartan and HCTZ, respectively [figure 3]. The percentage of patients who coughed on HCTZ were not different from those of patients on losartan. Both were similar to the percentage of "non-coughers" on lisinopril, about 30%. These incidences could be interpreted to indicate the background incidence of cough (or noncough) in a study of this unique design and in highly selected, cough-sensitive population. Similar results were observed in a study of similar design in patients receiving an ACE inhibitor, a renin-inhibitor, or placebo [84]. It is therefore anticipated that the incidence of cough with patients on losartan, will be similar to that of placebo. A relevant observation in the present study is that about 30% of patients who had been challenged and dechallenged twice did not responded by coughing for a third time. Could this challenge dechallenge "in itself" modify the future response to ACE inhibitors and be a sort of challenge tachyphylaxis? Support for such a hypothesis comes from the findings of Reisin et al. [85] that cough attributed to ACE inhibitors disappeared after continued treatment in approximately half of the patients. Perhaps other explanations exist. For instance, ACE inhibitors may promote the continuance of a cough only after a cough stimulus has occurred. This would be consistent with a previous report that

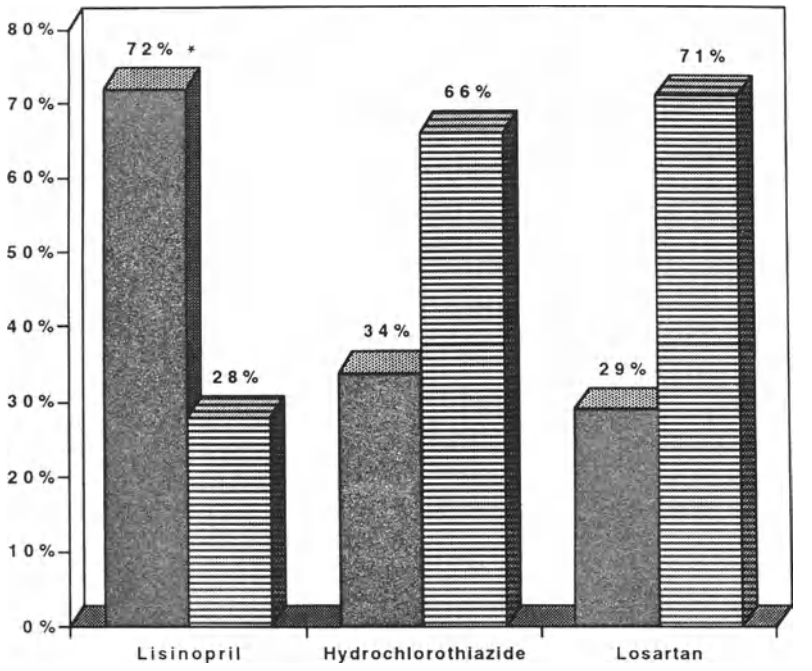


**Figure 3.** Proportions of patients with dry cough by treatment group in the Losartan Cough Study: ▨ "YES" for dry cough; ▤ "NO" for dry cough. *$p < 0.01$, versus other groups.

36% of patients who developed prolonged cough related to ACE inhibitors did so following a common cold [50].

The onset and time to resolution of ACE-inhibitor cough in the study population was approximately 3 and 4 weeks, respectively. However, the study design limited a complete evaluation of onset and resolution of cough; in the general population, some patients may take longer to develop cough and/or may take longer for cough to resolve. This study collected much information regarding "dry cough", including the identification of a plethora of ACE inhibitors that previously caused cough in these patients, which was reproduced with lisinopril treatment. This supports the theory that cough is a class effect of ACE inhibitors.

Of the patients enrolled into the study, 65% were women. This gender distribution is not typical of hypertension studies where usually 30–40% of the study population are women. This observation is consistent with the theory that women develop and/or report cough more commonly than men [31,56,86], regardless of the antihypertensive agent, but to a greater extent with ACE inhibitors. The reason for the apparent higher reporting rate of cough by women has not been elucidated.

Although the specific mechanism by which ACE inhibitors as a class cause cough is not firmly established, increased levels of mediators outside the RAAS cascade are

likely to be the mechanism of cough [87]. These mediators include kinins such as bradykinin, substance P, and two bronchial inflammatory agents derived from arachidonic acid—prostaglandins and leukotrienes [72,88,89]. By showing that losartan—which reduces blood pressure through blockade of the $AT_1$ Ang II receptor—and HCTZ—which reduces blood pressure by altering blood volume—are associated with significantly lower incidences of cough, the data from the Losartan Cough Study support the hypothesis that cough associated with ACE inhibition is not due to blockade of Ang II formation, but rather, is due to inhibition of kininase II related factors. Furthermore, the results of the current study are consistent with two other studies with losartan that suggest that Ang II receptor antagonists are more specific inhibitors of the RAAS and have no notable effects on systems influenced by kininase II, i.e., bradykinin accumulation [90] and alterations in prostaglandin synthesis [91].

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## B. ANGIOTENSIN BLOCKADE AND CARDIOVASCULAR DISEASE

# ANGIOTENSIN II RECEPTORS $AT_1$ AND $AT_2$: NEW MECHANISMS OF SIGNALING AND ANTAGONISTIC EFFECTS OF $AT_1$ AND $AT_2$

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***Summary.*** A series of pharmaceutical successes in the treatment of not only essential hypertension but also vascular hypertrophic and hyperplastic diseases, congestive heart failure, and renal degenerative diseases, with angiotensin-converting enzyme inhibitors and angiotensin (Ang) II receptor antagonists indicates that angiotensin may play a pivotal role in the genesis and maintenance of high blood pressure and resultant stroke, atherosclerosis, and heart and kidney diseases. There is more than one form of Ang II receptors. Using expression cloning, we isolated the $AT_1$ cDNA from bovine adrenocortical cells from the kidney of spontaneously hypertensive rats and $AT_2$ cDNA from rat PC12W cells and we showed that it was not the mas oncogene product. Further, we showed that in rodents, $AT_1$ consists of two subtypes, $AT_{1A}$ and $AT_{1B}$, which share a high degree of sequence homology in their coding regions, although mechanisms of their respective transcriptional control seemed to be different. By computer-assisted modeling and site-directed mutagenesis, we have delineated the docking site of Ang II. $AT_{1a}$ (and $AT_{1b}$) serves most of the commonly recognized actions of Ang II. In addition, this G protein-coupled receptor (GPCR) also activates a tyrosine kinase mechanism that may be an underlying cause of Ang II-mediated hypertrophic and hyperplastic changes of cardiovascular tissues. In the vascular system, the phospholipase C (PLC) activated by Ang II seems to be PLC-$\beta$ rather than PLC-$_{\gamma 1}$.

Interestingly, we found that Gq-activated PLC-$\beta$ activates p21 ras and mitogen-activated protein kinase (MAPK) in rat vascular smooth muscle cells. The mechanism of the cross-talk between $AT_1$ and the tyrosine kinase system is triggered by $Ca^{2+}$, but does not involve protein kinase C.

Studies using targeted gene deletion indicated that Ang II is intimately involved in nephrogenesis. Mice lacking angiotensinogen showed an abnormality in the formation of renal papilla, retardation in glomerular maturation, marked hypertrophy of small arteries of

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the kidney, and tubular dilatation, whereas targeted deletion of the $AT_1$ receptor resulted in small arterial wall hypertrophy. Blood pressure of $AT_{1A}$-deleted mice was markedly reduced (−45 mmHg).

The role and mechanism of action of $AT_2$ was not clear. We have recently produced $AT_2$ gene null mice and $AT_{1a}$ knockout mice by targeted gene deletion. $AT_2$-deleted mice had a higher blood pressure, whereas $AT_1$-deleted mice showed lower blood pressure. Deletion of the $AT_2$ gene also showed reduced exploratory activity. The most conspicuous action of the $AT_2$ receptor is seen in its salt-retaining action in the renal tubule. Under a constant renal blood flow condition an $AT_2$ antagonist markedly increased the urine volume and concomitant natriuresis. These effects are completely abolished in $AT_2$ deleted mice. The molecular and cell biological studies of the angiotensin receptors are needed.

Despite the complexity and often mutually antagonistic actions of $AT_1$ and $AT_2$, Ang II, working through $AT_1$ and $AT_2$ of the kidney work in the same direction to retain salt and water. These observations, as well as the effects of Ang II, indicate that the most fundamental role of Ang II is its role in the development of the salt-retaining organ, the kidney, and Ang II is uniquely related to the kidney in that both $AT_1$ and $AT_2$ receptors work for the retention of salt.

The presence of two different angiotensin (Ang) II receptors had been suggested because of the differential stability to dithiothreitol; the isoform-specific nonpeptidic (losartan, PD123177, PD123319) and peptide (CGP42112) antagonists demonstrated two clearly different isoforms of Ang II receptors, which were termed Ang II type 1 ($AT_1$) and type 2 ($AT_2$) receptors (see reviews for [1,2], figure 1).

$AT_1$ was further shown to consist of subtypes designated as $AT_{1A}$ and $AT_{1B}$ [3–6]. However, these $AT_1$ subtypes are limited to rodents (rat and mouse). Higher mammals have only one subtype, $AT_1$. Further studies showed practically all of the actions traditionally ascribed to Ang II can be explained by $AT_1$. These actions include contraction of the smooth muscle, inotropic effect on cardiac myocytes, stimulation of aldosterone release from the adrenal cortex, facilitation of catecholamine release from nerve endings, hypertensive action of centrally administered Ang II, hypertrophic actions on vascular smooth muscle cells (VSMC), mitogenic effects on some fibroblast cells, and activation of tyrosine-kinase pathway and mitogen-activated protein kinase (MAPK) and renal tubular effect in sodium reabsorption [1,2]. Thus, few physiological functions seemed to be left for the $AT_2$ receptor to perform. Its abundant expression in fetal mesenchymal renal, and brain tissues seemed to suggest its role in fetal development and organ morphogenesis, but $AT_1$ is equally abundant in various fetal tissues. $AT_2$ expression declines rapidly after birth, and only limited types of adult tissues express $AT_2$ mRNA at a level detectable by in situ hybridization, northern blot analysis, or RNase protection assay. These are rat adrenal medulla, cortex, kidney, heart, uterine myometrium and vasculature, ovarian granulosa cells, pancreas, and certain brain nuclei such as locus ceruleus, inferior olive, few thalamic nuclei and cerebellum [7,8]. There are cell lines expressing $AT_2$ exclusively like mouse R3T3 fibroblast cells [9], PC12W is a subline of pheochromocytoma cells [10], preadipocyte mouse 3T3-L 1 cells [11], mouse fetal fibroblast cells, and neuroblastoma-glioma hybrid cells, NG108-15 [12]. These


**Figure 1.** Comparison of amino acid sequences of rat angiotensin II type 1 ($AT_1$) and type 2 ($AT_2$) receptors. They show a 32% amino acid sequence identity. (Compiled from data in Ref. 49 and 50, reproduced with permission).

cells do not express the $AT_1$ receptor. However, other cell lines express both $AT_1$ and $AT_2$, such as neuroblastoma cells, N1E 115 [13], and the transformed rat pancreatic acinar cell line, AR42J [14]. On the other hand, cultured vascular smooth muscle cells express $AT_1$, but not $AT_2$ [15].

For the action of $AT_1$, current studies that we are pursuing on the cross-talk between the $AT_1$ receptor and MAPK activation, which involves protein tyrosine kinase mechanisms, a pathway usually found in growth hormone-stimulated signaling pathway, will be discussed.

For $AT_2$, we obtained $AT_2$ and $AT_1$ gene null mice and will mainly discuss the roles of $AT_2$ that have remained elusive.

The activation of the tyrosine kinase system by 7-transmembrane domain receptors (G protein-coupled receptors, GPCR) have been noted; its mechanisms have not been clear. Since the tyrosine kinase activation by Gi-coupled receptors seems to be different from that of Gq-coupled receptors [16], we have focused our studies on Gq-coupled angiotensin type 1 receptor $AT_1$ in vascular smooth muscle cells, which in the absence of growth factors such as serum, platelet-derived growth factors (PDGF-BB), or basic fibroblast growth factor (bFGF) do not undergo proliferation. However, Ang II induces hypertrophic changes, but not mitogenic changes (cell proliferation), even if it stimulates MAPK activation.

The $AT_1$ is coupled to the heterotrimeric G protein, Gq, to activate phospholipase C$\beta$, which generates inositol trisphosphate ($IP_3$) in initiating a calcium-

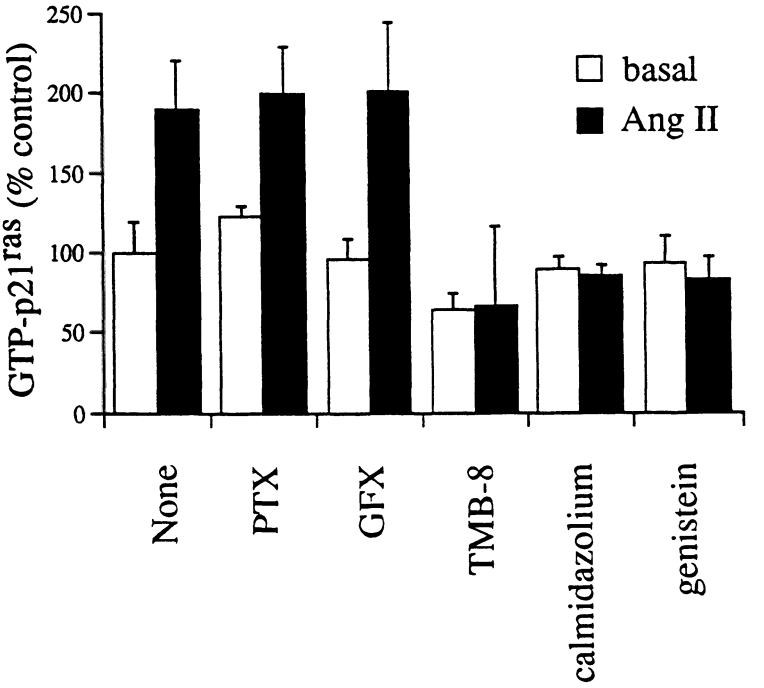


**Figure 2.** Activation of mitogen-activated protein kinase (MAPK) in quiescent rat vascular smooth muscle cells in culture as represented by the activation of $p21^{ras}$. Note that $Ca^{2+}$ alone can activate MAPK as shown by cytosolic $Ca^{2+}$ chelator. TMB-8 blocks the activation completely, but the inhibition of protein kinase C by GFX or inhibition of Gi protein by pertussis toxin TPX had no effect. The calmodulin antagonist calmimidazolium and tyrosine kinase inhibitor genistein also inhibit the pathway of $AT_1$-ras-MAPK-kinase.

mediated signaling pathway. On the other hand, growth factor receptors such as PDGF-R or epidermis growth factor (EGF)-R do not use the heterotrimeric G protein. Instead, they activate PLCγ1 directly by phosphorylation of a tyrosyl residue.

Morrero et al. reported that in their rat aortic VSMC, Ang II via $AT_1$ activates PLCγ1 instead of a PLC-β [17]. Further, they reported results indicating that this cascade which leads to MAPK is initiated or needs to be mediated by a low molecular weight cytosolic tyrosine kinase, Src60 [18,19]. The mechanism involved in the activation of Src60 was not clear. The hypothesis that the activation involves direct association of the protein tyrosine kinase Src60 with $AT_1$ did not prove correct [19].

By using rat VSMC with abundant $PLC\beta_1$ and $\beta_3$, we showed that the increase in cytosolic Ca is sufficient for the activation of the RAS (figure 2), Raf-1, and MAPK system, as well as its upstream component Grb2 and Sos. Increase in cytosolic $Ca^{2+}$ by a PLC-β or $Ca^{2+}$ ionophore elicited MAPK activation [20].

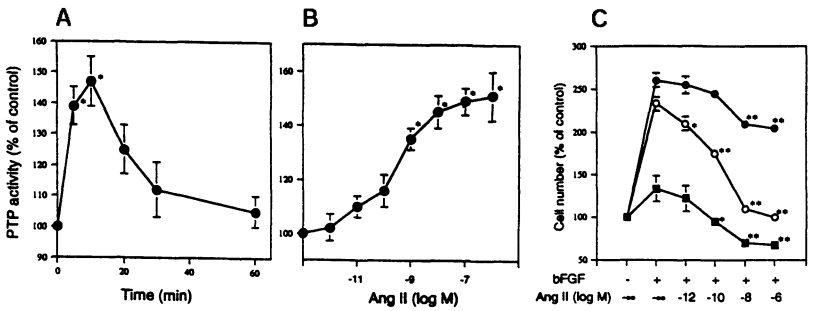


**Figure 3.** Transient activation of phosphotyrosine phosphatase and suppression (*A*, *B*) and basic fibroblast growth-factor stimulated cell proliferation (*C*) at various bFGF concentration. (*A*) Time dependency of the stimulation of phosphotyrosine phosphatase by $10^{-7}$M Ang II; (*B*) concentration dependency; and (*C*) increase in cell number, stimulated by a ■, 0.25; ○, 02.5; ●, 25.0ng/ml basic fibroblast growth factor, was inhibited by $10^{-7}$M Ang II.

Interestingly, it did not involve protein kinase C (PKC). Activation of PLCγ was not needed. Thus, the system became more amenable to explanation using the existing signaling system except for the link connecting elevated cytosolic $Ca^{2+}$ to a tyrosine kinase system. Ang II-stimulated (via $AT_1$) recruitment of Grb2-Sos or Shc-Grb2-Sos suggests that the presence of some tyrosine kinase mediates a Ca-calmodulin-dependent tyrosine kinase system. Thus, in addition to the well known $Ca^{2+}$-mediated contractile response, the $AT_1$-induced cytosolic $Ca^{2+}$ elevation seems to be intimately involved in the tyrosine kinase activation and MAP kinase activation, via cross-talk between GPCR and tyrosine kinase. How $Ca^{2+}$ activates a tyrosine kinase cascade is the subject of intensive investigation. It seems to be distinct from the Gi-coupled GPCR, which seems to involve Gβ-Gγ subunits of Gi proteins along with phosphatidyl inositol 3-kinase (PI3K) [21].

An important aspect of $AT_2$ function also seems to be involved in the signaling of the phosphotyrosine cascade or its turn off. Bottari and his associates showed that $AT_2$ in the pheochromocytoma cell line PC12W activates phosphotyrosine phosphatase (PTP) [22]. This cell line expresses only $AT_2$, not $AT_1$. We [23] and Mukoyama et al. [24] cloned $AT_2$ cDNA from rat cells and tissues and examined the PTP activity in PC12W cells and COS-7 cells expressing the cloned $AT_2$. Cell membranes were rigorously freed from plasma component by ultracentrifugation at 100,000 × g. In this system, $AT_2$ inhibited a PTP, presumably bound tightly by a transmembrane domain [23]. On the other hand, when a membrane-associated fraction in the postnuclear fraction was rapidly isolated from mouse R3T3 cells (which express only $AT_2$, not $AT_1$), it showed an activation of PTP [24] (figure 3a & b). The PTP activity was detectable both by using nitrophenylphosphate (pNPP) or the peptide substrate Raytide containing a phosphotyrosine residue. Further, this activity was inhibited by the PTP specific inhibitors Na-orthovanadate [25]. These

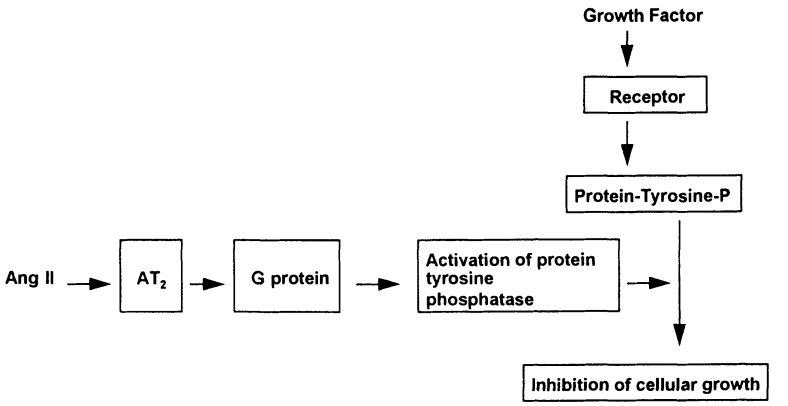


**Figure 4.** Illustration of $AT_1$-promoted mitogenesis and $AT_2$-promoted suppression of cell growth.

results suggest that cytosolic PTP, which is not a part of a receptor, is activated by $AT_2$. Important to note is that the growth stimulation of the fibroblast cells R3T3 by bFGF was suppressed by Ang II in a dose-dependent manner (figure 3c). The dose dependence ($IC_{50} \sim 0.3$ nM) coincided well with the dose dependence (0.5 nM) of PTP activation. These results indicate that $AT_2$ mediates activation of PTP, which affects the growth of R3T3 cells [24,25].

In cells expressing $AT_1$, Ang II activates MAPK, which leads to mitogenic or hypertrophied response through the activation of a tyrosine kinase system [26–28]. On the other hand, $AT_2$ activates PTP and inhibits cell growth, thus undoing the proliferative effect of $AT_1$, as shown in figure 4. Nakajima et al. showed a similar mechanism in vascular smooth muscles (VSMC) expressing the $AT_2$ receptor [29]. Since VSMC in serum or growth factor-containing medium do not express $AT_2$, cells were transfected with a chimeric $AT_2$ gene fused to the 5′-flanking region of smooth muscle myosin. The rat carotid artery expressing transfected $AT_2$ showed a marked reduction in the neointima formation following balloon catheterization. Cultured VSMC transfected with the same $AT_2$ expression vector showed a marked reduction in MAPK activity. These results indicated that $AT_2$ may reduce MAPK activity, which, in turn, will reduce the neointimal smooth muscle cell growth [29]. In these studies, $AT_2$ was stimulated only by endogenous Ang II. Janiak et al. reported the selective activation of $AT_2$ as an effective approach in the suppression of neointima formation following the balloon catheterization [30]. However, the expression of $AT_2$ in the neointimal tissues in rat carotid artery or aorta is minimal [31]. Thus, transfection of $AT_2$ may be required for a therapeutic use of $AT_2$.

Prolonged serum depletion elicits programmed cell death of R3T3 as evidenced by internucleosomal DNA degradation. This process of apoptosis was enhanced by

a 48 hr treatment with Ang II [32]. PC12W cells also undergo apoptosis upon depletion of their specific growth factor, nerve growth factor (NGF). This process is also accelerated by Ang II. Both R3T3 and PC12W cells express $AT_2$, but not $AT_1$. Thus, signals from $AT_2$ may enhance the apoptotic process initiated by the withdrawal of the growth factors [32].

The mechanism of the action of $AT_2$ that participates in this process seems to be due to the reduced activity of MAPK, which seems to be elicited by the activation of MAPKinase phosphatine 1 (MKP-1), which is a protein tyrosine phosphatase that inactivates MAPK by dephosphorylating the tyrosine phosphate group in MAPK. The inactivation of MAPK and accompanying enhancement of DNA fragmentation by Ang II in these $AT_2$-bearing cells are reversed by pertussis toxin (PTX) and orthovanadate. Yamada et al. [32] provided strong evidence that $AT_2$ signaling involves Gi or Go and activation of PTP. Recently Hayashida et al. [33] showed that a synthetic peptide with the 22 residue sequence of the third cytosolic loop of rat $AT_2$ suppresses MAPK activity of the VSMC upon transfer into VSMC by lipofectamine-liposome and this inhibition are reversed by PTX or orthovanadate. These results indicate that the third cytosolic loop can play a part in the activation of a Gi-mediated PTP, which inhibits MAPK. Thus, these receptor studies show that the action of $AT_2$ is to inactivate MAPK, and MAPK activity can be used as a sensitive index of $AT_2$ action, rather than directly determining PPT activity, which have to cope with a high background contributed by several PTPs. These approaches seem to provide a positive answer to the longstanding question as to whether a G protein is involved in the $AT_2$ action.

The demonstration of the direct binding of $AT_2$ to immunoprecipitated Gi$\alpha$-2 and Gi$\alpha$-3, by Zhang and Pratt [34], provide a direct and general basis for answering this question.

## TARGETED GENE DELETION OF THE $AT_2$ GENE

To determine the overall physiological role of $AT_2$, Ichiki et al. [35] and Hein et al. [36] eliminated the gene encoding $AT_2$ by targeted deletion in mice. The resultant $AT_2$-null mice showed elevated pressor sensitivity to an intravenous infusion of Ang II and elevated basal diastolic and systolic blood pressure by about 25 mmHg compared with the $AT_2$-intact $F_2$ hybrid mice (129 Ola X C57BL/6). The elevation in mean arterial basal blood pressure was not seen by Hein et al., possibly because of various technical problems, including heterogeneity in genetic background. (In the gene-deleted mice, the genome of embryonic stem cells from the 129 Ola strain is mixed with the genome of C57BL/6 mice.) It is interesting to note, however, the opposite effects of the action of $AT_1$ and $AT_2$ on blood pressure regulation. Targeted deletion of the gene encoding $AT_1$ resulted in blood pressure lowering of about 45 mmHg [37,38], whereas deletion of the gene encoding $AT_2$ led to an elevation of about 25 mmHg [35]. Angiotensinogen gene deletion resulted in a decrease in blood pressure of about 20–25 mmHg [39,40], which accounts for the balance of actions of $AT_1$ and $AT_2$ as the effect of Ang II is completely removed.

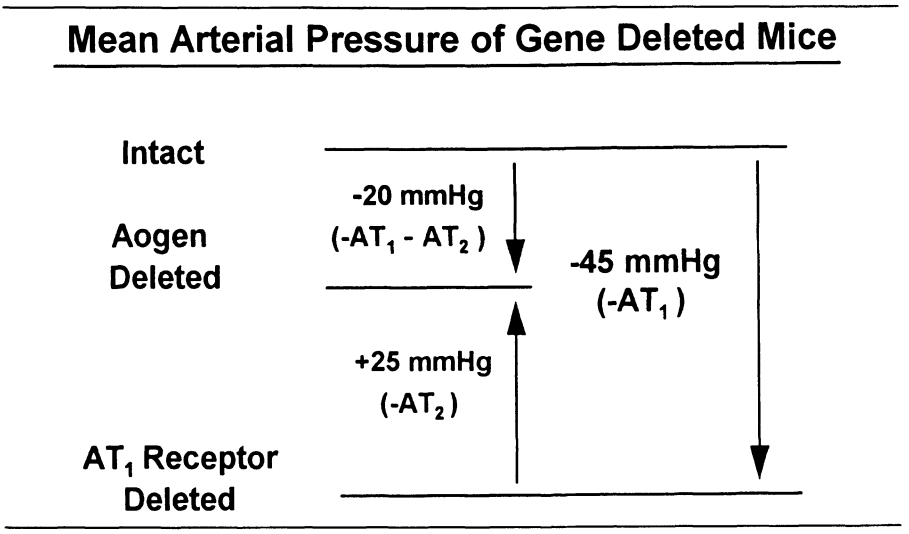


**Figure 5.** Positive contribution of $AT_1$ and negative contribution of $AT_2$ on the blood pressure as examined with $AT_{1a}$ and $AT_2$ gene knockout mice. The angiotensinogen gene knockout mice represent the overall effect of the total loss of Ang II. (Compiled from data in Refs. 35,37,38,39,40.)

Given the general observation arising from many targeted gene deletion experiments that receptor subtypes seldom undergo compensatory changes, the simple arithmetics of figure 5 shows that $AT_1$ ($AT_{1a}$) is a dominant pressor receptor, whereas $AT_2$ appears to be a depressor receptor that operates by an, as yet, unknown mechanism.

$AT_2$ is expressed in the kidney, and earlier studies suggest that $AT_2$ antagonist have diuretic effects [41]. Lo et al. isolated tubular function from hemodynamic function by maintaining a constant renal blood flow using the method of Roman et al. [42] and observed that intravenous infusion of the $AT_2$ blocker PD123319 markedly and rapidly increased diuresis and natriuresis from the rat kidney [43]. Conversely, the $AT_2$ agonist CGP42112A suppressed diuresis and natriuresis, indicating the very interesting function of renal tubular $AT_2$ in sodium retention. We were able to confirm this observation in rats and mice by using $AT_2$ antagonists and, further, by using $AT_2$-gene deleted mice to ascertain that the site of antagonist binding is indeed the $AT_2$ receptor since the $AT_2$ knockout mice did not show a diuretic response to PD123319. In recent studies, Siragy and Carey [44], however, did not observe a similar natriuretic effect of PD123319 in conscious rats. Only the $AT_1$ blocker losartan showed natriuresis. Furthermore, they reported increased cGMP levels in the renal interstitial fluid as renal $AT_2$ was stimulated, a result which is opposite to that reported earlier when Ang II-stimulated $AT_2$ was shown to lower cGMP levels [22,45]. These results are intriguing because the existing observations suggest that $AT_2$ attenuates the pressor and mitogenic response of $AT_1$ to Ang II, whereas both $AT_1$ and $AT_2$ work in concert to retain salt, albeit via different

mechanisms. These results may suggest a primary and intimate role of Ang II in the retention of salt.

Another area where $AT_2$ expression is clearly seen is in the central nervous system, which includes brain stem (locus ceruleus, inferior olive), several thalamic nuclei, lateral septum, and amygdala (central amygdaloid nucleus and medial amygdaloid nucleus) [46,47]. $AT_2$-null mice generated by targeted gene deletion show markedly lowered exploratory ambulation in a new environment and markedly increased avoidance of light area, indicating emotional instability or fearfulness [35]. Such behavioral changes may reflect action of amygdala and locus ceruleus, which sends off long projections to the cerebral cortex. It does not seem to reflect counteraction to the well-known central action of $AT_1$ in the control of blood pressure, water drinking, and vasopressin release through $AT_1$ in circumventricular organs and hypothalamic nuclei.

## OVERVIEW

It is clear that there are two main categories of the Ang II receptor, $AT_1$ and $AT_2$. In recent years, there has been increased attention focused on the $AT_2$ receptor in order to determine its structure, signaling mechanism, and function. Whereas much remains to be determined, it appears that the actions of Ang II via the $AT_2$ receptor are generally suppressive in nature, whereas function via the $AT_1$ receptor are more commonly stimulatory. In so far as biological activity is concerned, there is now evidence that Ang II via the $AT_2$ receptor can suppress cellular proliferation. Gene deletion studies in animals suggest that the $AT_2$ receptor may subserve a vasodepressor function, although its mechanism remains to be determined. $AT_2$ agonist and antagonist studies indicate that this receptor might subserve antidiuretic and antinatriuretic actions, at least in animals. Animals lacking $AT_2$ receptors have impaired drinking responses and behavioral alterations [46,47].

Details of the biological importance of the $AT_2$ receptor remain unclear, and further information is awaited. This matter is clearly potentially relevant to the therapeutics of cardiovascular disorders, especially heart failure and hypertension but also coronary heart disease. For example, recent studies by Levy et al. [48] indicate that aortic fibrosis elicited by chronic infusion of Ang II is prevented by the $AT_2$ antagonist PD123319 rather than the $AT_1$ blocker losartan. This finding may imply another hitherto unknown role of $AT_2$ in the cardiovascular tissue.

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# ANGIOTENSIN RECEPTOR ANTAGONISTS AND CARDIOVASCULAR REMODELING

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***Summary.*** Angiotensin (Ang) II is one of the key hormones involved in cardiovascular homeostasis. It has potent vasoconstrictor effects and is directly involved in the vascular and cardiac remodeling observed in response to chronic or acute hypertension. Two major Ang II receptor subtypes, $AT_1$ and $AT_2$, have been described, and their roles in vivo and in vitro have now been investigated using nonpeptidic antagonists, losartan and PD123319, which block $AT_1$ and $AT_2$ receptors respectively. The aim of this review is to focus on in vivo data to better define the respective functions of the $AT_1$ and $AT_2$ receptor subtypes. In adult rats, the $AT_1$ receptor subtype does not trigger trophic effects directly in cardiomyocytes, whereas the $AT_2$ receptor may have a major trophic role in smooth muscle cells.

## INTRODUCTION

Angiotensin (Ang) II is one of the key hormones involved in the regulation of cardiovascular homeostasis. It has potent vasoconstrictor effects and is directly involved in the vascular [1] and cardiac [2] remodeling observed in response to chronic or acute hypertension.

Two major Ang II receptor subtypes, $AT_1$ and $AT_2$, have been described [3–5]. $AT_1$ receptor appears primarily responsible for many of the physiological actions of Ang II, but the functional properties of the $AT_2$ subtype, only recently cloned, remain elusive and controversial [review in 3]. Both $AT_1$ and $AT_2$ receptors contain a seven-transmembrane domain but only the former is coupled to G protein. The properties of the two Ang II receptor subtypes have been characterized using two specific antagonists, the benzylimidazole derivative losartan ($AT_1$-specific) and PD123319 ($AT_2$-specific) [4,5]. The $AT_1$ receptor is predominent in adult mamma-

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lian tissue, including vessels [6–9]. However, the percentage of $AT_1$ and $AT_2$ receptors varies according to the cell type or tissues considered. For example, $AT_1$ receptors in the rat aorta are 60% of the receptors, whereas $AT_1$ receptors in the rat heart are 90% of the angiotensin receptors [10]. At the cellular level, adult cardiomyocytes express $AT_1$ subtype exclusively [11], whereas fibroblasts express both subtypes [12]. In pathophysiological situations, such as cardiac hypertrophy secondary to stenosis of the ascending rat aorta, $AT_2$ receptor expression increases to around 60%, whereas the $AT_1$ receptor is downregulated [7]. However, Wolf et al. [8] observed no alteration in left ventricular $AT_1$ and $AT_2$ receptor mRNA levels in response to aortic coarctation. Conversely, the development of cardiac hypertrophy after myocardial infarction was associated with upregulation of the $AT_1$ receptor [11]. Haywood et al. have recently shown that human myocardial levels of $AT_1$ receptor mRNA decreased in heart failure whereas the level of $AT_2$ mRNA remained unchanged [13]. In addition, the ratio of $AT_1$ to $AT_2$ receptors is reversed in experimentally induced vascular injury [14] or wound healing [15]. Based on in vivo and in vitro experiments, the $AT_1$ receptor is considered the major mediator of Ang II-induced cardiovascular effects [5], whereas $AT_2$ receptor activation is involved in the control of cell differentiation, proliferation, and apoptosis [14–18]. In vitro, $AT_1$ receptors mediate hypertrophy of both neonatal cardiomyocytes and smooth muscle cells [review in 3,19], whereas inhibition of endothelial cell replication seems to be $AT_2$ receptor-dependent [17]. However, the respective roles of each receptor in vivo remains uncertain. Therefore, the present review will focus on in vivo recent data concerning the cardiovascular effects of $AT_1$ and/or $AT_2$ receptor blockade with the aim of providing new insights into the function of each receptor.

## CARDIAC HYPERTROPHY

Cardiac hypertrophy in response to hypertension reflects two main processes: the hyperplasia of nonmuscle cells, such as fibroblasts, and the hypertrophy of cardiomyocytes [20].

As cited above, nonmuscle cell hyperplasia correlates with the development of fibrosis during the myocardial hypertrophy process. The beneficial effects of ACE inhibitors on the prevention or regression of fibrosis has been clearly demonstrated [review in 21]. Nonmuscle cells express both $AT_1$ and $AT_2$ receptors. In vitro $AT_1$ receptors favor collagen synthesis, whereas $AT_2$ receptor activation inhibits collagenase activity [12,22]. In R3T3 cells, Tsuzuki et al. have shown that activation of the $AT_2$ receptor subtype inhibits cell proliferation and activates tyrosine phosphatase [23]. More recently, Chassagne et al. observed that $AT_2$ stimulation inhibits replication of rat fibroblast transfected with $AT_2$ receptor human sequence. This process is associated with both inhibition of expression of the protooncogenes c-fos and c-jun and induced expression of mitogen-activated protein kinase (MAPK) phosphatase 1 [24].

Cardiomyocyte enlargement is a cardiac adaptative response to increased mechanical overload. The mechanisms triggering the development of hypertrophy are multifactorial and likely include mechanical, hormonal, and humoral factors [25].

Over the last decade, Ang II has been directly implicated in the development of cardiomyocyte hypertrophy based on (1) data obtained in cultured neonatal ventricular cardiomyocytes [19] and the isolated beating rat heart [26] and (2) the beneficial in vivo effects of ACE inhibitors [21,33]. The in vitro trophic effects of Ang II were blocked by losartan, indicating that these effects were mediated via the $AT_1$ receptor (review in 19). However, Booz and Baker, who found that $AT_1$ and $AT_2$ receptor subtypes were present in equal proportions in neonatal cultured cardiomyocytes, observed that $AT_2$ blockade (PD123177) or (CGP42112) enhanced Ang II stimulation of protein synthesis [27]. It should be noted that neonatal rat myocytes maintain some characteristics of an immature phenotype in culture [28], whereas adult rat cardiomyocytes in short-term culture (<4 days) maintain properties very close to those observed in adult rodents in vivo [29,30].

In order to determine whether Ang II has the same effect on adult cardiomyocytes as on neonatal cardiomyocytes, we investigated the direct effect of Ang II on the rate of protein synthesis in adult cardiomyocytes. We found that Ang II (concentrations up to 10-6 M for 3 days) had no significant effect on the rate of total protein synthesis evaluated by 14C Phe incorporation [55]. This result contrasts with the effects of Ang II observed in neonatal cardiomyocytes and suggests that (1) Ang II has a very minor trophic effect per se on differenciated adult cardiomyocytes and (2) the in vivo effect of the peptide is triggered via more complex pathways, including cellular cross-talk and/or other paracrine systems.

Recently, we and others have investigated the differential roles of angiotensin receptor subtypes in various in vivo rat models of cardiac hypertrophy and/or failure. We used a model of chronic Ang II infusion (120 ng/kg/min for 23 days) that induces arterial hypertension and left ventricular hypertrophy [31,32]. When angiotensin receptor antagonists were coadministered with Ang II, cardiac hypertrophy was prevented with losartan (10 mg/kg/day), a specific $AT_1$ antagonist of $AT_1$, but not PD 123319 (30 mg/kg/day), an $AT_2$ antagonist. It should be noted that losartan normalized systolic pressure at the concentration used, whereas PD 123319 had no hemodynamic effect. In addition, normotensive rats chronically treated with PD123319 (30 mg/kg/day) developed mild but significant cardiac hypertrophy which was prevented by coadministration of losartan [31]. These data suggest that (1) $AT_2$ receptor activation is not involved in the development of cardiac hypertrophy and (2) $AT_1$ receptor activation and/or other mechanically-induced pathways trigger the process of myocyte enlargement. Indeed, in this study we were unable to distinguish between effects mediated specifically via $AT_1$ receptor and those dependent on mechanical factors, since $AT_1$ blockade normalized systolic pressure.

In another rat model of left ventricular hypertrophy induced by persistent systolic pressure overload secondary to ascending aortic stenosis, there is a clear upregulation of the cardiac renin-angiotensin system and a change in angiotensin receptor subtype expression [7]. Beneficial effects of long-term ACE inhibition on hypertrophic remodeling in this model has been attributed to inhibition of cardiac ACE activity [33]. Interestingly, Weinberg et al. [34] proposed that the $AT_1$ receptor is not the

major transduction pathway for myocardial hypertrophy in response to the sudden pressure overload in this model since a long-term receptor $AT_1$ blockade was not associated with regression of left ventricular hypertrophy. In parallel, Liu et al. [35] analysed the respective roles of the kinin B2 receptor and angiotensin receptor subtypes in triggering the functional and tissular alterations during cardiac failure after myocardial infarction in the rat. They proposed that some of the beneficial effects of $AT_1$ blockade were triggered by $AT_2$ receptor activation and also mediated in part by kinins [35]. This later work illustrates that $AT_1$ blockade in vivo probably interferes with autocrine/paracrine regulation, involving the cardiac renin-angiotensin and kallicrein-kinin systems. Therefore, several lines of evidence now suggest that the $AT_1$ receptor does not directly trigger the enlargement of adult mammalian myocytes during pathological conditions.

## ARTERIAL MEDIA THICKENING

Arterial media thickening reflects the adaptation of arterial smooth muscle cells to increased systolic pressure [36]. This thickening depends mainly on the systolic pressure level [36,37], although humoral factors such as Ang II may also be involved [38].

In vitro, Ang II triggers vascular smooth muscle cell (VSMC) hypertrophy [39–41]. This effect is mediated via the $AT_1$ receptor, the only angiotensin receptor expressed in these cells in culture (review in 3). Furthermore, Bardy et al. demonstrated that mechanical factors stimulate local synthesis of Ang II in rabbit aorta, which in turn induces an increase in synthesis of proteins, such as fibronectin, via the $AT_1$ receptor [42,43]. It should be noted that rabbit aorta expresses the $AT_1$ receptor only, whereas both receptor subtypes are expressed in rat arteries [5].

Therefore, we investigated the role of $AT_1$ and $AT_2$ receptors in the production of vascular wall hypertrophy in the rat. Vascular wall hypertrophy was characterized by morphometric analysis combined with either immunohistochemistry or conventional histological staining. Conventional morphometry [32] and image analysis after smooth muscle α-actin (SMα-actin) immunolabeling [31] allowed the evaluation of media thickness in the aorta and coronary arteries respectively. Prolonged Ang II infusion (23 days) induced significant thickening of the media in both aorta and coronary arteries, independent of luminal diameter (figure 1). Treatment of Ang II-induced hypertensive rats with either losartan or PD123319 induced a decrease in media thickness, although PD123319 treatment was more effective [31,32].

It is noteworthy that the effects of $AT_2$ blockade were independent of changes in systolic pressure, whereas those of $AT_1$ blockade were indistinguishable from pressor effects. On the other hand, increased medial thickness of aorta and coronary artery was observed in response to chronic $AT_1$ blockade in normotensive rats. This process was inhibited by concomitant blockade of $AT_2$ receptors (figure 1).

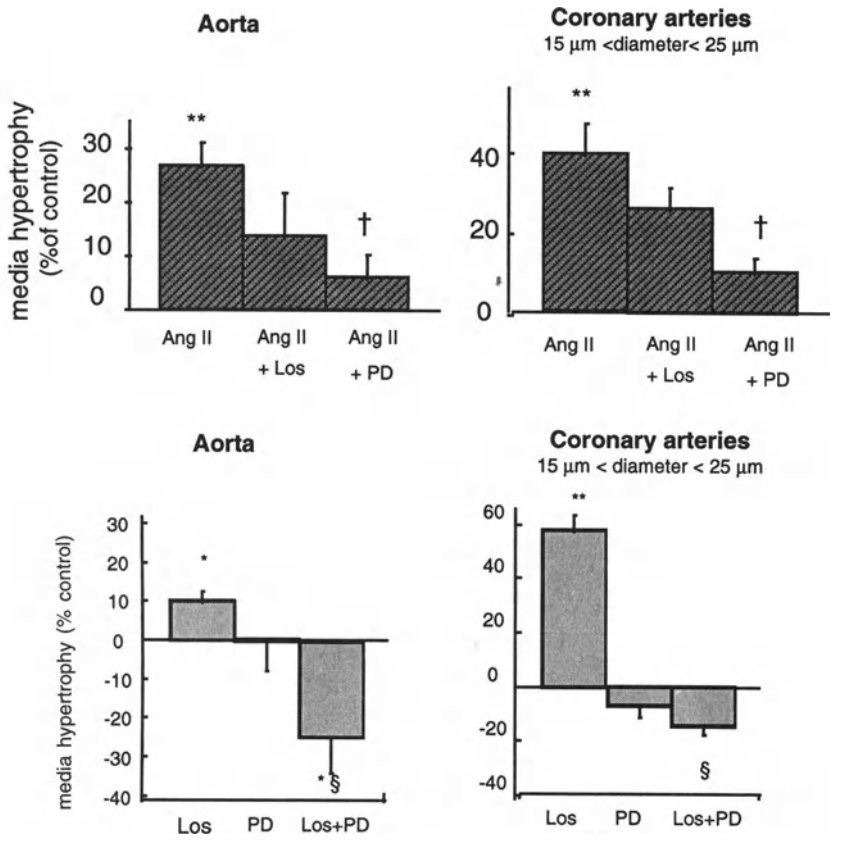


**Figure 1.** The hypertrophy of the media in the different experimental groups. Media thickness of aorta and coronary arteries is expressed as percent (%) of control values. In both type of vessels, Ang II induced a media thickening that was prevented by losartan and PD123319 treatment. Administration of losartan to normal rats also induced a medial hypertrophy, which is very important in the small coronary arteries, whereas combination of losartan and PD123319 treatment decreased the media thickness. The values are means ± SEM. *=$p < 0.05$; **=$p < 0.01$ vs. control. †=$p < 0.05$ vs. Ang II group. (Bonferoni-test analysis).

The finding that treatment of normotensive rats with losartan alone had no effect on blood pressure but induced medial hypertrophy that was prevented by additional treatment with PD123319 strongly suggests that an increase in systemic Ang II concentration, secondary to $AT_1$ receptor blockade (44, B. Levy unpublished data), activates the $AT_2$ receptor and unmasks its trophic effect. Taken together, the data show that $AT_2$ receptor activation triggers medial hypertrophy in rat arteries. The trophic effect of $AT_2$ receptor stimulation appears specific to VSMC and independent of arterial type (i.e., conductive or resistive). Whether this is a direct effect or mediated via other cascade(s) has not been investigated. However, recent in vitro

data indicate that the $AT_2$ receptor may be expressed in differentiated SMC, its activation triggering an increase in total RNA synthesis [45].

Therefore, results obtained in vivo and in vitro support the hypothesis that the $AT_2$ receptor activation plays a major role in the development of vascular hypertrophy in the rat, when VSMCs are not in an active proliferative stage.

## CHANGES IN SMOOTH MUSCLE CELL PHENOTYPE

In hypertensive mammals, medial hypertrophy is associated with (1) an increase in collagen and elastin content [46,47] and (2) changes in smooth muscle cell (SMC) phenotype-characteristic of an immature or secretory type [48–50]. Changes in SMC phenotype include (1) a shift in myosin heavy-chain gene expression, characterized by the reexpression of nonmuscle myosin (NM myosin) [48,50], which is normally expressed during fetal life, and (2) an increased expression of the extracellular matrix protein, cellular fibronectin (cFN), which is normally expressed in the intima layer only. Fibronectin may be expressed within aortic and coronary arterial media in response to acute or chronic hypertension [51,52]. Although the expression of an immature phenotype is independent of the artery type and the hypertension etiology, some differences in the precise pattern of expression of the above proteins have been described [52].

In normal rat artery, the typical pattern of immunolabeling is as follows: SM α-actin antibodies stain all medial smooth muscle cells, the labeling being homogeneously distributed, whereas cFN and NM myosin are restricted to the intimal layer of the aorta and completely absent from coronary arterial media.

In Ang II-induced hypertension, the medial distribution of SM α-actin within VSMC is qualitatively similar to normotensive controls, and the most obvious changes observed are in the distribution of NM myosin and cFN (figure 2). In aorta, cFN immunolabeling extends to cells of the inner half of the media (corresponding to a threefold increase in surface area), whereas the number of NM myosin positive cells increases dramatically throughout this layer (figure 2). The codistribution of NM myosin and SM α-actin identified these cells as SMC. In arterial media of Ang II-treated animals, NM myosin immunolabeling is markedly induced and positive cells are randomly distributed throughout the media. Accumulation of cFN in the coronary arterial media is also observed. SMCs exhibiting this immature phenotype are found in more than 65% of the coronary arteries in Ang II treated animals and in less than 15% in controls. Losartan, but not PD123319, totally prevents the expression of cFN and NM myosin in Ang II-treated rat SMC. The pattern of immunolabeling is similar to that of the control group in all arteries, irrespective of type and size (table I). Administration of PD123319 alone induces a small but significant increase in cFN and/or NM myosin in both aorta and coronary arteries, which was prevented by combined administration of losartan. Finally, losartan alone does not affect the phenotype of VSMCs in normotensive rats. The finding that $AT_2$ receptor blockade in normotensive rats was associated with a shift of VSMC towards an immature phenotype suggests the unmasking of an $AT_1$ receptor transactive

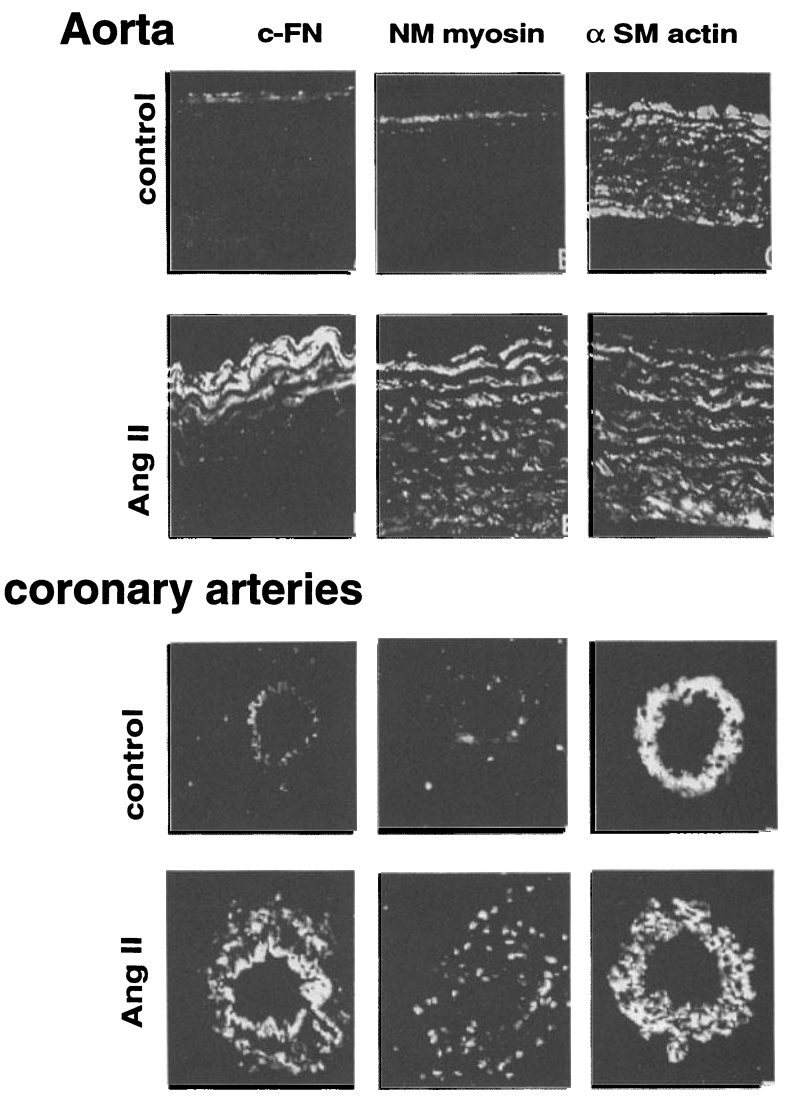


**Figure 2.** Distribution of c-FN, NM myosin, and SM α-actin in aorta and in coronary arteries of control, Ang II, treated rats. Note that cellular FN and NM myosin expression increased in Ang II treated rats when compared to controls.

pathway that mediates phenotypic changes [31]. Such an $AT_1/AT_2$ receptor interaction has been recently suggested by Siragy and Carey who proposed a shift of Ang II action to the $AT_1$ receptor in the presence of $AT_2$ receptor blockade to enhanced $PGE_2$ production in the rat kidney [53].

In conclusion, the qualitative and quantitative changes in aortic and coronary

**Table 1.** Cardiovascular changes secondary to Ang-induced hypertension and/or angiotensin II receptor antagonists

| | control | Ang II | Ang II + LOS | Ang II + PD | LOS | PD | LOS + PD |
|---|---|---|---|---|---|---|---|
| systolic blood pressure | = | ↑ | = | ↑ | = | = | ↓ |
| ventricular hypertrophy | − | + | − | + | − | + | − |
| media thickening | − | + | − | − | + | − | − |
| immature phenotype of VSMC | − | + | − | + | − | + | − |
| systolic blood pressure | = | ↑ | = | ↑ | = | = | ↓ |

Note: Summary of the qualitative and quantitative changes observed in the heart and vasculature of the different experimented groups. Ang II induced hypertension, ventricular hypertrophy, a shift of the VSMC phenotype towards an immature type, and medial thickening. These processes were prevented by $AT_1$ receptor antagonist. The $AT_2$ antagonist specifically prevented medial thickening. Note that the treatment of control rats with the $AT_1$ antagonist induced medial hypertrophy.

−, absence; +, presence; control, untreated rats; Ang II, Ang II-treated rats; Ang II + LOS, rats treated with both Ang II and Losartan; Ang II + PD, rats treated with both Ang II and PD123319; LOS, Losartan rats treated with Losartan; LOS + PD, rats treated with both Losartan and PD123319.

arterial phenotype secondary to Ang II-induced hypertension appear to be triggered by two independent pathways (figure 3): (1) Ang II induces medial hypertrophy of both aorta and coronary arteries, via the $AT_2$ receptor, independent of changes in blood pressure and (2) phenotypic changes in VSMC are controlled through $AT_1$ receptor activation and/or blood pressure elevation.

## CONCLUSIONS AND PERSPECTIVES

In this review we have analyzed the differential role of $AT_1$ and $AT_2$ receptors in the response of the myocardium and vessels to arterial hypertension. It emerges that

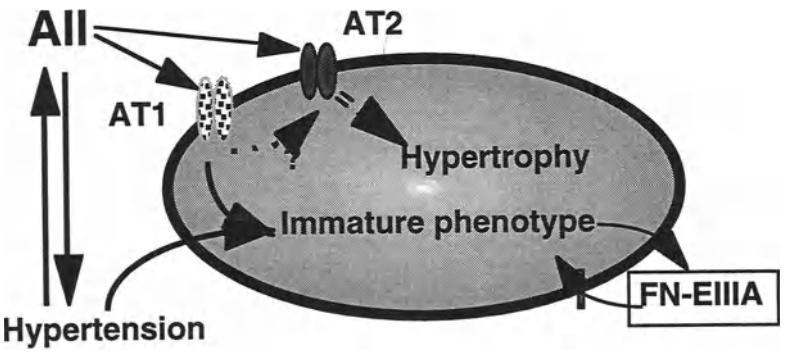


**Figure 3.** Triggers of qualitative and quantitative changes of vascular smooth muscle cells during hypertension in the rat.

the relative involvement of each receptor depends on cell type. Indeed, $AT_2$ has no effect on cardiomyocyte hypertrophy but triggers arterial medial thickening. On the other hand, the data reported herein (table I) contrast with previous studies that demonstrated $AT_1$-triggered cell growth and/or hypertrophy, whereas $AT_2$ mediated inhibition of cell replication (review in 3). It is noteworthy that the antiproliferative function of the $AT_2$ receptor has been observed in nonmuscle cells [17,23] and in SMCs actively replicating after endothelial injury [54,14]. The main goals for the forthcoming years will be to determine (1) the precise role of angiotensin receptors in different cell types and different stages of differentiation, (2) the transduction pathway used by the $AT_2$ receptor, and (3) the possible interactions between the 2 receptor subtypes.

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# INTERSUBJECT VARIABILITY IN THE PHARMACOKINETICS OF LOSARTAN

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***Summary.*** The use of orally effective angiotensin II receptor type 1 ($AT_1$) antagonists represents a new strategy for the management of hypertension and congestive heart failure. The development of losartan, a biphenyltetrazole derivative, was based on a carefully managed, rational drug design aimed at improving the low-oral bioavailability normally observed with peptide antagonists. The drug was also designed to preserve selectivity at the $AT_1$ receptor site without introducing intrinsic agonist activity. Losartan itself is an active $AT_1$ antagonist, but clinically relevant angiotensin blockade is mediated mainly by EXP3174, an active metabolite formed by a specific cytochrome P450 isozyme, CYP2C9. Therefore, dosage adjustment is dictated by variability in plasma concentrations of the metabolite rather than by variability in plasma concentrations of losartan. The present review describes both the development and the major pharmacokinetic characteristics of losartan, the first orally effective, nonpeptide $AT_1$ antagonist administered to man.

## INTRODUCTION

The search for specific antagonists of angiotensin (Ang) II receptors started more than twenty-five years ago. One of the first promising Ang II receptor antagonists to be discovered was the peptide saralasin [1]. However, the use of saralasin was limited by its poor absorption following oral administration, its short elimination half-life, and its substantial Ang II agonist properties [2,3]. The scientific community had to wait another ten years before Takeda Chemical Industries disclosed patents describing a series of nonpeptide 1-benzyl imidazole-5-acetic acid derivatives with Ang II antagonist properties [4,5]. Subsequently, four major states of development,

*N.S. Dhalla, P. Zahradka, I. Dixon and R. Beamish (eds.). Angiotensin II Receptor Blockade: Physiological and Clinical Implications.* 

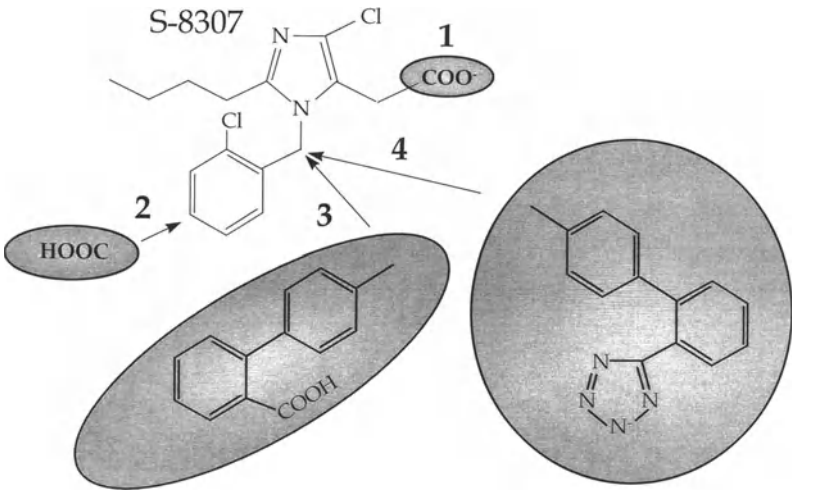


**Figure 1.** Major states of structural substitutions culminating in the synthesis of losartan.

involving important structural substitutions, were completed, culminating in the synthesis of losartan (figure 1) [6–8]. The decision to incorporate such structural substitutions resulted from:

1. the recognition that only C-terminal fragments of Ang II display significant biological activities [9]. It was further hypothesized that a carboxylic acid would be required for binding to the receptor;
2. the recognition that the substitution of a second acidic group in the para-position of the phenyl moiety of benzylimidazole acetic acid derivatives improved the potency tenfold without introducing intrinsic or nonspecific effects [10];
3. the recognition that enlargement of the molecule by replacement of the phenyl carboxylic acid group by a biphenyl carboxylic acid moiety produces orally effective agents with another tenfold increase in binding affinity [8];
4. the recognition that replacement of the biphenyl carboxylic acid moiety by biphenyltetrazole derivatives further improved bioavailability [11].

Thus, following all the structural substitutions described above, the compound obtained was the prototype biphenyltetrazole, losartan (DuP 753 or MK954), the first potent, nonpeptide, orally effective, competitive and selective antagonist of Ang II type I ($AT_1$) receptors to be made clinically available.

## PHARMACOKINETICS OF LOSARTAN

Absorption of losartan is rapid following oral administration, and peak plasma concentrations are attained within 30 to 60 minutes [12]. Based on measured and

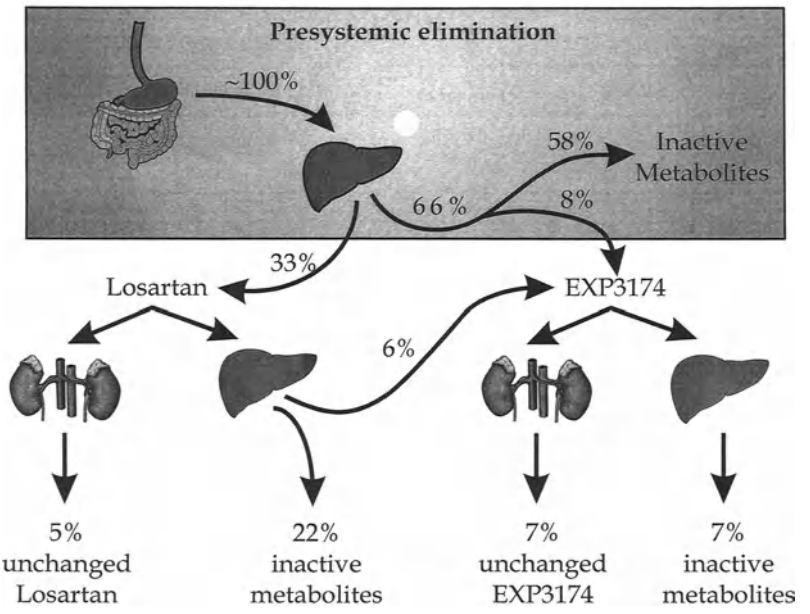


**Figure 2.** Schematic disposition of losartan and its active metabolite, EXP3174, in man.

predicted bioavailability from the hepatic extraction ratio, absorption of losartan from the gastrointestinal tract is considered to be complete [13]. Food slows the absorption of losartan and decreases its maximum plasma concentration, but it only has minor effects on the mean plasma concentrations of losartan [14]. Losartan undergoes extensive first-pass metabolism in the liver, and only about 33% of the dose reaches the systemic circulation unchanged (figure 2) [13]. Thus, upon its first exposure to liver enzymes, approximately 2/3 of the dose of losartan is transformed into several metabolites. In vitro drug metabolism studies performed with either liver slices or human liver microsomes are consistent with these observations [15–17]. In drug metabolism studies, at least 5 metabolites of losartan could be identified (figure 3).

Sequential oxidation of the alcohol side-chain of losartan into an aldehyde and a carboxylic acid moiety results in a metabolite, EXP3174, that is 10–40 times more potent as an $AT_1$ receptor antagonist than the parent compound [18]. The structure-activity relationship described in the development of orally active $AT_1$ receptor antagonists predicted that a carboxylic acid moiety overlapping with the C-terminal region of Ang II would be associated with more potent binding to the receptor [19]. Nevertheless, losartan is not a pro-drug because it is itself a potent $AT_1$ receptor antagonist [6,7]. In the dog, where very little losartan is transformed into EXP3174, sustained blockade of the Ang II effects can still be achieved [20,21]. Furthermore, numerous in vitro and ex vivo studies have demonstrated the activity of losartan

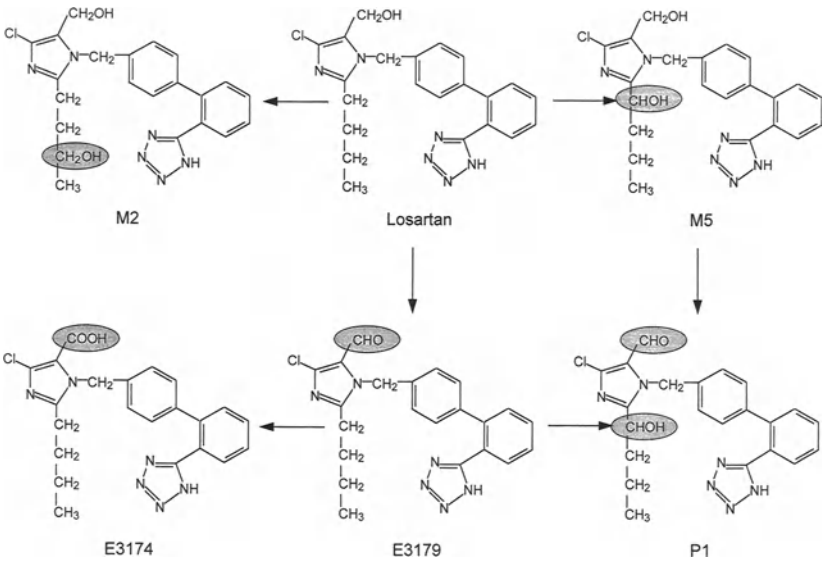


**Figure 3.** Scheme for oxidative biotransformation of losartan to its major metabolites.

which clearly does not have to be converted to EXP3174 in order to block Ang receptors [8,22,23]. However, the clinical relevance of EXP3174 in humans during chronic oral treatment with losartan needs further discussions.

The total clearance of losartan entering the systemic circulation is very high, approximately 610 ml/min [13]. Consequently, the drug is rapidly eliminated from the blood. Its terminal elimination half-life is approximately 2 hours [13]. The kidneys contribute very little to the overall excretion of losartan (renal clearance is ≈70 ml/min). Less than 5% of an oral dose of losartan is excreted unchanged in urine [13]. In contrast, circulating losartan is extensively metabolized into several metabolites, and total metabolic clearance is ≈540 ml/min [13].

As already indicated, extensive metabolism of losartan (≈66% of the dose) takes place upon its first exposure to the liver. During this first pass, approximately 8% of the dose is transformed into EXP3174 (figure 2) [13]. However, during subsequent passages through the liver, another 6% of the drug that is present in the blood stream is transformed into EXP3174 (figure 2) [13]. When the fraction of the dose of systemically available losartan metabolized into EXP3174 (6%) is added to the fraction formed by presystemic metabolism (8%), it can be estimated that 14% of an orally administered dose of losartan is transformed into EXP3174 [13]. The systemic clearance of EXP3174 is about 10 to 15 times slower (47 ml/min) than that of losartan [13]. The kidneys (renal clearance is 26 ml/min) and the liver (metabolic clearance is 21 ml/min) contribute equally to its elimination (figure 2). In fact, an amount of EXP3174 equivalent to 7% of the losartan dose is recovered in urine

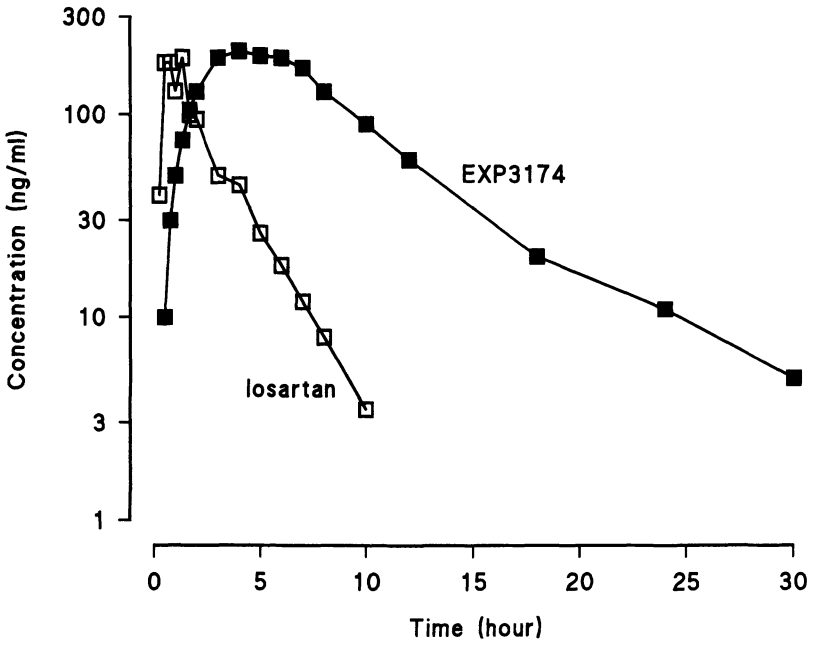


**Figure 4.** The profile of mean plasma concentrations of losartan (○) and EXP3174 (●) in man following administration of a single 50 mg oral dose of losartan (Adapted from Lo et al., 1995 [13]).

while the remaining EXP3174 undergoes sequential metabolism into various other metabolites. The terminal elimination half-life of EXP3174, which is much longer than that of losartan, is 6–9 hours [13].

Similar peak plasma concentrations of losartan and its major active metabolite, EXP3174, are achieved following oral administration of losartan (figure 4) [13]. Maximal plasma concentration of EXP3174 is reached at about 3.5 hours, which is slightly later than that of losartan [13,24]. However, the area under the plasma concentration-time curve for EXP3174 is almost five times larger than that of losartan [13]. Pharmacodynamic studies have demonstrated that plasma levels of EXP3174 parallel the profile of Ang II receptor blockade more closely than do plasma levels of losartan [24]. Therefore, it is not surprising that effective Ang II receptor blockade by losartan in humans is largely determined by the more potent active metabolite of losartan, EXP3174 [18,24].

Two major isoforms of the cytochrome P450 enzyme, namely, CYP2C9 and CYP3A4, appear to be involved in the sequential oxidation of losartan to EXP3174 [15,16]. In vitro metabolic studies with human liver microsomes and microsomes containing various recombinant human liver cytochrome P450 isozymes have demonstrated that formation of EXP3174 is inhibited by characteristic inhibitors of CYP2C9 and CYP3A4 as well as by antibodies directed against these specific

isoforms [15,16]. On the other hand, pharmacokinetic studies in healthy volunteers failed to demonstrate a significant interaction between losartan and ketoconazole, a well known inhibitor of CYP3A4 [25]. In contrast, subjects with genetically determined deficient CYP2C9 activity (1% of the Caucasian population) excrete minimal amounts (<1%) of a losartan dose as EXP3174 [26]. These recent clinical findings indicate that CYP2C9 is probably the major enzyme involved in the formation of EXP3174 in humans.

The efficacy of losartan in subjects with deficient CYP2C9 activity was not reported [26]. Based on the pharmacological properties of losartan and EXP3174, decreased efficacy would be expected since very little of the active metabolite is formed. However, toxicity from losartan accumulation should not be expected since several other metabolic pathways, independent of CYP2C9 activity, are most likely active in these subjects. Therefore, modulation of CYP2C9 activity by specific inducers or inhibitors would expectedly result in intersubject variability in the pharmacokinetics, as well as the efficacy, of losartan.

A fivefold increase in the plasma concentrations of losartan has been observed in patients with hepatic impairment because of alcohol cirrhosis [14]. This is due to a significant decrease in the metabolic clearance of losartan, which accounts for more than 85% of the total clearance of the drug [13]. Nevertheless, significant amounts of EXP3174 are still formed in these subjects, which suggests that CYP2C9 activity is partially preserved, even under these conditions of decreased liver function. On the other hand, the elimination of EXP3174 is significantly impaired, and mean plasma concentrations of the metabolite increase about twofold [14]. Consequently, a 50% dosage reduction is recommended in patients with alcohol-induced cirrhosis of the liver.

Age is another factor known to affect the pharmacokinetics of several drugs. This is due to decreased liver and kidney functions as well as changes in muscle and fat distribution [27,28]. Although data are limited, no significant difference in the innocuity of losartan was observed between elderly and young patients in clinical trials. This suggests that plasma concentrations of the active metabolite EXP3174 in elderly patients are similar to those measured in younger individuals.

Both losartan and EXP3174 are highly bound to plasma albumin (98.7% for losartan vs. 99.8% for EXP3174) [29]. However, neither losartan nor EXP3174 are significantly displaced in vitro by therapeutic concentrations of highly protein-bound drugs, such as naproxen, ibuprofen, diazepam, or warfarin [29]. This suggests that clinically significant drug interactions caused by displacement from binding sites are unlikely to occur. High protein binding to plasma albumin limits the diffusion of losartan and EXP3174 to peripheral organs. Consequently, the steady-state volumes of distribution of these compounds are quite small (34 l for losartan and only 12 l for EXP3174) [13].

The renal clearance of EXP3174 accounts for 50% of total EXP3174 clearance [13]. Therefore, one would expect metabolite accumulation in patients with renal

insufficiency, which would necessitate a decrease in the dosage of losartan. In contrast, in patients with moderate to severe, end-stage kidney disease (creatinine clearance is 10–29 ml/min), a proportional decrease in creatinine and renal clearance of losartan or EXP3174 is observed [30]. Plasma concentrations of EXP3174 do not increase but remain stable, and no dosage adjustment is recommended [30]. However, plasma concentrations of losartan are almost doubled in these patients [31]. These observations indicate that alterations in the routes and/or mechanisms of elimination of EXP3174 and losartan and/or an alteration in the amount of EXP3174 formed occur in patients with renal insufficiency. Similarly, in patients undergoing dialysis, plasma concentrations of losartan are higher than those measured in patients with normal renal function, while EXP3174 concentrations are comparable to those measured in the same [14]. Overall, these observations suggest that there is no need to adjust losartan dosages in patients with compromised renal function. Nevertheless, extreme caution is recommended in patients with either renin-angiotensin-dependent renal impairment or fixed renal blood flow, such as unilateral or bilateral renal artery stenosis [32].

Finally, no major drug-drug pharmacokinetic interaction has been identified for losartan. Only minor changes in mean plasma concentrations of losartan and EXP3174 were noticed during the coadministration of cimetidine to healthy volunteers [33]. Pretreatment with the cytochrome P450 inducer phenobarbital caused only a modest decrease in mean plasma concentrations of both losartan and EXP3174 [34]. In healthy volunteers, losartan did not alter the pharmacokinetics of intravenous or oral digoxin, and hydrochlorothiazide did not affect losartan pharmacokinetics, and vice versa [35,36]. Finally, the anticoagulant activity of warfarin was not found to be significantly affected by losartan [37,38]. This observation is very interesting since warfarin is a well known substrate of CYP2C9, the major isoform involved into the formation of EXP3174.

In summary, losartan is the first orally effective, nonpeptide, competitive antagonist of $AT_1$ to be marketed. Numerous in vitro and animal studies have demonstrated that losartan itself is a potent $AT_1$ antagonist. However, the profile of Ang II blockade observed in humans more closely parallels the plasma levels of one of its metabolites, EXP3174. Therefore, adjustments to the dosage of losartan are dictated by changes in the mean plasma concentrations of the metabolite observed under various clinical conditions. Clinical studies have demonstrated that dosage adjustments are required in patients with liver disease but are not necessary in elderly patients or in patients with renal insufficiency. To date, no major drug-drug interactions have been observed with losartan.

**ACKNOWLEDGMENTS**

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# FUNCTIONAL ANALYSIS OF TISSUE RENIN-ANGIOTENSIN SYSTEM USING "GAIN AND LOSS OF FUNCTION" APPROACHES: IN VIVO TEST OF IN VITRO HYPOTHESIS

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***Summary.*** The study of the effect of autocrine-paracrine vasoactive modulators (e.g., renin-angiotensin) on VSMC biology is very difficult in vivo because in vivo studies are limited. Recent progress in in vivo gene transfer technologies have provided us with the opportunity to study cellular responses to the manipulation of the individual components (i.e., by overexpression or inhibition). Currently, many researchers have developed many in vivo gene transfer techniques for cardiovascular application, including viral gene transfer and liposomal gene transfer. By using in vivo gene transfer approaches, the roles of the tissue renin-angiotensin system have been identified. Such an approach may increase our understanding of the biology and pathobiology of autocrine-paracrine system. This review has discussed the potential utility of in vivo gene transfer methods.

The study of the effect of autocrine-paracrine modulators (e.g., renin-angiotensin) is very difficult in vivo because in vivo studies are limited by (1) the multiplicity of coexisting variables, (2) the difficulties in manipulating individual components, and (3) the methodological limitations in studying the function of locally produced modulators in the absence of any contribution by the circulatory system. Gene transfer technology has provided us with the opportunity to study the cellular responses to the manipulation of the individual components (i.e., by overexpression or inhibition). For example, transfection of angiotensin-converting enzyme (ACE) vector into cardiovascular organs resulted in hypertrophy independent of hemodynamics and the circulating renin-angiotensin system (RAS) [1]. Alternatively, administration of antisense oligodeoxynucleotides (ODN) and angiotensinogen-gene activating element (AGE) 2 as "decoy" into the liver resulted in a significant

*N.S. Dhalla, P. Zahradka, I. Dixon and R. Beamish (eds.). Angiotensin II Receptor Blockade: Physiological and Clinical Implications.* 

transient decrease in blood pressure [2,3]. Such an approach may increase our understanding of the biology and pathobiology of autocrine-paracrine system, especially the RAS.

## IN VIVO GENE TRANSFER TECHNIQUES

Although it is important to develop in vivo gene transfer into the cardiovascular organs, few in vivo gene transfer methods are suitable for such purposes. Many in vitro gene transfer methods such as calcium phosphate precipitation and electroporation can not be applied to in vivo gene transfer because of significant cell injury and because they pose significant problems to the investigation of the role of potential autocrine mediators (e.g., angiotensin). Currently, in vivo gene transfer techniques for cardiovascular applications include viral gene transfer such as retrovirus, adenovirus, and HVJ (Hemagglutinating Virus of Japan: Sendai virus) and liposomal gene transfer such as cationic liposome (Lipofectin) [4,5]. Overall, the current in vivo methods for cardiovascular gene transfer are limited by the low efficiency and by the potential toxicity.

### Retroviral method

The retroviral method is well described. It has generally a high-transfer efficiency and can integrate transferred genes into the genome [6]. This method has been used for in vivo gene transfer into blood vessels (see below). However, the efficiency for vessel wall transfer is very low because retroviruses do not work in nonreplicating cells. This characteristic should be considered when selecting retrovirus as the vector for specific organs.

### HVJ-mediated method

The HVJ method appears to possess many ideal properties for in vivo gene transfer such as (1) efficiency, (2) safety, (3) easy handling, (4) brevity of incubation time (5) no limitation of inserted DNA size. In this method, foreign DNA is complexed with liposomes, a nuclear protein, and the viral protein coat of HVJ (figure 1). The HVJ method has been successfully employed for gene transfer in vivo to many tissues including liver, kidney, and vascular wall [1–3,7–10]. This method is also suitable for transfer of antisense oligonucleotides. HVJ method can result in a significant increased stability and effectiveness of antisense and cis-element decoy ODN [11,12].

### Adenoviral method

Adenoviruses do not require cell replication for transfer. The adenoviral method is a highly efficient transfection method, but it has the potential disadvantages of viral infection and viral antigen-induced immunity, and it has limitations in the inserted DNA size (but capacity is probably up to 7 kb pairs). Unlike retroviral transfer, this system may not integrate the inserted DNA. Further studies are needed to prove the

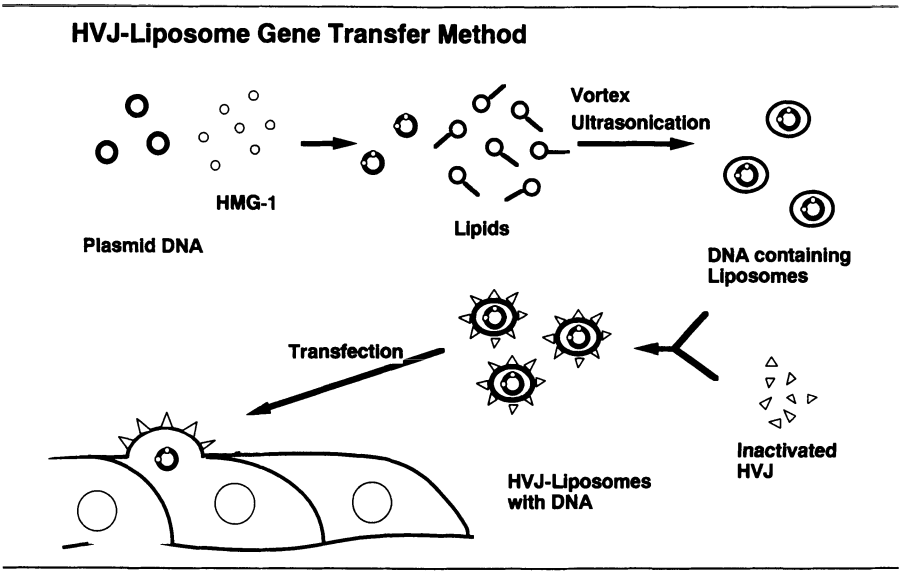


**Figure 1.** Schematic summary of HVJ-liposome transfer method.

safety, e.g., antigenicity, in human gene therapy, but in vascular biology research, this method seems to be one of most useful tools by high efficiency.

### Lipid-mediated gene transfer

The liposomal method is safe and easy to handle. The cationic lipid-mediated method seems to be efficient of in vitro, but not in vivo, transfer of DNA. Although Lim et al. reported the successful transfection of DNA into intact coronary and peripheral arteries in vivo [13], the transfection efficiency was low and the incubation time needed was long.

## IN VIVO ANALYSIS OF RENIN-ANGIOTNESIN SYSTEM USING GENE TRANSFER TECHNIQUES

### Role of angiotensin in the cardiovascular remodeling

Initial most important findings on the role of tissue RAS has been analyzed using transgenic technology. Transgenic rats harboring murine renin gene showed continuous hypertension and higher expression levels of tissue renin and angiotensin (Ang) II [14]. Transgenic/gene targeting technology provide us with many advantages such as (1) to study the specific gene function as systemic and developmental effects and (2) to test the specific gene function chronically, etc. [15]. Nevertheless, several disadvantages of this technology are that (1) it is time consuming and costly, (2) the effect of the overexpressed transgene is exerted throughout development, (3) it is difficult to target the transgenic expression to only local tissues, and (4) it is

**Table 1.** Comparison of transgenic/gene targeting technology and in vivo gene transfer technology

| Transgenic/gene targeting | In vivo gene transfer |
|---|---|
| 1. Systemic delivery<br>Hemodynamic effects<br>Developmental effects | Local delivery autocrine/paracrine effects |
| 2. Chronic effects | Acute effects |
| 3. Limitation in animal species<br>mainly mice, probably rat, rabbit<br>(gene targeting—only mouse) | No limitation rat, mouse, rabbit, porcine, human? |
| 4. Low copy number of transgene | High copy number of transgene |
| 5. Time consuming | No time consuming |
| 6. Expensive | Cheap |
| 7. Permanent expression | Transient expression |

difficult to exclude the potential contribution of the systemic effect of transgene expression. If the targeted gene can cause lethal effects, it is impossible to test the specific functions by transgenic or gene targeting techniques (table 1). In those cases, gene transfer approach may be ideal. Thus, local gene transfer approach may be more effective for studying the role of autocrine/paracrine mediators.

Receng data suggest that Ang II may be generated locally in many tissues. Components of the RAS have been shown to be present in the heart, blood vessel, adrenal, kidney, brain, and elsewhere [16–19]. Using the HVJ-liposome method, we initially analyzed the role of renin that was locally synthesized in liver. Direct transfection of human renin gene into hepatocytes in vivo resulted in transient hypertension associated with an increased plasma Ang II level [20]. Elevated blood pressure was reduced by specific human renin inhibitor and Ang II receptor antagonist. This study demonstrated that (1) transfected renin can be processed in local tissues and (2) local renin production is a rate-limiting step in the pathogenesis of hypertension. Given the importance of tissue RAS, we have focused upon the role of vascular RAS, especially ACE. Vascular renin-angiotensin is of particular interest to cardiovascular investigators since its existence may have important implications in the pathophysiology and pharmacology of diseases such as hypertension, atherosclerosis, restenosis after angioplasty, and congestive heart failure. Although renin enzymatic activity [21], angiotensin peptides [22], ACE activity [23] and angiotensin receptors [24] have been detected in blood vessels, debate still remains on the origin and the relative importance of the various components. Discrepancies between reports exist on renin mRNA expression in the vessel wall, the regional localization of angiotensinogen, and the presence of ACE in medial smooth muscle cells. Such discrepancies may reflect differences in animal strains, experimental design, and pathophysiological states. It has been also observed that after vascular injury, the neointimal smooth muscle cells express abundant angiotensinogen [25] and ACE [26]. These data suggest that: (1) the expression of vascular renin-angiotensin is dependent on the pathophysiological milieu, (2) the smooth muscle cell has the capability of expressing the components of the RAS, given the appro-

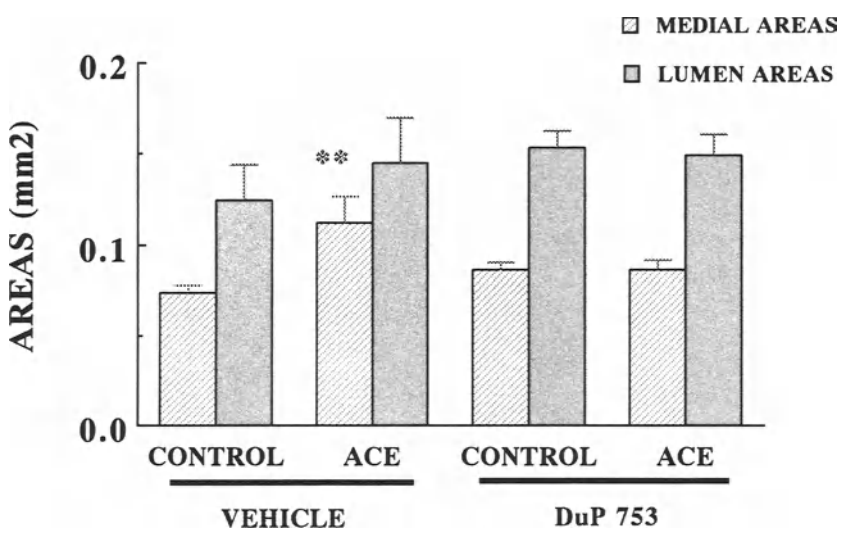


**Figure 2.** In vivo transfer of human ACE gene resulted in vascular hypertrophy independent of hypertension and circulating renin-angiotensin system. (Reprinted with permission of [1].)

priate conditions, and (3) the expression of the RAS in smooth muscle cells may have functional significance.

However, characterization of the role of vascular angiotensin in vivo is limited by the difficulty in manipulating individual components of the RAS as well as by the methodological limitations in studying the function of a local RAS in the absence of any contribution by the circulating RAS. In vivo gene transfer technology provides us with the opportunity to study the physiological responses to the in vivo manipulation of the individual components of the vascular RAS (i.e., by overexpression or inhibition) without changes in the circulating system. We tested our hypothesis by (1) transfecting ACE vector locally into intact rat carotid arteries in vivo and (2) studying the biochemical and physiological consequences of overexpression of ACE within vessel wall using in vivo gene transfer technique. Our data demonstrate that increased local expression of ACE within the vessel wall promotes autocrine/paracrine Ang II-mediated vascular hypertrophy in vivo (figure 2).

Moreover, in vivo gene transfer technique is a very powerful tool to elucidate unknown function of angiotensin receptors. As functions of the Ang II type 2 $AT_2$ receptor were unclear, it was apparently increasingly important to dissect the novel functions of the $AT_2$ receptor. In vivo transfection of the $AT_2$ receptor gene into the balloon-injured carotid arteries resulted in a significant inhibition of neointimal formation after angioplasty [27]. These data demonstrated that the $AT_2$ receptor works antiproliferative actions on vascular smooth muscle cell (VSMC) growth. More recently, Yamada et al. reported that $AT_2$ receptor anticipates in the regula-

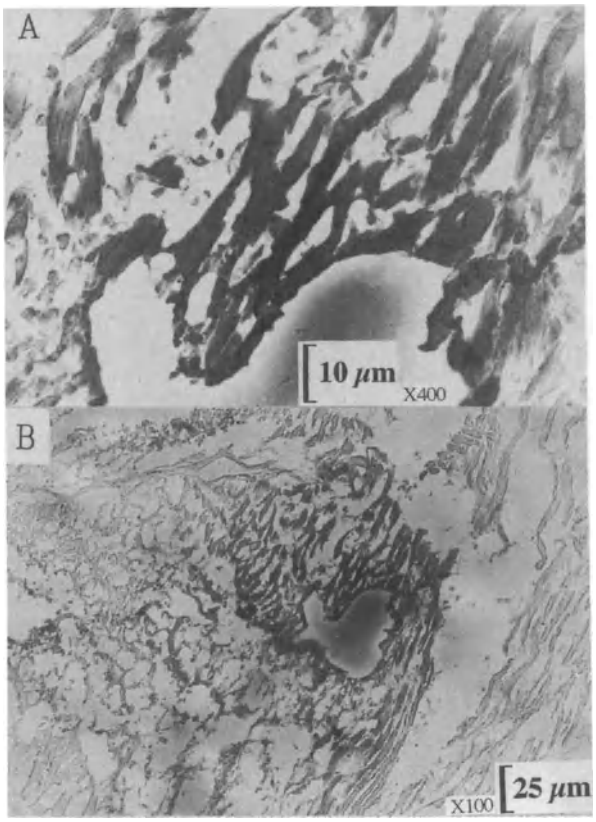


**Figure 3.** Staining for β-galactosidase after in vivo transfection of HVJ-liposome complex containing β-galactosidase (10 μg/ml) by direct injection into rat heart (*A*, × 400; *B*, × 100).

tion of VSMC growth through apoptosis using gene transfer approach [28]. Overall, in vivo gene transfer technology has the following advantages: (1) the target gene can be transfected into a local segment of a blood vessel, thereby avoiding a systemic effect, (2) this transfected vascular segment can be compared to adjacent untransfected segments or to the contralateral control blood vessel, which are subject to the same hemodynamics and circulating humoral factors, and (3) the consequences of local overexpression within the physiological/pathophysiological range of the target gene may be studied.

More importantly, tissue ACE also plays an important role in the cardiac remodeling, as in vivo transfection of human ACE vector into the rat heart results in a significant increase in the size of cardiac myocytes, which results in cardiac hypertrophy. As shown in figure 3, HVJ-liposome method is also useful for in vivo transfection into the heart [29–32]. There are few reports of successful gene transfer in the heart in vivo because it is very difficult to transfect efficiently into cardiac

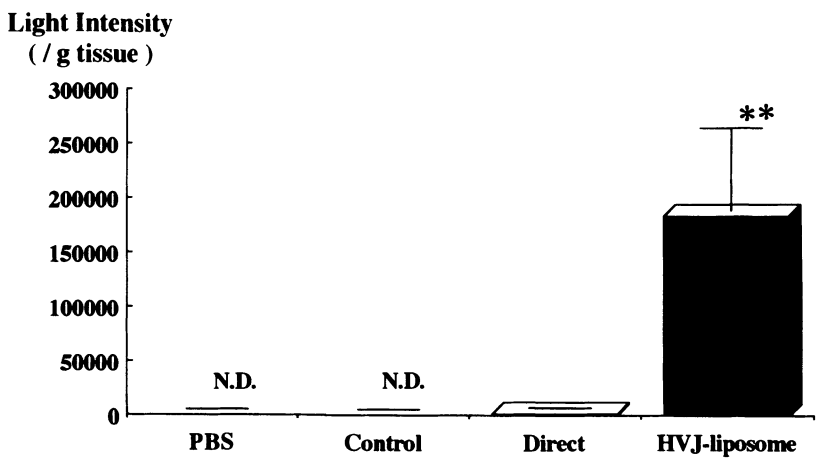


**Figure 4.** Luciferase activity in hearts transfected with luciferase vector or control vector with HVJ-liposome method or injection of "naked" plasmid by direct injection.
HVJ-liposome = hearts transfected with luciferase vector with HVJ-liposome method (10 ug/ml); Direct = hearts transfected with injection of "naked" luciferase vector (100 ug/ml); Control = hearts transfected with control vector with HVJ-liposome method (10 ug/ml); PBS = hearts transfected with vehicle (PBS).
N.D. = not detected. **$P < 0.01$ vs. other groups.

myocytes in vivo as well as in vitro. Many researchers demonstrated in vivo gene transfer into the heart using direct injection of "naked" DNA [33–37]. However, this approach is relatively inefficient, resulting in gene transduction in less than 1% of the cells in the area of DNA injection [33–37]. Therefore, the application of this method is apparently far. To overcome these problems, some investigators have recently focused on the adenoviral gene transfer method [38–42]. The adenoviral vector seems to be very efficient when applied via direct injection or coronary infusion [38–40], but there are some theoretical disadvantages [1,2]. With all these concerns taken into consideration, the current methods have several theoretical disadvantages. In contrast, luciferase activity in hearts transfected by HVJ-liposome method was significantly higher than that in hearts transfected by direct "naked" plasmid transfection (figure 4) [32]. Moreover, incubation of HVJ-liposome complex containing β-galactosidase vector within the pericardium resulted in widespread staining of cardiac myocytes and fibroblasts, mainly located in several surface layers beneath the pericardium and in the middle of the myocardium around the vasa vasorum. Alternatively, direct infusion of HVJ complex containing β-galactosidase vector into coronary artery also resulted in widespread staining of β-galactosidase in cardiac myocytes around the microvasculature. The widespread transgene expression using the HVJ-liposome method suggests that this method may be useful in introducing plasmids into cardiac myocytes to study cardiac function as well as to treat cardiac diseases by gene therapy.

## Role of angiotensin in blood pressure regulation

Angiotensinogen, which is mainly produced in the liver, is a unique component of the RAS because angiotensinogen is the only known substrate for Ang I generation. Recent findings of genetic studies suggest that the angiotensinogen gene is a possible determinant of hypertension [43,44]. To clarify the role of angiotensinogen in blood pressure regulation, we employed antisense strategy to block circulating angiotensinogen selectively. Antisense ODN are widely used as inhibitors of specific gene expression because they offer the exciting possibility of blocking the expression of a particular gene without changing functions of other genes [45]. Therefore, antisense ODN are useful tools in the study of gene function and may be potential therapeutic agents. However, antisense ODN have many unsolved problems such as their short half-life, low efficiency of uptake, and degradation by endocytosis and nucleases [45]. Recently we have developed an efficient gene transfer method mediated by viral liposome complex (HVJ-liposome method) [8]. This delivery system also enhances the efficiency and prologns the half-life of antisense ODN in vitro and in vivo [11,12]. In this study, we reasoned that circulating angiotensinogen is a rate-limiting step in systemic blood pressure regulation. Indeed, in vivo transfection of antisense ODN against rat angiotensinogen into the portal vein resulted in a transient decrease in plasma angiotensinogen level and high blood pressure in spotraneously hypertensive rats (SHR). These data suggest that angiotensinogen is an important determinant in the regulation of blood pressure. Importance of circulating angiotensinogen in blood pressure regulation is also true in the normotensive rats, since administration of antisense angiotensinogen ODN into the normotensive Wistar rats also resulted in a significant transient decrease in blood pressure [46].

Given the importance of angiotensinogen regulation in the pathogenesis of hypertension, it is of importance how the aniotensinogen gene is regulated. The angiotensinogen gene has been suggested to be regulated by novel transcriptional factors such as angiotensinogen gene-activating factor (AGF) 1–3 in cultured human hepatocytes (HepG2 cells) in vitro [47]. However, the molecular mechanism(s) of angiotensinogen regulation in vivo has not yet been clarified. In this study, we examined how hepatic angiotensinogen gene expression is regulated in vivo. To determine the critical transcriptional regulator of hepatic angiotensinogen production in vivo, we utilized synthetic double-stranded ODN as "decoy" cis-elements to block the binding of nuclear factors to promoter regions of the targeted gene, resulting in the inhibition of gene transactivation [48–51]. Using this strategy, we examined whether the angiotensinogen-gene-activating elements (AGE) 2 and 3 in the promoter region of the angiotensinogen gene have a pivotal role in the regulation of circulating angiotensinogen production in vivo. A classical approach to define the role of transcriptional factors in the regulation of genes is to use promoter-reporter gene transfection experiments such as chloramacetyltransferase (CAT) and luciferase constructs. This approach is very useful to identify cis- and trans- acting element interactions, but it has some disadvantages as follows: (1) it is costly and time consuming to make a series of constructs, (2) it cannot analyze endogenous gene regulation, and (3) it is hard to determine the specific elements.

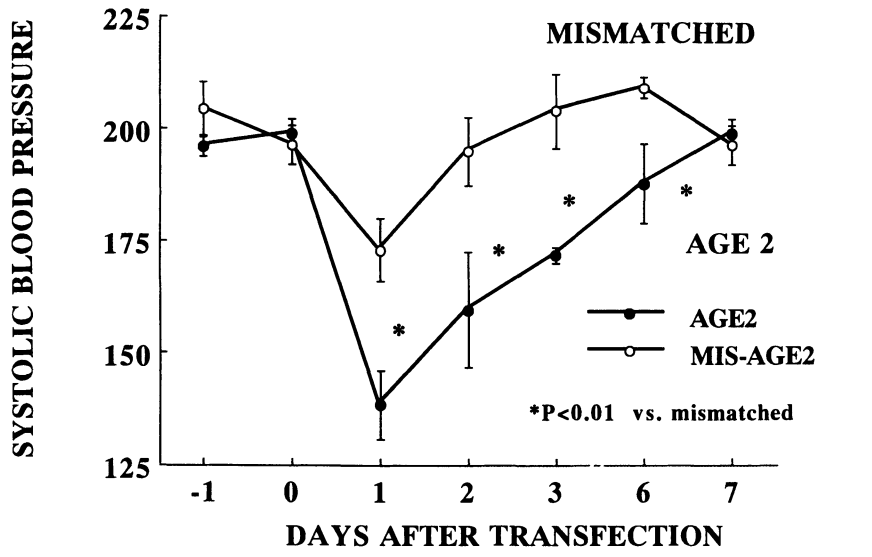


**Figure 5.** Systolic blood pressure of SHR (20 weeks old) injected with either AGE 2 decoy or mismatched decoy ODN by HVJ-liposome method.
*$p < 0.01$ versus SHR treated with mismatched ODN. AGE 2 = SHR transfected with AGE 2 decoy ODN; MIS-AGE 2 = SHR transfected with mismatched AGE 2 decoy ODN. (Reprinted with permission from [3].)

In contrast, the decoy approach has many advantages: (1) decoys are easily synthesized, (2) endogenous gene regulation and pathophysiological roles can be studied, and (3) the specific cis-elements can be determined, even if the specific regulatory cis-elements have not yet been clarified. Our previous results demonstrated that AGE 2, but not AGE 3, plays an important role in the regulation of hepatic angiotensinogen gene expression in the liver because transfection of AGE 2, but not AGE 3, decoy ODN decreased high blood pressure of SHR (figure 5) [3]. More importantly, AGE 2, rather than AGE 3, also plays a pivotal role in the regulation of angiotensinogen, thereby regulating blood pressure even in the central nervous system, as intracerebral administration of AGE 2 decoy ODN also resulted in a significant decrease in blood pressure in SHR [52]. Overall, we revealed the utility of gene transfer and decoy technology for hypertension research, especially to evaluate the specific functions of transcriptional factors of target gene regulation.

## FUTURE DIRECTION OF HYPERTENSION RESEARCH

As discussed above, in vivo gene transfer and transgenic/gene targeting techniques provide us the opportunity to test the in vitro hypothesis related to hypertension. Interestingly, recent progress in genetics have focused on the RAS in the pathogenesis of hypertension. Now ACE and/or angiotensinogen are candidates for hypertension because their genotypes are closely correlated with phenotypes. However, it

is very difficult to prove the hypothesis in genetics. Probably in vivo gene transfer may address the questions in genetics.

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# EXPLORING THE DIFFERENCE BETWEEN ANGIOTENSIN CONVERTING ENZYME INHIBITORS AND ANGIOTENSIN II-RECEPTOR ANTAGONISTS. A FOCUS ON BRADYKININ

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***Summary.*** Nineteen ninety-six certainly was the year of bradykinin regarding its assault on the minds of clinicians interested in cardiovascular disease. This assault was further amplified by the introduction, in Canada, of the first angiotensin receptor antagonist, an agent that appeared to be devoid of activities involving bradykinin. Intriguingly, this assault was also fueled by some pharmaceutical companies who had ACE inhibitors, but no angiotensin receptor antagonists. The suggestion was that bradykinin was good, not bad.

The closer one gets to the field of vascular regulation, the more complicated the situation becomes. There are now many defined vasoactive substances, but even with the use of the probes and scientific methods of today, a comprehensive understanding will not likely emerge. The bottom line will quite likely depend upon well-conducted, randomized clinically controlled studies that would compare agents with a bradykinin effect, namely, ACE inhibitors, with those without a bradykinin effect. Thus far, the trials have shown great similarity between the agents in the field of hypertension and those of cardiac failure.

In late 1995, the first angiotensin (Ang) II receptor antagonist, losartan potassium, was released for clinical use in Canada. Angiotensin-converting enzyme (ACE) inhibitors, on the other hand, have been widely used for about 15 years. They have been used extensively in hypertension because they are generally effective and well tolerated. In addition, they have been the subject of much interest because of several large randomized control trials that have shown enormous clinical benefit in a variety of conditions. The various conditions treated with ACE inhibitors in the following trials include congestive cardiac failure studied in the CONSENSUS [1]

*N.S. Dhalla, P. Zahradka, I. Dixon and R. Beamish (eds.). Angiotensin II Receptor Blockade: Physiological and Clinical Implications.* 

and SOLVD trials [2], myocardial infarction investigated in the SAVE study [3] and the AIRE study [4], and mediators of renal protection investigated in the Captopril Type I Diabetic nephropathy study [5]. To clinicians, the burning question was, Would angiotensin receptor antagonists be the same as, better than, or worse than, ACE inhibitors, and in what circumstances?

At its release, losartan potassium was labelled as an agent for the treatment of hypertension only, and it still is. In prerelease clinical studies that included some 3000 patients, determinations of efficacy and safety in blood pressure reduction were undertaken. There were no studies of long-term cardiovascular benefit. Notwithstanding this, one study suggested that losartan reduced blood pressure and worked as well as the ACE inhibitor enalapril [6], the βeta blocker atenolol [7], and the dihydropyridine calcium channel blocker felodipine ER [8]. It is usually taken once daily with acceptable trough to peak characteristics. Studies had shown it to be remarkably well tolerated with only dizziness having an incidence greater than that of placebo in the aforementioned comparative studies. In addition, it seemed devoid of the problematic cough. Losartan had an incidence of cough comparable to placebo in comparative studies and similar to hydrochlorothiazide in the losartan versus lisinopril cough study [9].

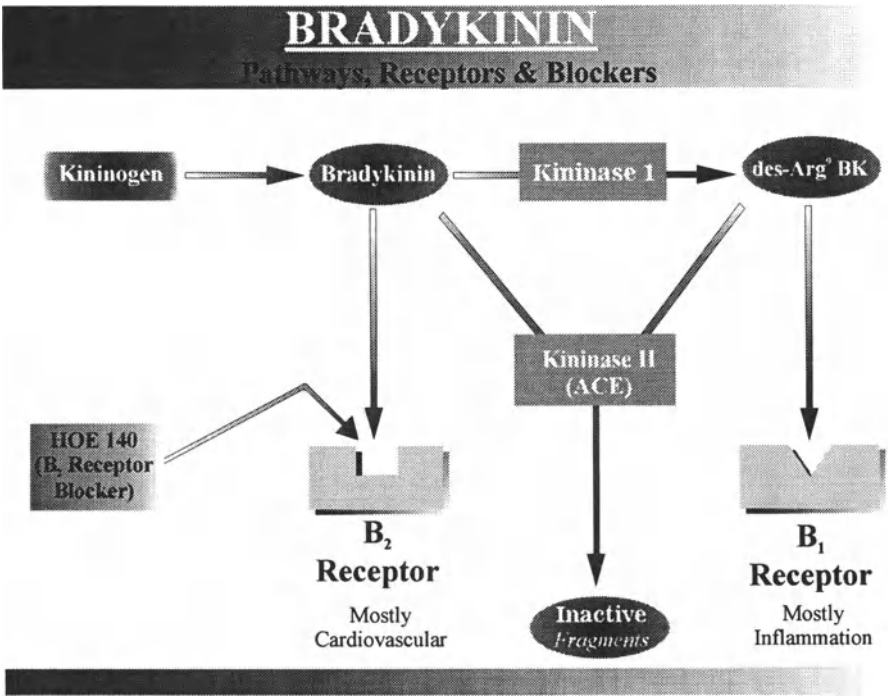
Preliminary studies done in congestive cardiac failure looked promising with effects of losartan again similar to ACE inhibitors [10,11]. Long-term rat experiments showed good long-term renal protection when looking at the end point of glomerular sclerosis [12].

Adding to the interest of exploring the differential properties of ACE inhibitors and Ang II receptor antagonists, alternate mechanisms of Ang II production were observed, particularly in the heart. The enzyme chymase of the human heart was reported to catalyze the conversion of Ang I to Ang II bypassing ACE [13,14]. In humans and dogs, substantial amounts of Ang II are generated in the myocardium by non-ACE enzymes, namely, human heart chymase. However, all of its production in the rat seemed to depend upon the ACE system.

Another role of ACE, in which it was called kininase II, is its degradation of bradykinin and des-Arg$^9$ bradykinin into inactive fragments. Blocking this enzyme with an ACE inhibitor may well increase the systemic or local concentrations of bradykinin, a substance which is a vasodilator, and may therefore augment some of the cardiovascular properties of the ACE inhibitor.

The pivotal question then became, Might ACE inhibitors have additional cardiovascular effects apart from their effect on Ang II, which stem from increased concentrations of bradykinin and other vasodilators. Alternatively, does blockade of the $AT_1$ receptor by substances like losartan provide more complete protection from Ang II, a substance which many claim to be a cardiovascular villain? Clearly as clinicians attempt to answer this question, the considerable literature now available in the bradykinin field will be reviewed in greater detail.

Nineteen ninety-six saw an increase in the interest in bradykinin. The increased interest was fuelled by both a better scientific understanding of the substance and the availability of stable blockers of bradykinin receptors. Also some major pharmaceu-




tical companies that did not have Ang II receptor antagonists, but did have ACE inhibitors, were attempting to expose the potentially beneficial properties of bradykinin as an extension of their marketing philosophy.

Simply, bradykinin is a very potent nona-peptide, present in the blood in subnanomolar concentrations. It has a very short half-life of approximately 30 seconds. It is produced locally, and perhaps only local production has any physiological relevance. It has been extremely difficult to measure until recently. The blood levels accepted today are a hundredfold lower than those that were thought to be relevant just a few years ago. Its injection into the skin causes the classic features of inflammation, namely, redness, pain, swelling, and warmth. Its action is to produce an endothelium-dependent vasodilatation and small vessel leakage. Additionally, it releases nitric oxide and $PGI_2$ [15], both vasoactive substances.

Figure 1 illustrates that bradykinin is produced by the cleavage of kininogen by kallikreins. It also shows the two receptors that have been characterized, as well as degradation pathways. The B2 receptor is probably the receptor of the cardiovascular system, is probably preformed in many tissues, and is responsible for vasodilation. A specific stable peptide antagonist Hoe 140 (icatibant) has been available for a number of years [16]. Hoe 140 has been used quite widely in cardiovascular

experiments in animals and in some human studies. The B1 receptor is probably the receptor of inflammation. It is probably not preformed, but is rapidly expressed following injury. The B1 receptor can be demonstrated in the cardiovascular system, but only in heavily instrumented animals; therefore, its physiological significance must remain unclear for the present time. Both of these receptors are G protein-coupled surface receptors, with a variety of agonists and antagonists available for both types of receptors.

A logical question would be, Is there a role for bradykinin in cardiovascular regulation? Normal human volunteers given Hoe 140 had only a trivial rise of systemic blood pressure [17]. However, when Hoe 140 was infused into the coronary circulation of 15 subjects without significant stenosis, a significant reduction of coronary flow resulted [18]. These individuals were heavily instrumented upstream in the circulation; therefore, one cannot necessarily deduce that bradykinin is being continuously produced. Further, since this effect is dependent upon an intact endothelium, it may well be limited by the presence of atherosclerosis [19]. This type of dependance has been described in other coronary artery studies, such as the TREND study [20].

Clearly bradykinin is a vasodilator, but is this property of therapeutic importance involved in hypertension? Ferner et al. demonstrated that when enalapril was given orally, the intensity of the skin reaction that occurs following the skin injection of bradykinin is augmented [21]. This interestingly did not occur with captopril or with placebo. Further, captopril potentiated the hypotensive effect of intravenous bradykinin [22]. However, in most studies, there is no change of venous bradykinin or des-Arg$^9$ bradykinin following the oral administration of ACE inhibitors. We have previously said that probably only local production of bradykinin and its local action are important. Since tissue bradykinin is impossible to measure, many important questions in this area are far from being answered.

If an important change of the bradykinin environment following ACE inhibition is hypothesized, one might expect to see a differential effect between ACE inhibitors and Ang II receptor antagonists. In most clinical situations, this is not the case, where there appears to be equivalence of effect [6–8,11]. The addition of an ACE inhibitor to an Ang II receptor antagonist only has additive hypotensive effect when the volume status of the subject is manipulated with a potent diuretic. However, in the salt depleted dog, the fall of blood pressure in response to enalaprilat was not modified by the intravenous injection of the B2 receptor antagonist B5630 [23]. The rat experiments in this area are also confusing. Studies in a wistar rat model of renal artery stenosis, with two kidneys and one clip, reveal about a 30% blunting of the hypotensive effect of the ACE inhibitor by the B2 receptor antagonist Hoe 140 [24]. However, Hoe 140 had no effect upon the hypotensive effect of an ACE inhibitor in the spontaneously hypertensive Brown Norway rat [25,26].

If the evidence does not strongly support that bradykinin is important in human hypertension and that its manipulation likely offers no clinical advantage, is there any suggestion that it may have important therapeutic effects in other vascular conditions, such as heart failure, post MI, reversing or preventing left ventricular

hypertrophy, the neointimal hyperplastic response to injury, as well as in states of progressive renal disease, such as diabetic nephropathy?

In congestive cardiac failure, the long-term effects of losartan and similar agents are unknown. Crozier and colleagues saw hemodynamic and symptomatic improvement in a placebo-controlled, multidose double-blind study over 12 weeks [10]. Dickstein and coworkers, in a 8-week study, randomly assigned patients to losartan or enalapril and concluded that they were of comparable efficacy [11]. The hemodynamic and antiproteinuric effects of losartan were compared to those of enalapril in 11 nondiabetic, proteinuric patients [27]. Two-dose levels of each agents were used, each level for 4 weeks. The agents were found to be of comparable efficacy. Proteinuria, however, is a surrogate endpoint of a process which often ends in glomerular obsolescence. While the long-term human studies with losartan are not available, long-term rat studies have suggested efficacy in retarding the development of glomerular sclerosis [12].

The need to demonstrate equivalence or the lack thereof in a number of these pathological states has been driven by scientific curiosity. It has proceeded because of a better understanding of bradykinin and its receptors and the availability of specific blockers.

There have been extensive animal studies using a variety of agonists and antagonists in a number of fields. Three areas will be discussed: (1) studies in the prevention of myocardial hypertrophy in response to a coarctation model of hypertension, (2) studies in limiting infarct size following the interruption of coronary blood flow and subsequent reperfusion, and (3) studies in the neointimal hyperplastic response to balloon-induced endothelial injury.

Linz et al. [28] studied the progression of left ventricular hypertrophy induced by aortic banding in the Sprague-Dawley rat. Control animals were compared to animals treated with ramipril or losartan. In this study, ramipril at low doses prevented the development of cardiac hypertrophy, whereas losartan did not. In addition, ramipril at an antihypertensive dose and losartan at an antihypertensive dose produced significant regression of the cardiac hypertrophy induced in this experiment. Linz et al. used this same model to study the effects of nonantihypertensive and antihypertensive doses of ramipril, alone and with a specific B2 receptor antagonist, Hoe 140 [29]. They suggested that ramipril at both dosage levels prevented the development of left ventricular hypertrophy and that Hoe 140 abolished this effect. Rhaleb et al., using the same animal model with both antihypertensive and nonantihypertensive doses of ramipril, showed that there was a modest but statistically insignificant antihypertrophic effect by ramipril at a low dose [30]. This became significant at the antihypertensive dose, but Hoe 140 did not block this phenomenon.

Different groups, therefore, produced diametrically opposed results. In fact, Rhaleb et al. concluded that a decrease of Ang II and a fall of blood pressure might well explain the results that they had seen.

Several studies have looked at the areas of intimal lesions and the vessel wall response to injury induced by balloon catheterization of the carotid artery of the rat.

deBlois et al. [31] looked at the effect of ramipril, both with and without Hoe 140, in Sprague-Dawley rats. In this study, ramipril significantly inhibited the intimal lesion formation, and Hoe 140 completely prevented the inhibitory effect of ramipril. Hoe 140, when given alone, caused a modest though insignificant increase in the size of the lesions. Kauffman et al. [32] looked at the effect of high- and low-dose losartan on intimal lesions. Both dosing levels of losartan produced a significant (50%) reduction of blood pressure. The low dose produced an insignificant (23%) reduction in cross-sectional area, and the higher dose produced a significant (48%) reduction of the intimal lesions. There was, however, no relationship between the effect on intimal lesions and the amount of blood pressure lowering. Prescott et al. [33] compared benazeprilat with losartan. They looked at both the areas of intimal response and smooth muscle cell migration. An intermediate dose of losartan (10 mg/kg/day) reduced both smooth muscle cell proliferation and migration in response to balloon injury, whereas the ACE inhibitor only inhibited smooth muscle cell migration. Using the same model, Farhy et al. [34] looked at (1) two ACE inhibitors, namely, ramipril and enalapril, (2) these ACE inhibitors combined with Hoe 140, and (3) losartan. These studies also included the nitric oxide synthase inhibitor L-NAME. They concluded that both ramipril and losartan significantly reduced neo-intimal formation, but the ramipril had a more marked effect than losartan. Hoe 140 reduced this effect by 75% in the case of ramipril and by 62% in the case of enalapril. The residual effect was similar to that of losartan. Further, L-NAME also blocked the inhibitory effect of ramipril, which suggests not only a contribution from ACE inhibitors due to Ang II production and kinin degradation, but also a role for nitric oxide. In this series of studies, when using antihypertensive doses, ACE inhibitors inhibit the intimal thickening following balloon-induced injury. A similar effect is seen with losartan, but at higher dosages. The effect of losartan is dose-dependent, and higher doses are required to cause an effect comparable to those of ACE inhibitors. In addition, the inhibitory effect of Hoe 140 is also dose dependent, and there is a small and insignificant growth promoting effect of Hoe 140 when used alone. This phenomenon has quite marked clinical relevance in the field of post-angioplasty endothelial injury and restenosis. A recent study, the MERCATOR study [35], showed that the ACE inhibitor cilazapril could not prevent restenosis and had no impact upon the clinical outcome. These studies have had a narrow focus, and none have been able to probe the possible contribution of changes in the tissue concentrations of Ang II or any possibly inhibitory and growth-modulating effect that the $AT_2$ receptor might have on this important area. There has been interesting work regarding the role of $AT_2$ in growth modulation and in the response to injury [36]. The $AT_2$ receptor is very floridly expressed during fetal life and is rapidly downregulated after birth. It is, however, up regulated following vascular and other injuries. In studies of vascular endothelial receptor expression, both $AT_1$ and $AT_2$ receptors are more avidly expressed following injury, and losartan often reduces the growth response, whereas the specific $AT_2$ receptor blocker PD 123319 promotes it.

Turning to the question, Do ACE inhibitors limit myocardial infarct size? reveals

that there have been a number of studies, but unfortunately the experimental technique has varied from study to study. Miki et al. [37] looked at infarct size following 30 minutes of occlusion and 72 hours of reperfusion in rabbits. They compared captopril, ramiprilat, and Hoe 140 and found that neither ACE inhibitors nor the B2 receptor antagonist had any major effect in this area. Using both rabbits and a 30 minute occlusion again, but 120 hours of reperfusion, Hartman et al. [38] looked at ramipril, ramipril with Ang II, Ang II alone, and losartan. In this model, neither Ang II stimulation or receptor antagonism altered the degree of myocardial necrosis, whereas ramiprilat protected against ischemia and reperfusion-related myocardial damage. Looking at the effect of intra coronary injections of bradykinin, ramipril, and Hoe 140 in mongrel dogs, Martorana et al. [39] showed that bradykinin had a cardioprotective effect similar to ramiprilat, but ramiprilat, when given with Hoe 140, had no effect. Richard et al. [40], using dogs with 90 minutes of occlusion and 4 hours of reperfusion, compared enalaprilat with EXP 3174. Both were administered intravenously. Neither the active metabolite of enalapril (enalaprilat) nor that of losartan (EXP 3174) were shown to have any effect upon infarct size.

The rabbit studies are contradictory, where Miki et al. and Hartmann et al. reached dissimilar conclusions. Similarly, the dog experiments are contradictory, whereby Martorana et al. observed a significant effect of the ACE inhibitor and exogenous bradykinin, whereas Richard et al. failed to demonstrate any effect of the ACE inhibitor on infarct size.

Clearly this is a very confusing field and currently provides no clinically applicable wisdom.

Most clinicians first became interested in bradykinin because of the "captopril cough". Approximately 10% of all patients exposed to an ACE inhibitor experience a dry and irritating cough. Similarly, bradykinin has been cited as the cause of the angioedema, observed much less commonly than the cough, but potentially life-threatening. In this sense, the role of bradykinin seems to make sense and be somewhat intuitive since bradykinin causes a profound vasodilatation and blood vessel leakage but, of course, there is no direct evidence that this is the case. In the area of the cough, there is some more powerful evidence. Bradykinin is known to be a powerful irritant of the airways. NPC 567, a BK receptor antagonist, has been effective in protecting against the bronchospasm induced in sheep with allergic asthma [41]. Three hundred patients with moderately severe asthma were studied in a multicenter, double-blind, randomized, placebo-controlled trial which showed that after four weeks, nebulized Hoe 140 led to a dose-dependant improvement of the pulmonary function test [42]. However, patients given 10 mg of ramipril did not have an exaggerated response to aerosolized bradykinin or histamine [43]. Both inhaled citric acid and capsaicin are standard tussigenic stimuli. Male guinea pigs were placed in a plexiglass box and exposed to aerosols before and after two weeks of captopril in the drinking water [44]. Coughs were counted by trained observers. Captopril increased this response, and it was blocked by Hoe 140.

Clearly there is a huge amount of work to be done further in this area. There is

a need to dissect the roles of Ang II as it relates to both the $AT_1$ and $AT_2$ receptors. We must further define the roles of the bradykinin B2 and B1 receptor using currently available and future receptor blockers, as well as be able to ascribe vascular responses to prostanoids and nitric oxide separately from those resulting from Ang II and bradykinin. Clearly bradykinin is capable of changing vascular tone, but its role in regulation is unknown. It appears that ACE inhibitors effect the degradation of bradykinin and might possibly augment its vasodilatory effect. Further, bradykinin might be responsible for some of the adverse effects of ACE inhibitors.

In the major clinical areas of hypertension, there appears to be no difference between the available angiotensin receptor antagonist and ACE inhibitors at the present time. Similarly, in the initial heart failure and renal protective endeavours, equivalence of effect is nearly always found. The animal work looking at left ventricular hypertrophy and endothelial responses to injury, as well as infarct size, however, reveals a possible contribution of bradykinin towards the salutary responses of these agents to ACE inhibitors, but this is often quite confusing. The enormous amount of interest in this field is exciting, and potentially a lot rides on it as far as patient care and the pharmaceutical industry are concerned.

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# ROLE OF CYTOKINES IN SEPTIC CARDIOMYOPATHY

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***Summary.*** The interrelations of cytokine and nitric oxide (NO) metabolism with heart function have been best documented for the heart in sepsis. The existence of human septic myocardial depression in intensive care patients was only unequivocally proved in the 1980s by the group of Parrillo, utilizing a nuclear imaging technique. Septic cardiomyopathy is frequently masked by a seemingly normal cardiac output. However, relative to the lowered systemic vascular resistance in sepsis, resulting in a reduced afterload, cardiac outputs and ventricular ejection fractions of septic patients are often not adequately enhanced. Septic cardiomyopathy involves both the right and the left ventricle; global as well as regional contractile disturbances occur and systolic pump as well as diastolic relaxation failure. Septic cardiomyopathy is potentially reversible. In response to volume substitution, the hearts can be considerably enlarged. The disease is not primarily hypoxic in nature, as coronary sinus blood flow is high and as coronary vessels are dilated. Difficult situations may arise, when septic cardiomyopathy develops in patients with pre-existing coronary heart disease. The severity of myocardial depression correlates with a poor prognosis, heart failure accounting for about 10% of fatalities from sepsis and septic shock. Septic cardiomyopathy is prevalent in Gram-positive, Gram-negative, fungal and viral sepsis, and left ventricular stroke work indices are compromised to a similar degree independent of the causative germ, pointing at the relevance of the final mediator pathways as opposed to the specific virulence factors.

The etiology of the disease is multifactorial. Several candidates with potential pathogenetic impact can be addressed: bacterial toxins, cytokines and mediators including nitric oxide, cardiodepressant factors, oxygen reactive species, catecholamines. Using cultures of neonatal rat cardiomyocytes, several "negative inotropic cascades" were identified. Experimentally supported concepts include the endotoxin-induced release of cytokines with cardiodepressant

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action, primarily tumor necrosis factor α (TNFα) and interleukin-1; induction of inducible nitric oxide synthase (iNOS) in cardiomyocytes was shown for endotoxin and interleukin-1; TNFα has pleiotropic effects including a concentration-dependent iNOS-induction, a decreased synthesis of precursors of the phosphoinositide pathway, interference with the β-adrenoceptor/G-protein/adenylyl cyclase-pathway and a decrease in calcium transient; a cardiodepressant factor (CDF) isolated from blood of patients in septic/cardiogenic shock blocks calcium current into cardiomyocytes.

## INTRODUCTION

### Link of the renin-angiotensin system to cytokine biology

Apart from its pharmacological activities, angiotensin II also has cytokine-like activities. It acts as a growth factor in the cardiovascular system and has been implicated in angiogenesis [1]. Blocking angiotensin-converting enzyme (ACE) can be beneficial in the treatment of patients with chronic heart failure [2], post myocardial infarction [3,4], and may also improve endothelial vasomotor dysfunction in normotensive patients with coronary artery disease [5]. Part of these effects is linked to vasodilatation secondary to a diminished breakdown of bradykinin, which is known to enhance endothelial NO release [6]. Although the NO releasing potential of ACE inhibitors is very well documented in the vasculature, little is known about the possible interaction of cardiac angiotensin, bradykinin, and NO metabolism and whether there is a pharmacological impact of ACE inhibition on NO in the heart. In neonatal rat cardiomyocytes, the bradykinin receptor subtype 2 was detected and characterized by radioligand studies and found to mediate a negative inotropic effect [7]. Experimental data indicate that the local tissue activity of ACE regulates the expression of endothelial nitric oxide synthase (ecNOS), which thus contributes to chronic modulation of endothelial function [8]. Recent studies provided evidence that ecNOS is also expressed in cardiomyocytes of human atrial myocardium [9] and that ACE inhibitor treatment of patients upregulates the expression of ecNOS in the human atrial myocardium [10]: The Holtz group studied myocardial specimens of patients undergoing cardiac surgery and demonstrated that patients who had been under pretreatment by ACE inhibitors had an elevated expression of ecNOS at the level of mRNA, protein, and enzyme activity. This ACE inhibitor-induced upregulation of ecNOS expression did not appear because of treatment-induced shifts in NYHA classification of heart failure. The functional consequences of this increase in ecNOS activity are to be elucidated, and it remains to be established whether cardiac NOS could be a target of pharmacotherapy in heart disease.

### NO in the heart—physiology and pathophysiology

A physiological and pathophysiological role of NO in the regulation of cardiac contractility has by now been elaborated (reviewed in [11–13]). Apart from the constitutive, calcium/calmodulin-dependent ecNOS (NOS3), which produces picomolar quantities of NO and L-citrulline from L-arginine, the expression of an inducible form, (iNOS) (NOS2), within cardiac myocytes is evidenced under

various pathological conditions. Neuronal, calcium-dependent NOS (NOS1) is found in intracardiac sympathetic neurons and specialized cardiac conduction tissue, but not in cardiac myocytes.

By its iron-chelating properties, NO was shown to increase the activity of a soluble form of the guanylyl cyclase of the cardiomyocytes and probably also increases the activity of a particulate form of the guanylyl cyclase of the cardiomyocytes, with a consecutive rise in cyclic guanosine monophosphate (cGMP). cGMP compromises systolic and diastolic heart function. An inhibition of calcium handling probably is involved via

activation of a cGMP-dependent cAMP phosphodiesterase,
a cGMP-dependent protein kinase regulating L-type calcium channels,
and a cGMP-dependent protein kinase altering calcium sensitivity of myofilaments.

The role of ecNOS in the cardiomyocyte is emerging:

The endogenous constitutive NO pathway was shown to be involved in muscarinergic cholinergic signal transduction via enhancement of cGMP;
An increase in cGMP by ecNOS or iNOS can antagonize the inotropic properties of the β-adrenoceptor-G protein-adenylyl cyclase system, which thus interferes with β-adrenergic signaling;
Cardiac myocyte ecNOS can be activated by increases in time-averaged $[Ca^{2+}]_i$ provoked by higher beating frequencies;
Preceding the expression of iNOS, high concentrations of cytokines may lead to a rapid NO-dependent negative inotropy, presumably via ecNOS activation.

iNOS is an enzyme of the bactericidal arsenal of the mammalian organism and has a capacity to synthesize NO in nanomolar quantities, which thus promotes not only bacterial killing, but also detrimental cytotoxity of mammalian cells which eventually leads to apoptosis. iNOS is not subject to regulation by calcium/calmodulin, but rather the amount of NO produced depends on the number of enzyme molecules. In numerous experimental models, iNOS induction by cytokines and endotoxin was verified.

Data from several groups are now available concerning the induction of iNOS in heart disease, however, the number of patients studied still is limited:

The group of de Belder and Moncada [14,15] found, studying human myocardium, that iNOS was expressed in dilative cardiomyopathy, myocarditis, and postpartum cardiomyopathy, but found that, in seven patients with ischemic heart disease, a clear predominance of cNOS activity was measurable;
Haywood et al. [16] found iNOS expression in specimens of failing myocardium in 67% of patients with dilative cardiomyopathy (16/24), 59% of patients with ischemic heart disease (10/17), and 100% of patients with valvular heart disease (10/10);

Habib et al. [17] showed strong histochemical immunoreactivity for iNOS in myocytes in human dilative cardiomyopathy and less in ischemic heart disease;
Thoenes et al. [18] reported iNOS expression in four out of six hearts of septic patients only, but not in hearts failing from dilative (n = 9) or ischemic cardiomyopathy (n = 7), Becker muscular dystrophy (n = 2), or mitoxantrone-induced toxic cardiomyopathy (n = 1);
Wildhirt et al. [19] studied hearts of patients with lethal myocardial infarction and found iNOS expression in nonnecrotic cardiomyocytes within or close to the infarcted region;
Lewis et al. [20] found that iNOS induction in human cardiac allografts is associated with contractile dysfunction.

Currently, the pathophysiological impact of NO in heart disease is a matter of intensive research. The role of NO in pathogenesis has so far best been studied in

**Table 1.** Terminology

*Infection*: A microbial phenomenon characterized by an inflammatory response to the presence of microorganisms or the invasion of normally sterile host tissue by those organisms.

*Bacteriaemia*: The presence of viable bacteria in the blood.

*SIRS* (systemic inflammatory response syndrome): The systemic inflammatory response to a variety of severe clinical insults, including infection, pancreatitis, ischemia, multiple trauma and tissue injury, hemorrhagic shock, immune-mediated organ injury, and exogenous administration of inflammatory mediators such as tumor necrosis factor or other cytokines. SIRS is manifested by (but not limited to) 2 or more of the following conditions:

1. temperature: >38.0°C or <36.0°C;
2. heart rate: >90 beats/min;
3. respiratory rate: >20 breaths/min or $P_aCO_2$ < 32 mm Hg;
4. white blood count: >12,000 cells/$mm^3$, <4000 cells/$mm^3$, or >10% immature (band) forms.

*CARS* (compensatory anti-inflammatory response syndrome): Anti-inflammatory reaction, manifesting clinically—following a pro-inflammatory phase—as anergy, an increased susceptibility to infection, or both.

*MARS* (mixed antagonistic response syndrome): Antagonistic reaction syndrome with multiple surges of SIRS and CARS.

*Sepsis*: The systemic response to infection. This response is identical to SIRS except that it must result from infection.

*Severe sepsis*: Sepsis associated with organ dysfunction, perfusion abnormalities, or hypotension. Perfusion abnormalities may include (but are not limited to) lactic acidosis, oliguria, and an acute alteration in mental status.

*MODS* (multiple organ dysfunction syndrome): Presence of altered organ function in an acutely ill patient such that homeostasis cannot be maintained without intervention.

*Sepis-induced hypotension*: Systolic blood pressure <90 mm Hg or reduction of >40 mm Hg from baseline, in the absence of another known cause for hypotension.

*Septic shock*: Sepsis-induced shock with hypotension (as defined above) despite adequate fluid resuscitation, in conjunction with perfusion abnormalities (as defined above). Patients who are on inotropic or vasopressor agents may not be hypotensive at the time that perfusion abnormalities are measured, yet may still be considered to have septic shock.

*Refractory septic shock*: Septic shock without rapid response to volume resuscitation and vasopressors.

*Acute septic cardiomyopathy*: Cardiac impairment in the scope of sepsis, resulting in a decreased pump function of the heart relative to systemic vascular resistance.

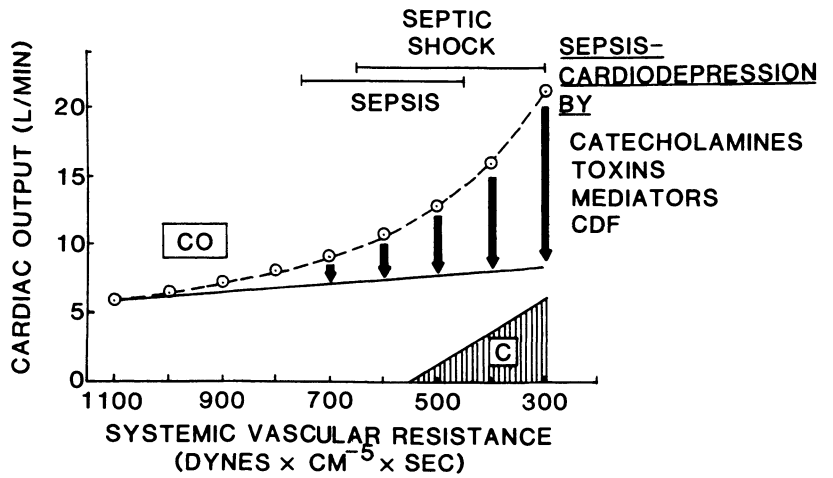


**Figure 1.** Myocardial depression in sepsis. To compensate for a progressive vasodilatation (decrease in systemic vascular resistance), the calculated rise in cardiac output (-----) should be necessary to maintain a mean arterial pressure of 90 mm Hg, according to the following equation: [mean arterial pressure—mean right atrial pressure (10 mm Hg)] = systemic vascular resistance (dynes × s × $cm^{-5}$; normal value 1100 ± 200) × cardiac output (2/min) [80]. The cardiac output measured in sepsis and septic shock (——), however, is usually lower than the calculated values, probabaly due to the cardiodepressant effects of catecholamines, bacterial toxins, sepsis mediators and cardiodepressant factor CDF. CO, cardiac output; C, catecholamines necessary to stabilize blood pressure. Adapted from [11].

the heart in sepsis (definitions see table 1), which may serve as a model for inflammatory disease of the heart and a model for investigating the consequences of an altered NO generation in the heart.

## SEPTIC CARDIOMYOPATHY: CLINICAL CHARACTERISTICS OF THE DISEASE

### Diagnosis in the clinical setting

Only in the early 1980s were Parker and Parrillo able to first unequivocally demonstrate the existence of human septic myocardial depression utilizing nuclear imaging technology [21]. Even now, the impairment of the heart within the scope of sepsis or multiple organ dysfunction syndrome is frequently underscored, since cardiac outputs of septic patients are seemingly normal or may even be enhanced. However, heart failure becomes best evident when cardiac output is considered in relation to the systemic vascular resistance, which is severely lowered due to sepsis-induced vessel damage and consecutive vasodilatation (figure 1). A healthy heart could compensate for the pathological fall in afterload by an increase in cardiac output up to 20 l/min, while the values observed in septic patients are considerably lower. Some evidence has accumulated in recent years about the etiology of myocardial depression in sepsis. The etiology of the disease is multifactorial. Several candidates with potential pathogenetic impact can be addressed: bacterial toxins,

cytokines, and mediators, including nitric oxide, cardiodepressant factors, oxygen reactive species, and catecholamines (for review see [11]). Using cultures of neonatal rat cardiomyocytes, several "negative inotropic cascades" were identified [22]. Some experimentally supported concepts will be presented in this paper.

The impressive impact of the pathologically reduced afterload in sepsis on heart function is conspicuous in figure 2, which gives several measurements of cardiac index of two patients recovering from septic shock, in whom heart function was at best slightly depressed. Cardiac index reached values of 9 l/min/$m^2$ BSA at low systemic vascular resistances, but declined in response to an increase in afterload. When judging heart function in critically ill patients, two things must be considered: (1) all parameters describing heart function are normalized to a systemic vascular resistance of 1100 dyn $\times$ $cm^{-5}$ $\times$ sec, and (2) reference values for hemodynamics or echocardiography have not been defined in patients, whose systemic vascular resistance is decreased. Thus, in the clinical setting, diagnosis is best confirmed by hemodynamic measurements in combination with radionuclid ventriculography. However the latter is only available in few intensive care units.

The clinical picture of septic cardiomyopathy (table 2) is best evident when left and right ventricular stroke work indices are calculated. Cardiac impairment is frequently masked by a seemingly normal cardiac output. However, relative to the lowered systemic vascular resistance in sepsis, which results in a reduced afterload, cardiac outputs and ventricular ejection fractions of septic patients are often not adequately enhanced. In septic cardiomyopathy, both right and left ventricle involvement, global and regional contractile disturbances, and systolic pump and diastolic relaxation failure occur. Septic cardiomyopathy is potentially reversible. Due to the increased ventricular compliance, the heart can be considerably enlarged, particularly in response to volume substitution. The disease is not primarily hypoxic in nature since coronary sinus blood flow is high and coronary vessels are dilated. Difficulties may arise when septic cardiomyopathy develops in patients with pre-existing coronary heart disease [23], where septic myocardial depression may overlap with myocardial ischemia. The increase in heart index in sepsis causes an increased myocardial oxygen demand, which, in the presence of coronary stenoses, can aggravate regional ischemia. On the other hand, an ongoing therapy with betablockers, nitrates, and calcium antagonists for coronary artery disease may deteriorate the labile cardiovascular situation of the septic patient. The "stiff" ventricle in coronary artery disease implies an increased sensitivity towards volume substitution, which yields a more rapid increase in left ventricular end-diastolic pressure.

When septic and cardiogenic shock coincide, hemodynamic parameters (cardiac index, systemic vascular resistance) are helpful in determining the dominant shock event.

**Prognosis and therapy of septic cardiomyopathy**

Septic cardiomyopathy may be completely reversible. A high left ventricular end-diastolic volume index and coherently low ventricular ejection fraction in the acute

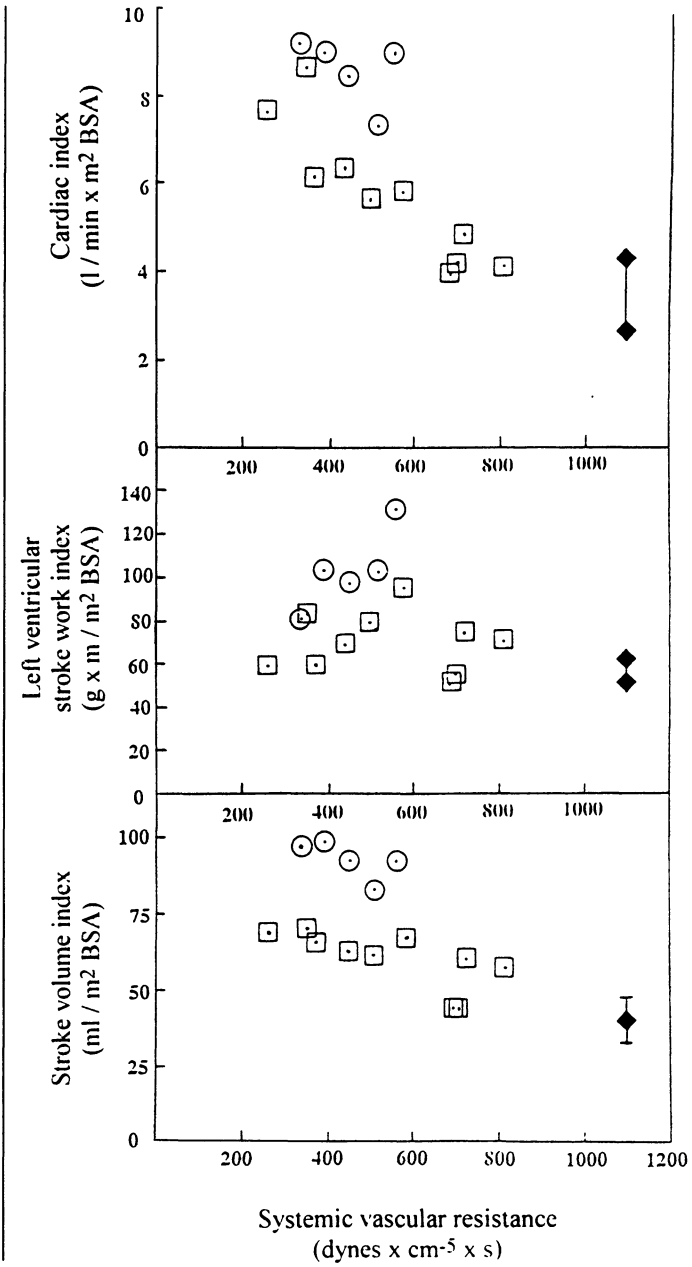


**Figure 2.** Cardiac function of two patients with septic shock: Influence of pathological afterload reduction. In both patients, parameters of heart function have been monitored over a period of 20 days (patient no. 1, O) and 8 days (patient no. 2, □), respectively, during the recovery phase from septic shock. Data are given in relation to the systemic vascular resistance. For comparison, values of healthy individuals with a normal vascular resistance of 1100 dynes × s × $cm^{-5}$ are given (◆). Adapted from [11].

**Table 2.** Clinical features of septic cardiomyopathy

- Inadequate rise in cardiac index, taking the lowered systemic vascular resistance into account
- No increase in stroke volume (LV, RV)
- Ejection fraction (LV, RV) decreased
- Regional and global cardiac dysfunction
- Considerable dilatation of the heart
- Increase in ventricular compliance
- Contraction and relaxation abnormalities
- Coronary arteries dilated, high coronary sinus blood flow
- Right ventricular failure due to ARDS-induced pulmonary hypertension

phase are considered to be favorable signs [24]. Nevertheless, septic cardiomyopathy accounts for about 10% of the fatalities in sepsis and septic shock [25], which constitutes a major cause of death. The severity of myocardial depression is associated with a poor prognosis [26]. Forty percent of fatalities from sepsis and septic shock result from intractable vasodilatation, and 50% result from irreversible multiorgan failure [25].

It is alarming to note that despite all of the new generations of antibiotics and the progress in critical care medicine, mortality of sepsis and septic shock is still as high today as mortality in the beginning of this century (figure 3) [11,27]. It can be inferred that fighting the causative microorganisms is not enough in this clinical situation.

At present, therapy of septic cardiomyopathy is merely symptomatic. No causal treatment regimen has been established for clinical routine, although some regimens are under investigation (reviewed in [11,27]). When considering treatment of myocardial depression, it must not be regarded as a condition isolated from the systemic disease, but rather should be considered in the context of the treatment of sepsis, septic shock, and MODS (for definitions see table 1, [28,29]). Therapy is based on several columns:

management of shock,
elimination of the infectious focus, antibiotics, and antiinfectious measures,
supportive treatment of multiorgan dysfunction syndrome,
additive therapy of systemic inflammatory response (interruption of the toxin-mediator network by neutralization, antagonization, and elimination),
treatment of the underlying disorder.

Detailed guidelines for sepsis therapy were published by the European Society of Intensive Care Medicine, Society of Critical Care Medicine, and the American College of Chest Physicians [28,30].

### Pathogenesis of septic cardiomyopathy: heart disease in the scope of systemic inflammatory response

It seems that the septic malfunctioning of organs, including the heart, that occurs in sepsis is not simply determined by the invading germs, but rather by the over-

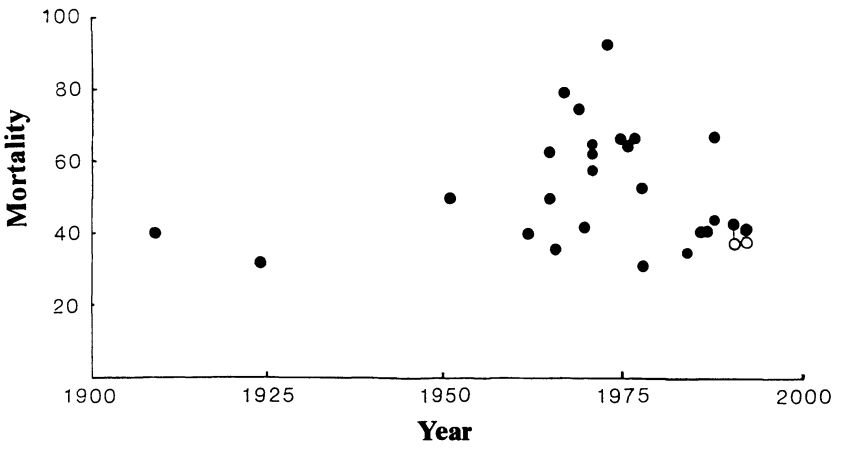


**Figure 3.** Mortality of sepsis and septic shock. Data of more than 3000 patients are collected from the literature, with every point (●) representing information from a large sepsis trial of the according year. In case of double-points (●-○), the results of placebo-controlled sepsis treatment trials (○ verum group; ● placebo group) are given. Adapted from [11].

whelming activation of endogenous cytokine and mediator cascades in response to the bacterial/viral/fungal invasion; septic cardiomyopathy is prevalent in Gram-positive, Gram-negative, fungal, and viral sepsis, and left ventricular stroke work indices are compromised to a similar degree independent of the causative germ, which points to the relevance of the final mediator pathways as opposed to the specific virulence factors (figure 4) [31]. This concept of a cardiomyopathy in the scope of a systemic inflammatory response (SIRS) implies that noninfectious stimuli also may trigger mediator cells to release primary and secondary mediators that are interrelated in a mediator network (figure 5) [11]. SIRS cardiomyopathy may be encountered in patients after severe trauma, heart surgery with the use of the cardiopulmonary bypass [32], pancreatitis, burns, ischemia-reperfusion, and other severe disorders.

Thus, there is a pattern of cardiac impairment common to all inflammatory stimuli, which may be modified by germ- and trauma-specific compounds.

However, SIRS evokes an anti-inflammatory counter-reaction, which is referred to as compensatory anti-inflammatory response syndrome (CARS). In the disease course, episodes of SIRS and CARS may alternate. This state of alternating episodes is known as mixed antagonistic response syndrome (MARS) (definitions and terminology see Table 1 [28,29]). Presently, research to elucidate these complex regulatory processes and the mechanisms of organ failure in this setting is limited to describing proinflammatory components.

### Pathogenesis of septic cardiomyopathy: the most attractive hypothesis

Current concepts concerning the pathogenesis of septic cardiomyopathy concentrate on the cardiodepressant effects of NO, which is thought to be generated in the

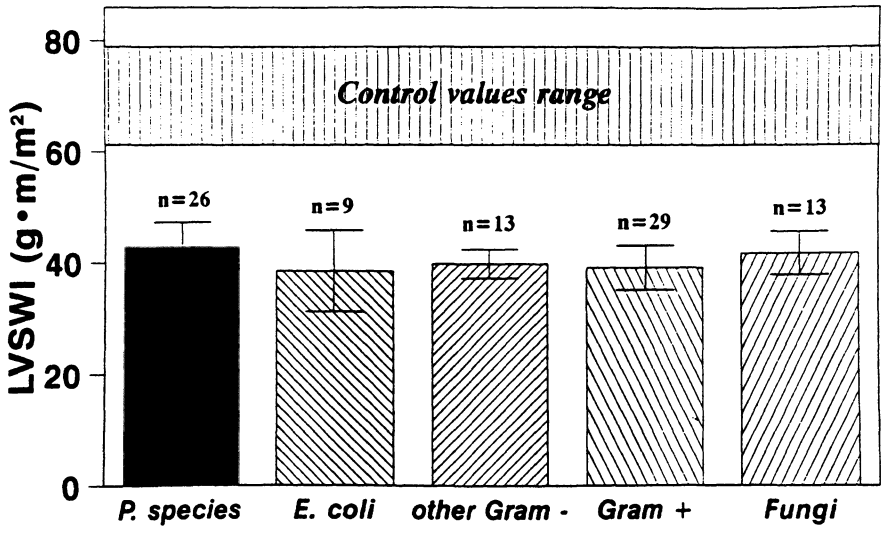


**Figure 4.** Decreased left ventricular stroke work indices in various forms of Gram-negative, Gram-positive, and fungal sepsis. Adapted from [23].

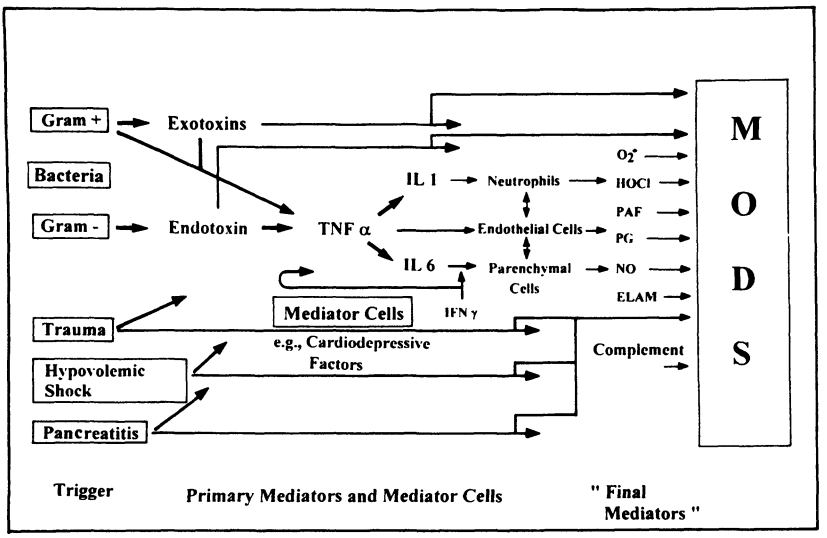


**Figure 5.** Development of multiple organ dysfunction syndrome. Adapted from [11].

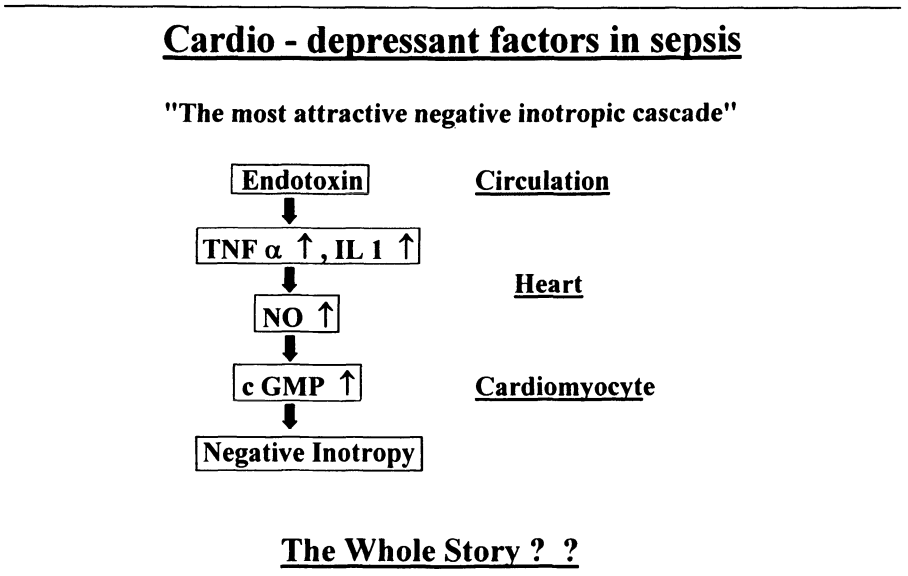


**Figure 6.** Plasma cardiotoxic factors in sepsis: the most attractive hypothesis. Adapted from [11].

septic heart at an accelerated pace and in large amounts [11–13]. The most attractive hypothesis postulates a cascade of reactions in response to circulating endotoxin (figure 6), which triggers mediator cells to release proinflammatory cytokines, like tumor necrosis factor α (TNFα) and interleukin-1 (IL-1). The latter is described as important [33] or less relevant [34]. These cytokines are thought to induce iNOS in the heart. The consequence is an accelerated release of NO from L-arginine, which results in enhanced cGMP-production, which then results in cardiodepression.

Numerous arguments favor this hypothesis, but data also infers that this may not be the whole story. In the following text, relevant clinical findings in humans will be presented and discussed.

*Endotoxin: a cardiodepressant substance in vivo*

Endotoxin consists of bacterial lipopolysaccharides (LPS) in the outer membrane of Gram-negative bacteria. The molecules' active moiety is the conserved part, named lipid A, which carries the biological activity of endotoxin [35].

Endotoxin was administered to healthy volunteers and produced a temporary cardiovascular impairment in these individuals, which mimicked acute septic cardiomyopathy, with a decrease in left ventricular stroke work index and an increase in heart size [36]. However, currently no endotoxin receptor mechanism has been identified in the heart. In contrast, it is well established that LPS activates macrophages and monocytes by binding to the membrane-bound CD14 molecule present on mononuclear cells (figure 7) [37,38]. The binding of LPS to CD14 is

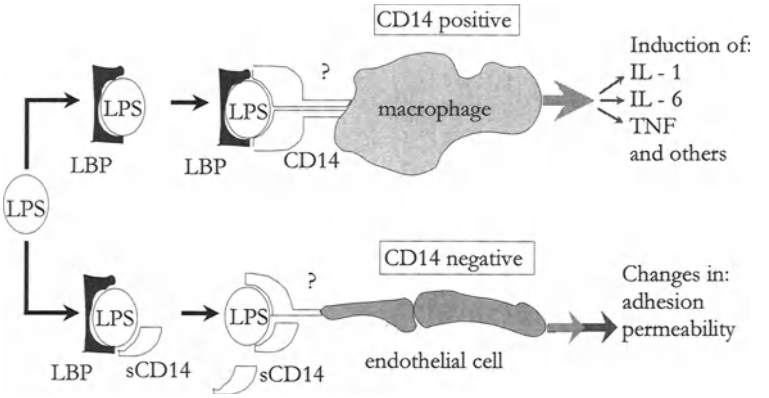


**Figure 7.** Endotoxin receptor mechanism in monocytes/macrophages carrying CD 14 and endothelial/smooth muscle cells devoid of CD14. Adapted from [40].

enhanced if LPS forms a complex with lipopolysaccharide binding protein (LBP), which is synthesized by the liver and present in the blood [39]. Endothelial cells [40] and vascular smooth muscle cells [41] devoid of membrane-bound CD14 are activated by endotoxin via a soluble CD14 molecule associating with LPS (figure 7). In response to the binding of endotoxin, both macrophage and endothelial cells produce large amounts of TNFα.

Consistently, individuals having received endotoxin exhibit high TNFα plasma levels [42]. It was tempting to speculate, therefore, that the rise in serum TNF levels might provoke the myocardial depression that is witnessed in sepsis.

*TNFα: a cardiodepressant substance in vivo*

Plasma and serum levels of TNFα were found to be elevated not only in sepsis and septic shock, but also in various forms of nonseptic heart disease, such as cardiac hypertrophy, severe heart failure, acute myocardial infarction, angina pectoris, ischemia-reperfusion injury, acute viral myocarditis, and heart allograft rejection (for review see [43]). Although cardiodepression by TNFα application, which mimicks the clinical and hemodynamic features of acute septic cardiomyopathy—like in the case of endotoxin administration [48–50]—has been evidenced in mammalian organisms, including humans [44–47], it is unclear whether enhanced TNFα levels in nonseptic pathological heart conditions are an epiphenomenon or whether they indicate a causal role of the cytokine [51]. Yet clinical septic cardiodepression was shown to improve after administration of TNFα antibodies [52,53].

Intricate knowledge is now at hand about the receptors mediating these TNFα effects [54]. They are present on nearly all cell types of the mammalian organism and are subject to regulation. There are two receptor subtypes, referred to as p55 and p75. After ligand coupling in trimeric formation, both mediate an activation of the

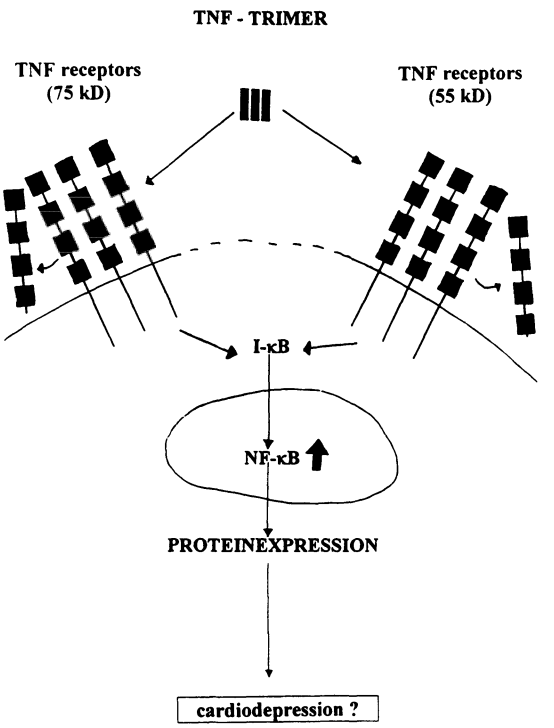


**Figure 8.** TNF receptor subtypes p55 and p75. NF-KB, nuclear factor-kappa B.

transcription factor nuclear factor kappa B. In consequence, a pleiotropic array of effects has been observed in target cells, including cardiodepression (figure 8).

In 1995, Torre-Amione et al. first reported the existence of both TNF receptor subtypes in the human myocardium on the human cardiomyocyte [55]. From experiments with feline cardiac myocytes, the authors inferred that the negative inotropic TNF effect is mediated by the p55 receptor, whereas the role of the p75 receptor in the human heart is unknown.

Efforts are being, and have been, taken by many groups to identify which signal eventually might intracellularly mediate negative inotropy by TNFα. The major candidate in focus is an induction of iNOS with a consecutive rise of NO: by applying very high TNFα concentrations in diverse experimental settings, several groups were able to induce a rapid, NO-dependent cardiodepressant effect [56–60]. Other groups, however, reported NO-independent cardiodepression by TNFα [60–63]. Thus, the negative inotropy by TNFα, as described in numerous experimental settings, varies considerably with respect to the TNFα concentrations applied, the kinetics of the process, the documented impairment of the contractile state in detail, and the NO dependency [61].

*NO: a cardiodepressant substance in vivo*

Cardiac effects of NO in physiological doses were studied by Paulus et al. [64]. They applied the NO donor sodium nitroprusside in patients undergoing cardiac catheterization for atypical chest pain without evidence of heart disease. A low-dose bicoronary infusion of sodium nitroprusside ($\leq 4\,\mu g/min$) did not significantly reduce ejection fraction or stroke volume but exerted potentially beneficial effects on left ventricular relaxation (earlier onset) and diastolic distensibility (increased); peak left ventricular systolic pressure was reduced, but there was no change in left ventricular $dP/dt_{max}$. Infusion of an identical dose of sodium nitroprusside into the right atrium failed to reproduce these effects, which indicates that they could not be attributed to systemic vasodilatation. These findings point to a modulation of left ventricular performance by paracrine NO.

Strongly increased production of NO in human sepsis and septic shock was demonstrated by enhancement of the stable end products nitrite and nitrate [65]. Furthermore, nitrite/nitrate levels of septic patients correlate with systemic vascular resistance and disease severity [66]. The impact of NO on heart function can be studied by use of antagonistic drugs in septic patients. Competitive, nonselective blockade of NOS in human septic shock was achieved in several noncontrolled studies, which uniformally led to an increase in systemic vascular resistance [13]. However, contractile performance of the left ventricle was reported to be either unaltered, increased, or impaired in these studies (for review see [67]). Methylene blue, an inhibitor of guanylyl cyclase and a probable inhibitor of other enzymes, was administered to septic patients. Mean arterial pressure increased; pulmonary artery pressure and cardiac filling pressures and output were not significantly affected; but left ventricular stroke work index after 60 min was enhanced [68]. However, methylene blue was not beneficial to the survival of these patients. In fact, nonselective blockade of NOS may potentially be harmful, e.g., by increasing preload of the left ventricle, by raising pulmonary artery blood pressure, or by decreasing coronary artery blood flow [67]. The combination of inhaled NO with systemic NOS blockade has been studied in an animal model [69]. Further efforts using selective inhibitors of iNOS may prove to be more helpful, although iNOS is not induced in all septic hearts [18].

## SEPTIC CARDIOMYOPATHY: INVESTIGATIONS ON CELLULAR MECHANISMS IN ISOLATED CARDIOMYOCYTES

The cardiodepressant profiles of endotoxin, TNFα, interleukin-1, and NO were investigated in neonatal rat cardiomyocytes, which allowed for a correlation of contractile performance on a single cell level with biochemical measurements [11,22,99,100].

### The place of isolated cardiomyocytes in researching mechanisms of septic cardiomyopathy

In clinical studies, animal experiments, and work with isolated heart preparations, direct effects of bacterial toxins or mediators involved in sepsis often interfere with

indirect effects and are difficult to discern. Primary effects on the coronary circulation and stimulation of local mediator cells or nerval effects superimpose direct negative inotropic and cytotoxic mediator actions. Although the methodological limitations are conspicuous, experiments with isolated heart muscle cells may help to pin down the various processes attacking the cardiomyocyte in systemic inflammatory response. Several negative inotropic cascades were thus identified.

**Materials and methods** [99,100]

Fetal calf serum, CMRL-1415-ATM medium, DMEM, horse serum, and collagenase (Worthington, CLS II): Biochrom (Berlin, F.R.G.); recombinant human tumor necrosis factor α, bovine insulin (24–25 I.U./mg), bovine serum albumin, dexamethasone, (−)-noradrenaline, timolol, (−)-isoproterenol, lipopolysaccharide (*Escherichia coli*), sodium nitroprusside, diethyl pyrocarbonate (DEPC), phenylmethylsufonyl fluoride (PMSF): Sigma (Deisenhofen, Germany); murine tumor necrosis factor α; Knoll AG (Ludwigshafen, Germany); interleukin-1β: Promocell (Heidelberg, Germany); HEPES, ampholytes (Servalyte, pH 2–4), sodium dodecyl sulfate, glycine, β-mercaptoethanol, and trypsin (1:250): Boehringer Ingelheim (Heidelberg, Germany); tobramycin: Lilly (Bad Homburg, Germany); urea, piperazine diacrylamide: Biorad (Munich, Germany); dithiothreitol: Biomol (Hamburg, Germany); WITAlytes: WITA (Teltow, Germany); guanidinium-isothiocyanate, caesium chloride, random primers, specific primers, Superscript Plus, 100 bp-DNA-ladder, agarose: Gibco/BRL (Eggenstein, Germany); RNase-inhibitor, Taq-DNA-polymerase: AGS (Heidelberg, Germany); dNTPs set, IPG dry strips (pH 4–7, 110 × 3.3 $mm^2$), pharmalytes, acrylamide, N,N′-methylenebisacrylamide, ammonium persulfate, N,N,N′,N′-tetramethylethylenediamine, bromophenol blue: Pharmacia (Freiburg, Germany); DNA Sequencing Kit: Perkin Elmer (Berlin, Germany). All other chemicals were of analytical grade and were purchased from Merck (Darmstadt, F.R.G.) or Boehringer (Mannheim, F.R.G.) or Sigma (Deisenhofen, F.R.G.). Culture flasks (25 $cm^2$) and multidish-6-well plates: Becton Dickinson Europe (Meylan, France).

*Monolayer cultures: preparation and cultivation*

Preparation and cultivation of monolayer cultures of spontaneously contracting neonatal rat heart myocytes from the hearts of one- to three-day-old Wistar rats were carried out essentially as described [70] by repeated tissue incubations in 0.12% trypsin-0.03% collagenase-salt solution ($Ca^{2+}$- and $Mg^{2+}$-free) at 37°C. Muscle cells were separated from nonmuscle cells by the differential attachment technique. The suspension of muscle cells in CMRL-1415-ATM medium (adjusted to pH 7.4 at room temperature) containing 10% fetal calf serum, 10% horse serum, 10 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid), and 0.02 mg/ml tobramycin was distributed into 25 $cm^2$ plastic culture flasks or multidish 6-well plates (1.0 to 1.5 × $10^5$ cells/$cm^2$). After 24 hours, when the cells formed a monolayer and contracted spontaneously, the serum-supplemented medium was

replaced by serum-free CMRL-1415-ATM medium containing 10 mmol/l HEPES, 0.1 μmol/l dexamethasone, 5 μmol/l insulin, 0.4 μmol/l iron-saturated transferrin, 0.4 μmol/l bovine serum albumin, and 0.02 mg/ml tobramycin, pH 7.4 (serum-free CMRL medium). When indicated, dexamethasone was omitted from the medium. The cells were cultured for up to 3 days. TNFα culture periods of 3 days were applied to achieve a maximum effect whenever α-adrenoceptor-mediated reactions dependent on the phosphoinositide pathway were studied [71]. The medium was replaced every 24 hours.

*Cardiac nonmuscle cells: preparation*

Cultures of cardiac nonmuscle cells were prepared as described [70]. Nonmuscle cells were cultured in CMRL-medium in the presence of 10% FCS. Medium was changed daily.

*Spontaneously or electrically driven contractions*

Spontaneous or electrically driven contractions of single cultured neonatal rat heart muscle cells within the monolayers were monitored using an electro-optical system as described previously [63,70]. When electrically-driven (60–120 beats/min) cells were studied, the myocytes seeded in 25 $cm^2$ culture plastic flasks were superfused (4 ml/min) with various solutions. The fluid in the culture flask could be exchanged within 2 to 3 min through syringe needles in the flask. Electrical stimulation was accomplished by two steel electrodes for external pacing connected to a Grass SD9 stimulator (Grass Instruments, Quincy, MA). For analysis of the α- and β-adrenergic-stimulated increase in pulsation amplitude and pulsation velocity of the cardiomyocytes, a standard protocol was employed, which consisted of superfusion with a standard serum-free, low-calcium (0.3–0.6 mM) superfusion CMRL-medium without additives, followed by superfusion with medium containing 100 μM noradrenaline and 10 μM timolol (α-adrenergic stimulation) or with 10 μM isoproterenol (β-adrenergic stimulation). For eliciting maximum contractile response, a medium containing a high calcium concentration of 2.4 mM was employed.

As parameters of contractile performance, pulsation frequency and baseline pulsation amplitude were registered in spontaneously beating cells [72,73]. Pulsation frequency, pulsation amplitude and velocity, and beating regularity were monitored in electrically triggered cells [74].

*Nitrite and protein content determination*

Nitrite concentration of cell culture supernatants was determined by means of the Grieß reaction as described previously [11,61]. Protein content of the cell monolayers was determined according to Lowry.

*Total RNA preparation*

Total RNA was prepared from cardiomyocytes after a defined culture period in the presence, or absence, of TNFα by lysing the cells in guanidinium-isothiocyanate

solution and centrifugation through a caesium chloride cushion as described by Sambrook et al. [75]. Integrity of the RNA was confirmed by agarose gel electrophoresis, and the concentration was determined by measuring the UV absorption at 260 nm.

*Reverse transcription of RNA samples*

Reverse transcription (RT) of RNA samples was accomplished using a standard protocol of SuperScript Plus RNase $H^-$ Reverse Transcriptase for 1 h at 42°C. The reaction mixture contained the following components: 1 µg RNA; 1 × RT-buffer; 0.5 mM each dNTP; 12 ng/µl random primer; 1 mM dithiothreitol; 0.54 U/µl RNase-inhibitor; 4 U/µl reverse transcriptase.

*Polymerase chain reaction*

For RT-polymerase chain reaction (PCR) primers for rat inducible nitric oxide synthase (iNOS) were used: TAC ATG GGC ACC GAG ATT GG (sense) and TGA AGG CGT AGC TGA ACA AGG (antisense). For detecting the cDNA of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal standard the following primers were used: CAT CAC CAT CTT CCA GGA GCG (sense) TGA CCT TGC CCA CAG CCT TG (antisense).

The PCR was performed in a reaction containing the following components: 5–10 µl RT-reaction; 1 × PCR-buffer (complete); 12 µM each dNTP; 5 pmol of each primer; 2 U Taq-DNA-polymerase. Using a thermo-cycler (Trioblock, Biometra, Göttingen, Germany) after 2 min of denaturation at 95°C, the PCR amplification was performed: 30 sec denaturation at 94°C, 30 sec primer annealing at 60°C, 30 sec extension at 72°C (35 cycles for iNOS, and 30 cycles for GAPDH). The PCR products were separated by agarose gel electrophoresis (expected lengths of the amplification products: 580 bp for iNOS and 443 bp for GAPDH) and isolated from the gel by electroelution. Nucleotide sequences of the PCR fragments were determined by automated sequencing with PCR primers and the DNA Sequencing Kit containing dye terminator cycle sequencing ready reactions. The sequencer was from Applied Biosystems (München, F.R.G.).

*Interleukin-6 activity*

Interleukin-6 (IL-6) activity in the cell culture supernatants was determined by the 7TD1-bioassay [76]: briefly, serial fourfold dilutions of samples or standards (10 ng recombinant IL6/ml) were prepared in flat bottom 96-well plates in 50 µl DMEM containing 10% fetal calf serum, $5 \times 10^{-5}$ M 2-mercaptoethanol, L-glutamine, and antibiotics. The same volume (50 µl) of medium containing IL-6-dependent target cells (2,000/well) was added. The cultures were then incubated for three days. To these cultures, MTT (2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; 0.5 mg/ml in phosphate buffered saline) was added for 4 hours. The cultures were incubated with dimethylformamide-solution (5% SDS in 50% dimethylformamide) for at least 2 hours, and absorption of the diazolium salt was

measured at 550 nm in an ELISA reader. The IL-6 activity in the tested samples was calculated by probit analysis [77] in reference to the recombinant IL-6 standard tested in parallel cultures. Each sample was measured three times.

*High-resolution, two-dimensional gel electrophoresis*

For high-resolution two-dimensional gel electrophoresis (2D-PAGE), protein sample preparations of rat heart muscle cells cultured in the absence or presence of TNFα (10 or 100 U/ml; 3 flasks per group) was performed as follows: after determination of the wet weights, the cell pellets (about $6 \times 10^6$ cells per sample) were suspended in buffer containing 50 mM Tris/HCL, pH 7.5, and 1 mM PMSF to obtain a final volume of 150 µl cell suspension per sample. Urea, dithiothreitol, and ampholytes were added to obtain a final concentration of 9 M urea, 70 mM dithiothreitol, and 2% ampholytes. Proteins were solved by carefully stirring for 30 minutes at room temperature. 2D-PAGE combining isoelectric focusing (IEF; first dimension) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; second dimension), as developed by Klose [78], was performed as described [79]. A middle-size gel technique was used. Proteins were focused in vertical tube gels with a diameter of 0.9 mm within an IEF gel chamber (WITA, Teltow, Germany) and separated in the second dimension in vertical slab gels (168 × 160 × 1 mm) within the DALT chamber (Serva, Heidelberg, Germany). 20 µl of the protein sample were applied to the anodic side of the IEF gel containing 4% acrylamide (w/v), 0.3% piperazine diacrylamide, and a total of 2% (w/v) carrier ampholytes WITAlytes, pH 2–11. The proteins were focused for 6800 Vh without cooling. After isoelectric focusing, the gels were equilibrated for 2 minutes in a buffer containing 125 mM Tris/phosphate, pH 6.8, 40% glycerol, 65 mM dithiothreitol, and 3% SDS. The IEF gels were applied onto the SDS-PAGE gels, containing 15% acrylamide (w/v) and 0.2% bisacrylamide. The SDS-PAGE system of Laemmli [80] was used, omitting the stacking gel. Proteins were detected by silver staining as described by Jungblut and Seifert [79].

At least three 2D-PAGE gels per sample were run, and the whole experiment was reproduced for three independent cardiomyocyte preparations. Visual comparisons between the protein pattern of controls and TNFα-treated samples were performed as described by Jungblut and Klose [81].

With three additional independent cardiomyocyte preparations and identical culture conditions, samples were prepared, and IEF was performed using an immobilized gradient of pH 4–7 in the Multiphore II (Pharmacia-LKB, Freiburg, Germany), followed by SDS-PAGE, as described in detail previously [11,82,83].

*Statistical analyses*

Values are given as mean ± SD. All experiments were performed with at least three independent cardiomyocyte preparations. The normality of distributions was analyzed with David's test. The unpaired two-tailed Student's t-test was used for groups with equal variances. In case of unequal variances based on Bartlett's test,

the Welch's approximate t-test was used. For determination of the regularity of beating, the variation coefficient was additionally calculated ($V = \sigma:\mu \times 100$). For multiple comparisons ANOVA was used. "*" indicates $p < 0.05$.

**Cardiodepressant profile of chronic endotoxin-exposure in cardiomyocytes**

Cardiomyocytes pretreated for 1–3 days in the presence of endotoxin (1–10 μg/ml) were compared with untreated control cells in the absence or presence of dexamethasone. The morphological appearance of the cells by phase contrast microscopy and spontaneous beating were unaltered.

By the end of the culture period, cardiomyocytes were challenged with β- or α-adrenoceptor agonists: The increase in pulsation amplitude conspicuous upon administration of the β-adrenoceptor agonist isoproterenol in control cells was absent in cardiomyocytes treated with endotoxin (10 μg/ml) (figure 9, upper graph), but the increase was regularly elicited when the medium had been supplemented with dexamethasone (0.1 μM) (figure 9, lower graph).

In contrast, α-adrenoceptor-induced arrhythmias and increased pulsation amplitude were not suppressed by pretreatment with endotoxin (10 μg/ml) (figures 10 and 11).

**Cardiodepressant profile of chronic TNFα-exposure in cardiomyocytes**

Cardiomyocytes were pretreated for 1–3 days in the presence of TNFα (10 U/ml) and challenged with inotropic stimuli (table 3). Basal pulsation amplitudes of spontaneously beating rat cardiomyocytes after 3 days of culture were not significantly different from control cells, neither in the presence nor in the absence of dexamethasone. In contrast, after a 3 day exposure with TNFα, α-adrenoceptor-induced increase in pulsation velocity, as well as $Ca^{2+}$-induced increase in pulsation velocity, was suppressed. β-adrenergic-stimulated increase in pulsation amplitude was blocked by TNFα (10 U/ml, 24 hours), both in the presence (table 3) and absence of dexamethasone (0.1 μM) (figure 12). After 3 days of culture (TNFα, 10 U/ml), a suppression of α-adrenoceptor-induced arrhythmias was detectable, which is regularly observed in control medium from the strong proarrhythmogenic effect of a high degree of α-adrenoceptor occupation in these cells [83] (table 4). $Ca^{2+}$-induced and isoproterenol-induced irregularity of beating was determined by beat-to-beat-analysis of the intervals between pulsations and comparison of the variances and variance coefficients of beating intervals in the presence of TNFα (10 U/ml, 24 hours), the variances and variance coefficients of beat-to-beat-intervals were considerably smaller than in nontreated controls, which thus indicates a more regular beating (table 4).

**Cardiodepressant effects of NO in cardiomyocytes**

Sodium nitroprusside is known to release the radical nitric oxide in aqueous solutions. Dissolved in synthetic culture medium, sodium nitroprusside (1 mM, 10 mM) leads to a linear, concentration-dependent increase in the nitrite concentration, as stable end product of nitric oxide, for at least 6 hours.

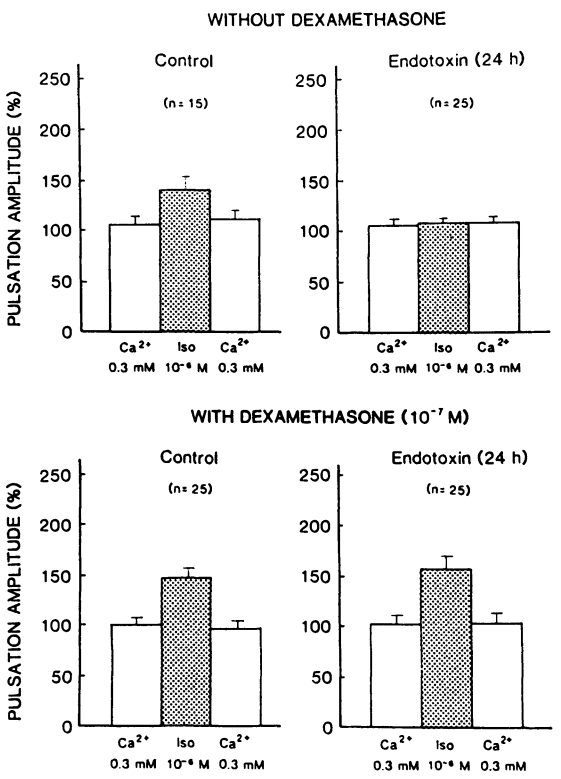


**Figure 9.** Endotoxin-induced depression of the stimulatory effect of isoproterenol on pulsation amplitude in neonatal rat cardiomyocytes: effect of dexamethasone. Neonatal rat cardiomyocytes were cultured for 24 hours in serum-free medium, in the presence or absence of endotoxin (10 μg/ml) and dexamethasone (0.1 μM). After the incubation period, cells were challenged with isoproterenol (1 μM). Cells were kept at a constant beating rate by extracellular electrical stimulation under continuous superfusion with a medium containing 0.3 mM Calcium ($Ca^{2+}$) and were challenged with a superfusion medium supplemented with 1 μM isoproterenol (Iso), followed by a washout phase. In untreated control cells, a significant increase in pulsation amplitude was reproducibly seen, reversible upon washout, whereas 24-hour pretreatment with endotoxin blocked the response to isoproterenol.

As previously reported by other groups, exogenous administration of nitric oxide to cultured cardiomyocytes is contractile-depressant [84]. When applying medium containing freshly dissolved sodium nitroprusside (10 mM) to both spontaneously beating and electrically triggered, superfused neonatal rat cardiomyocytes after 24 hours of culture in synthetic medium, a depressive effect on contractility is witnessed. Both the negative chronotropic effect documented in spontaneously beating cardiomyocytes and the decrease in contraction velocity shown in electrically stimulated cells were reversible upon washout of the substance after 15 to 20 minutes (figure 13). The fall in beating frequency and contraction velocity could be pre-

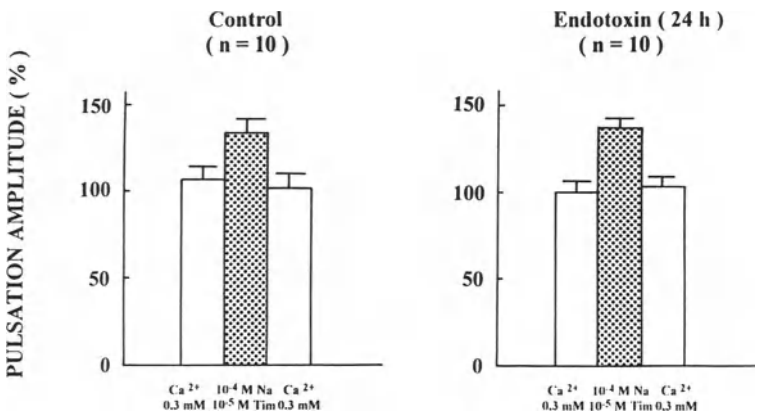


**Figure 10.** Endotoxin does not depress α-adrenoceptor-stimulated increase in pulsation amplitude in neonatal rat cardiomyocytes. Neonatal rat cardiomyocytes were cultured for 24 hours in serum-free and dexamethasone-free medium, in the presence or absence of endotoxin (10 μg/ml). After the incubation period, cells were challenged with a combination of noradrenaline (NA; 100 μM) and timolol (Tim; 10 μM). Cells were kept at a constant beating rate by extracellular electrical stimulation under continuous superfusion with a medium containing 0.3 mM calcium ($Ca^{2+}$).

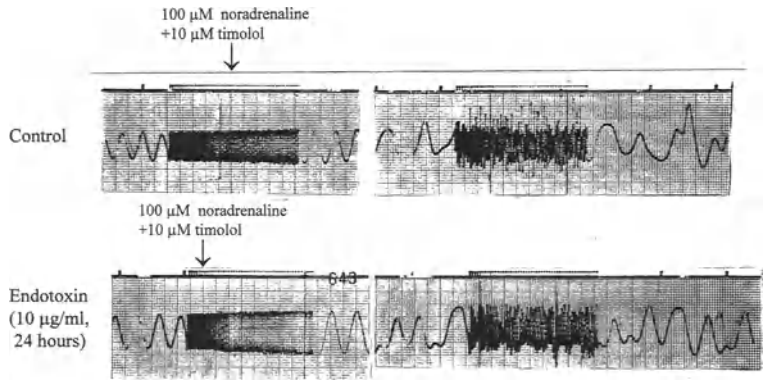


**Figure 11.** Endotoxin does not suppress α-adrenoceptor-provoked arrhythmias in neonatal rat cardiomyocytes. Neonatal rat cardiomyocytes were cultured for 24 hours in serum-free and dexamethasone-free medium, in the presence or absence of endotoxin (10 μg/ml). After the incubation period, cells were challenged with norepinephrine (100 μM) and timolol (10 μM).

vented by applying sodium nitroprusside in the presence of hemoglobin (10 mg/ml), which is known to bind NO extracellularly with high affinity. Contractility of cardiomyocytes was impaired when using concentrations of sodium nitroprusside from 1 mM.

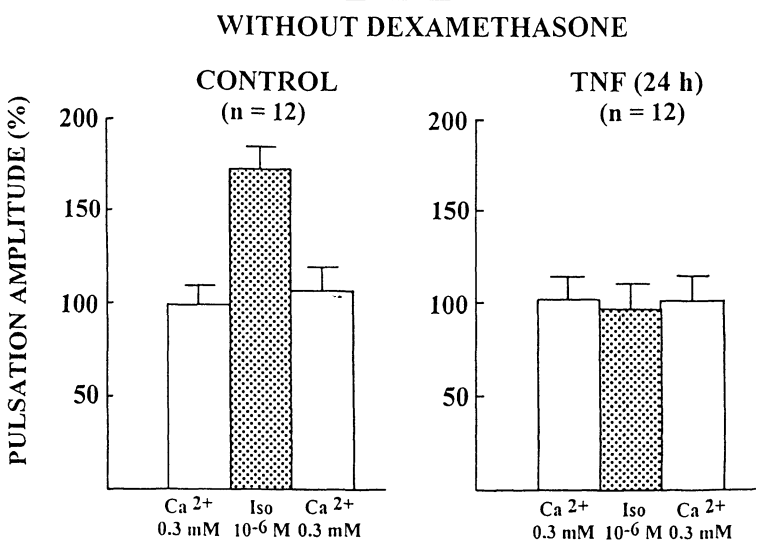


**Figure 12.** Effect of TNFα on β-adrenoceptor agonist induced increase in pulsation amplitude in neonatal rat cardiomyocytes. After culturing cardiomyocytes for 24 hours in the presence of TNFα, without dexamethasone, cells were challenged with 1 μM isoproterenol (Iso), followed by a washout phase. Cells were kept at a constant beating rate by extracellular electrical stimulation under continuous superfusion with a medium containing 0.3 mM calcium ($Ca^{2+}$) and were challenged with a superfusion medium supplemented with 1 μM isoproterenol (Iso), followed by a washout phase. In untreated control cells, a significant increase in pulsation amplitude was reproducibly seen, reversible upon washout, whereas 24-hour pretreatment with low-dose TNFα blocked the response to isoproterenol. Qualitatively the same effect was observed in the presence of dexamethasone (table 3).

**Table 3.** TNFα blocks the stimulatory effect of catecholamines and high calcium on contractility in neonatal rat cardiomyocytes

| | Control | TNFα (10 U/ml) |
|---|---|---|
| Baseline pulsation amplitude [μm], 3-day incubation period | 3.27 ± 1.54 (n = 29) | 3.73 ± 2.10 (n = 34) |
| $\alpha_1$-adrenoceptor-induced increase in pulsation velocity [% of control], 3-day incubation period | 142 ± 27 (n = 19) | 101 ± 7* (n = 10) |
| β-adrenoceptor-induced increase in pulsation amplitude [% of control], 1-day incubation period | 151 ± 14 (n = 11) | 89 ± 22* (n = 11) |
| $Ca^{2+}$-induced increase in pulsation velocity [% of contro], 3-day incubation period | 137 ± 24 (n = 12) | 119 ± 12* (n = 8) |

Note: Cardiomyocytes were cultured for 1 to 3 days in the absence or presence of TNFα. After the culture period, cellular contractile performance was measured in spontaneously beating cells (pulsation amplitude) or was electrically triggered challenged with α- or β-adrenoceptor agonists. *$p < 0.05$ in comparison to control.

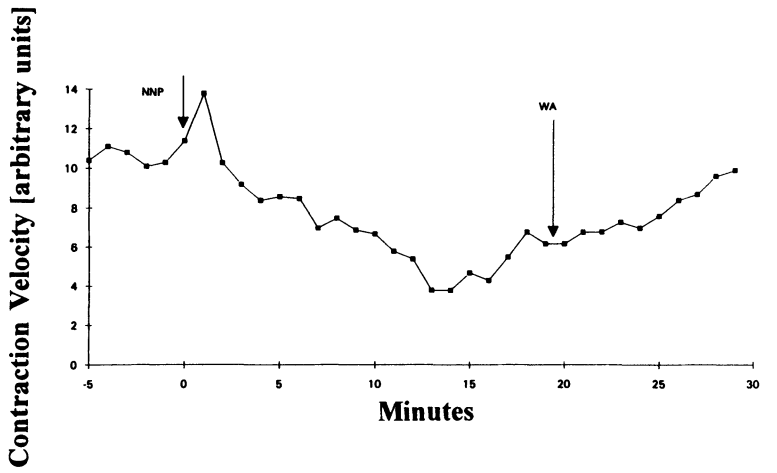


**Figure 13.** Effect of 10 mM sodium nitroprusside on contraction velocity of an electrically triggered neonatal rat cardiomyocyte. NNP, Sodium nitroprusside; WA, Washout.

**Table 4.** Impairment of the proarrhythmogenic action of catecholamines and high calcium by TNFα in neonatal rat cardiomyocytes

| | Control | INFα (10 U/ml) |
|---|---|---|
| $\alpha_1$-adrenoceptor-mediated induction of arrhythmias | 23/28 (82%) | 0/13 (0%) |
| β-adrenoceptor stimulation | | |
| mean beat-to-beat interval | 103 ± 25 | 98 ± 8 |
| (% of mean prestimulation value) | (n = 99) | (n = 39) |
| variation coefficient | 24.3 | 8.2 |
| $Ca^{2+}$-induced beating irregularity | | |
| mean beat-to-beat interval | 119 ± 57 | 91 ± 21* |
| (% of mean prestimulation value) | (n = 99) | (n = 45) |
| variation coefficient | 47.9 | 22.0 |

Note: Cardiomyocytes were cultured for 1 to 3 days in the absence or presence of TNFα. After the culture period, regularity of beating was evaluated in electrically triggered challenged with α- or β-adrenoceptor agonists. *$p < 0.05$ in comparison to control.

### Nitric oxide production of neonatal rat cardiomyocytes and nonmuscle cells in the presence of endotoxin and TNFα

Neither recombinant human nor murine TNFα (10, 100 U/ml) led to an increase in the nitrite content of cardiomyocyte (table 5a) and nonmuscle cell (table 5b) culture supernatants after 24 hours incubation, even when dexamethasone was omitted from the culture medium. TNFα (1000 U/ml) significantly increased the nitrite content only in the absence of dexamethasone. In contrast, lipopolysaccharide (endotoxin) (10 μg/ml) as a positive control potently stimulated nitrite release of both cardiomyocytes and cardiac nonmuscle cells (table 5a,b). Also interleukin-1β

**Table 5a.** Nitrite content [μM] of cardiomyocyte culture supernatants after a 24-hour incubation period

| | Control | rhTNFα 10 U/ml | murine TNFα 10 U/ml | rhTNFα 100 U/ml | murine TNFα 100 U/ml | rhTNFα 1000 U/ml | LPS 10 μg/ml |
|---|---|---|---|---|---|---|---|
| NO DEX | 5.97 ± 1.21<br>n = 35 | 6.17 ± 1.48<br>n = 29 | 6.44 ± 1.18<br>n = 9 | 6.13 ± 1.03<br>n = 27 | 6.78 ± 0.63<br>n = 9 | 9.44 ± 1.61*<br>n = 5 | 15.4 ± 5.8*<br>n = 36 |
| + DEX | 5.19 ± 1.09<br>n = 27 | 4.91 ± 1.22<br>n = 13 | | 5.07 ± 0.12<br>n = 3 | | 5.01 ± 1.64<br>n = 7 | 7.7 ± 4.0<br>n = 6 |

Note: Cardiomyocytes were cultured for 24 hours in synthetic medium without (NO DEX) or with dexamethasone (+DEX, 0.1 μM). Statistical comparisons were made between control and TNFα- or LPS-treated cells within the groups cultured in the absence or presence of dexamethasone, *$p < 0.05$. DEX, dexamethasone; LPS, lipopolysaccharide.

**Table 5b.** Nitrite content [μM] of cardiac nonmuscle cell culture supernatants after a 24-hour incubation period

| | Control | rhTNFα 10 U/ml | rhTNFα 100 U/ml | LPS 10 μg/ml |
|---|---|---|---|---|
| NO DEX | 5.75 ± 1.47<br>n = 17 | 5.45 ± 0.78<br>n = 14 | 5.39 ± 0.81<br>n = 15 | 19.6 ± 12.0*<br>n = 18 |
| +DEX | 4.39 ± 0.66<br>n = 9 | | | 5.9 ± 0.45*<br>n = 6 |

Cardiac nonmuscle cells were cultured for 24 hours in synthetic medium without or with dexamethasone (0.1 μM). Statistical comparisons were made between control and TNFα- or LPS-treated cells within the groups cultured in the absence or presence of dexamethasone, *$p < 0.05$. DEX, dexamethasone; LPS, lipopolysaccharide.

(IL-1β) (100 U/ml, 24-hour culture) was a strong stimulus for nitrite production (data not shown).

### Induction of iNOS in neonatal rat cardiomyocytes by endotoxin and interleukin-1β

In comparison to control, TNFα (1000 U/ml) in the absence of dexamethasone weakly induced the mRNA for iNOS after 24 hours incubation (figure 14). In the presence of 10 U/ml TNFα, iNOS-mRNA was not different from control after 24 hours. In contrast, IL-1β and endotoxin strongly induced iNOS in these cells.

### Proinflammatory effects of TNFα, endotoxin, and IL-1β

TNFα in the absence of dexamethasone elicited a specific proinflammatory response in the cardiomyocytes, documented by an increased release of IL-6 from the cardiomyocyte cultures, which was determined as IL-6 content in the culture supernatant. No morphological alterations were observed by phase contrast microscopy in TNFα (10–1000 U/ml)-treated cardiomyocytes in comparison to nontreated controls. Table 6 gives the values for low-dose TNFα (10 U/ml), compared to values for endotoxin and interleukin-1β. Interleukin-1β is the most potent stimulus for interleukin-6 release.

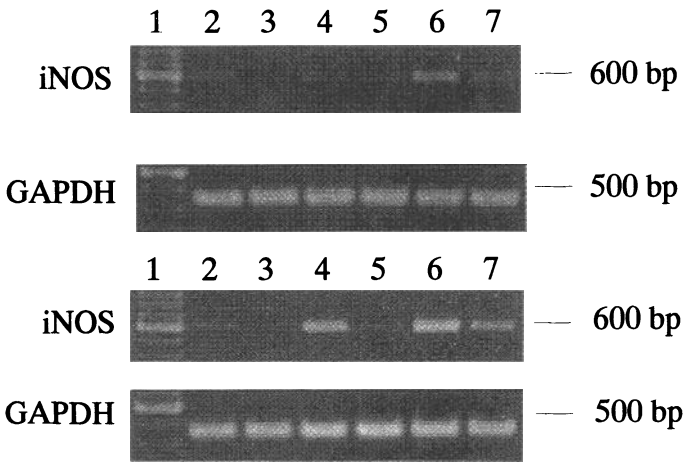


**Figure 14.** Analysis of expression of iNOS mRNA in neonatal rat cardiomyocytes by RT-PCR. After a 24-hour culture period with TNFα (10 or 1000 U/ml) (*A*), interleukin-1β (100 U/ml), or endotoxin (1 μg/ml) (*B*), with or without dexamethasone (0.1 μM), total RNA was isolated from cardiomyocytes and RT-PCR was performed for GAPDH and iNOS, and the products were resolved in an ethidium-bromide stained agarose gel. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; iNOS, inducible nitric oxide synthase. *A*: Lane 1, 100 base pair-DNA ladder; Lane 2, Control medium without dexamethasone; Lane 3, Control medium with dexamethasone; Lane 4, 10 U/ml TNFα without dexamethasone; Lane 5, 10 U/ml TNFα with dexamethasone; Lane 6, 1000 U/ml TNFα without dexamethasone; Lane 7, 1000 U/ml TNFα with dexamethasone. *B*: Lane 1, 100 base-pair-DNA-ladder; Lane 2, Control medium without dexamethasone; Lane 3, Control medium with dexamethasone; Lane 4, IL-1β without dexamethasone; Lane 5, IL-1β with dexamethasone; Lane 6, Endotoxin without dexamethasone; Lane 7, Endotoxin with dexamethasone.

**Table 6.** Proinflammatory effects of endotoxin, TNFα, and interleukin-1β in neonatal rat cardiomyocytes: increase in cell-associated interleukin-1 activity and released interleukin-6 activity

| Stimulus (24-hour period, serum-free) | DEX | Interleukin-6 (released) [pg/ml] | Interleukin-1 (released) [pg/ml] |
|---|---|---|---|
| Control | − | 7,016 ± 1525 | 0 |
| Control | + | 2,516 ± 348 | 0 |
| Endotoxin | − | 15,548 ± 4270 | 95 ± 14 |
| Endotoxin | + | 3,133 ± 1111 | 0 |
| TNFα | − | 15,254 ± 2110 | 0 |
| TNFα | + | 3,299 ± 0 | 0 |
| IL-1β | − | 69,163 ± 22,977 | 289 ± 63 |
| IL-1β | + | 10,634 ± 2311 | 219 ± 48 |

Note: Dexamethasone (DEX), 0.1 μM; Endotoxin, 1 μg/ml; TNFα, 10 U/ml; Interleukin-1β, 100 U/ml.

### Looking for NO-independent mechanisms of TNFα

In agreement with previous studies utilizing cultured cardiomyocytes in diverse experimental settings [22,56,59–63,83,85–87], the present study documents that TNFα blocks the inotropic response to catecholamines (α- and β-adrenoceptor

stimulation) and high extracellular calcium and attenuates α-adrenoceptor-induced arrhythmias. In the experiments reported here, the cardiodepressant profile of TNFα was characterized in detail by using chronic exposure (6–72 hours) at low, pathogenetically relevant concentrations, which allowed for possible alterations of gene expression to occur. The data show that basal pulsation amplitude is unaltered by TNFα pretreatment. Yet profound impairment of contractile response is evident upon stimulation of α- and β-adrenoceptors by either catecholamines or high calcium, both with regard to the increase in contractility and to the proarrhythmogenic activity. The experiments provide evidence that not only α-adrenoceptor-mediated arrhythmias are suppressed by TNFα but also that beating irregularities provoked by β-adrenoceptor stimulation and high extracellular calcium are mitigated in the presence of TNFα. On the other hand, Weisensee et al. [88] had reported the induction of arrhythmias in cardiomyocytes several minutes after treatment with a 1000-fold higher concentration of TNFα, so that response to an acute challenge with very high concentrations of TNFα has to be differentiated from chronic stimulation. Although the negative inotropic action of TNFα has been well documented in vivo, data concerning a possible effect on beating regularity are lacking. However, Song et al. [89] had found in rats that endotoxin applied in vivo, being a strong inductor of TNFα, protects against ischemia-induced ventricular arrhythmias.

Although TNFα profoundly depressed contractile performance of cardiomyocytes at low, clinically relevant concentrations (10 U/ml = 0.033 ng/ml, confer to the reported TNFα plasma levels [43]), this effect did not go along with an enhanced NO production of cardiac muscle and nonmuscle cells from using human or murine TNFα. Furthermore, no enhancement of iNOS transcription in cardiomyocytes could be measured under these conditions. Other groups had reported NO-dependent experimental cardiodepression by using TNFα at concentrations of 1000 U/ml [57] and 20 ng/ml [56], administering activated macrophage-conditioned medium to cardiomyocytes [59], or studying myocytes from endotoxemic animals [58]. Shindo et al. [90] reported no induction of iNOS or NO release in neonatal rat cardiomyocytes at a TNFα concentration of 10 ng/ml in serum-containing medium; the contractile performance was not documented. Weak induction of iNOS and slightly enhanced NO release was documented in our experiments under serum-free conditions when a TNFα concentration of 1000 U/ml (=3.3 ng/ml) was used. Thus, in the experimental setting used in this study, a NO-independent mechanism of cardiodepression has to be postulated for low TNFα concentrations.

*Protein pattern of neonatal rat cardiomyocytes under the influence of TNFα*

About 800 protein spots were counted on the 2D maps, and high reproducibility of position and intensity of the spots could be achieved. By visual comparison of the protein patterns of controls (figure 15A) and TNFα-treated cardiomyocytes (figure 15B,C), no reproducible differences in position and intensity of the protein spots could be detected. No induction of protein variants could be identified in the 2D protein maps of cells incubated with both 10 U/ml (figure 15B) and 100 U/ml

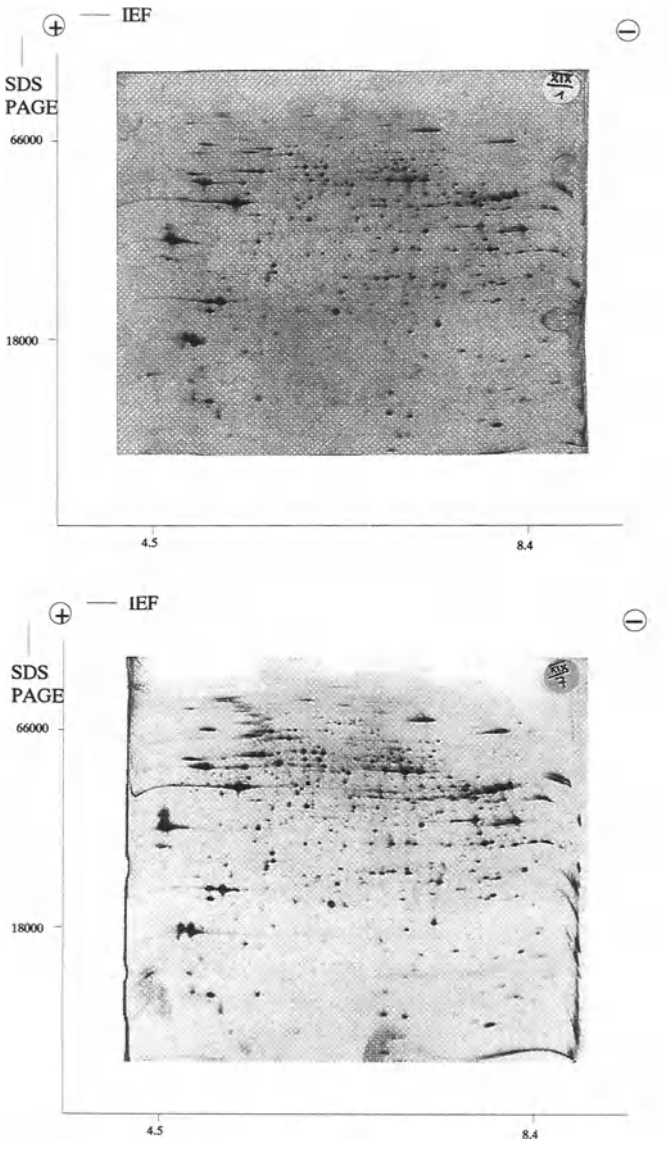


**Figure 15.** Effect of TNFα on protein pattern of neonatal rat cardiomyocytes. Cardiomyocytes were cultured for 48 hours in the absence (a) or presence of 10 U/ml TNFα (b) or 100 U/ml TNFα (c). After the culture period, the cells were harvested and lysed and the proteins were separated according to the isoelectric point (horizontally in the figure) and according to molecular weight (vertically in the figure). No gross alterations in cardiomyocyte protein patterns were observed.

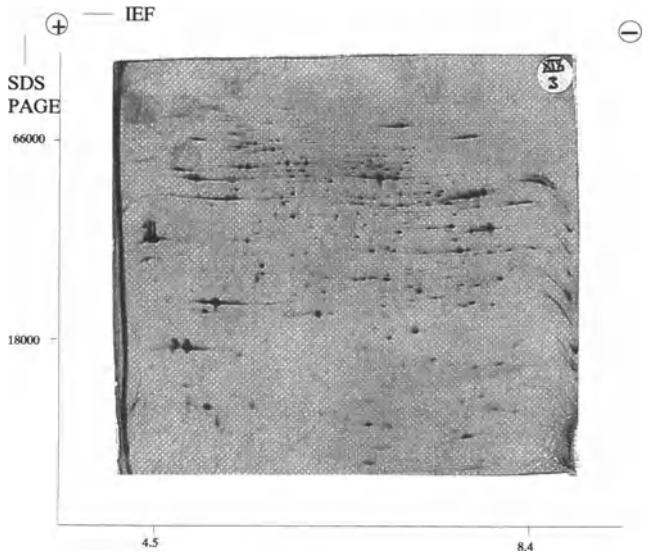


**Figure 15 (continued)**

TNFα (figure 15c). Qualitatively the same results were obtained when performing 2D-PAGE with immobilized pH-gradients.

Thus, mere cytotoxicity does not seem to be the underlying reason, since cells remain morphologically intact and protein patterns do not give proof of toxicity. Rather a specific cardiodepressive action of TNFα seems likely.

*Influence of TNFα on signal transduction*

An impairment of the β-adrenoceptor-adenylyl cylase system by TNFα can be excluded, since no inhibition of the adenylyl cyclase activity was observed [85] and a stimulation of adenylyl cylase was evidenced under experimental conditions identical to those chosen for this study [91]. Possible clues to NO-independent contractile disturbance come from (1) a study of Yokoyama et al. [60], who found a reduction of calcium transient in TNFα-treated myocytes that was not mediated by NO, and (2) findings that TNFα diminishes the phosphatidylinositol-bisphosphate ($PIP_2$) synthesis in cardiomyocytes because of diminished production of precursors, which is a consequence of the reduced activity of glycerol-3-phosphate dehydrogenase, a key enzyme of lipogenesis (figure 16). In view of the pleiotropic effects of TNFα (for review see [11]), it seems likely that impaired cellular calcium metabolism by TNFα because of interference with several signal transduction pathways (for review see [91,92]) apart from the iNOS-dependent cardiodepression at high concentrations could contribute to impairment of cardiac performance.

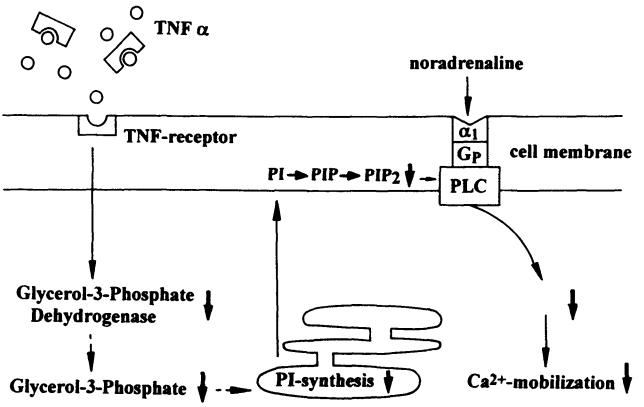


**Figure 16.** Effect of TNFα on phosphoinositide metabolism in neonatal rat cardiomyocytes. The positive inotropic and also the toxic, arrhythmogenic effects of α1-adrenoceptor stimulation in the heart are mediated at least in part by the phosphoinositide pathway. α1-adrenoceptor occupation activates the phosphoinositol (PI)-specific phospholipase C. The substrate of the enzyme is phosphoinositol bisphosphate ($PIP_2$). It is cleaved to diacylglycerol and the calcium mobilizing inositol triphosphate ($IP_3$), which leads to a release of calcium from the sarcoplasmic reticulum into the cytosol. This signal transduction is one of the targets of TNFα action in cardiomyocytes: both basal-and $\alpha_1$-adrenoceptor-mediated formation of $IP_3$ is reduced by chronic exposure (3 days) of cardiomyocytes to low TNFα concentrations. This reduction is due to a diminished synthesis of $PIP_2$. As a likely mechanism of TNFα-induced lowering of $PIP_2$ formation, inhibition of the activity of a key enzyme of lipogenesis, glycerol-3-phosphate dehydrogenase (GDH) was identified. Adapted from [83].

*Link to apoptosis?*

Both TNF receptor subtypes may induce apoptosis, but the p55 receptor seems to be mainly involved [54,93]. The APOl/Fas receptor inducing apoptotic cell death has high sequence similarities with p55-TNF receptor [93]. Both proteins carry a death-domain intracellularly. However, it is entirely unclear whether induction of apoptosis by cytokines may play a role in septic cardiomyopathy.

## SEPTIC CARDIOMYOPATHY: EXPERIMENTALLY SUPPORTED CONCEPTS

Presently, a uniform concept of the mechanism of septic myocardial depression is out of reach. However, some picture in diversity emerges.

Though endotoxin is thought to represent the main trigger toxin of cardiodepression in Gram-negative sepsis, the situation is much less clear in Gram-positive sepsis, where endotoxin is not required for the onset of the disease [94] and toxins acting as superantigens may play a dominant role. It is well-documented that the degree of myocardial depression in Gram-positive sepsis is similar to that in Gram-negative sepsis (figure 4). This is likely to be due to cytokine release, which is common to Gram-positive and Gram-negative infection. However, bacterial toxins, like toxic shock syndrome toxin-1 [95] and the alpha toxin of *Clostridium perfringers* [96], may contribute to pump failure by distinct mechanisms and may

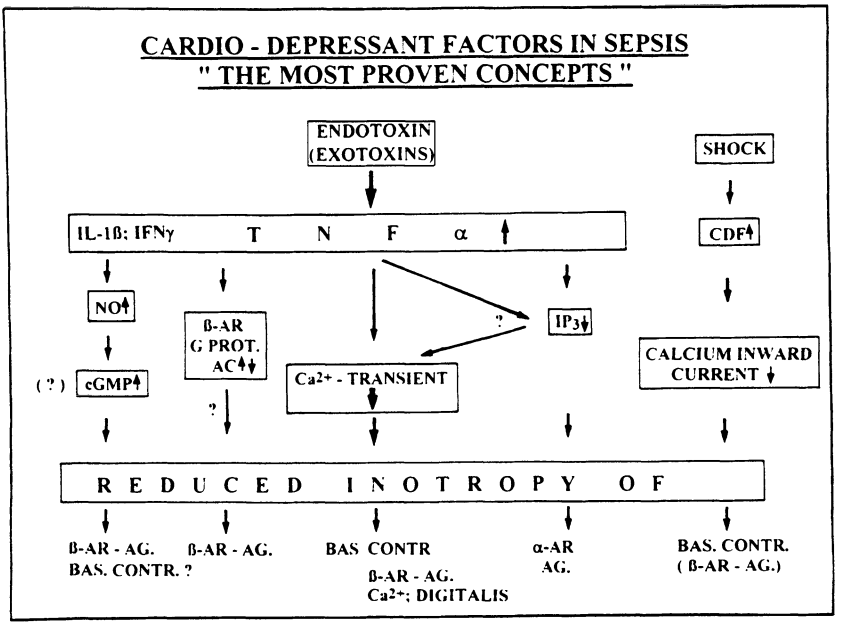


**Figure 17.** Cardiodepressant factors in sepsis: experimentally supported concepts. NO, nitric oxide; cGMP, cyclic guanosine monophosphate; G prot, guanine nucleotide binding protein; AC, adenylyl cyclase; AR-AG, adrenoceptor agonist; $IP_3$, inositol triphosphate; CDF, cardiodepressant factor; BAS CONTR, basal contractility. Adapted from [11].

directly inhibit myocardial function. In the cardiomyocyte model, Pseudomonas exotoxin A—an inhibitor of protein biosynthesis via ADP ribosylation of elongation factor 2 [97]—was shown to narrow the regulatory range of contractile performance [73]. In the clinical setting, the relevance of bacterial toxins for septic cardiomyopathy has not been sorted out.

How cytokines and endogenous mediators eventually exert their cardiodepressant action is the focus of research. It was found that several pathways, not a single cascade of events, mediated cardiodepression in various experimental settings [92], including the NO/cGMP pathway, the phosphoinositide pathway, the β-adrenoceptor/G protein/adenylyl cyclase axis, the calcium transient [60], and the calcium inward current [98] (figure 17). Consequently, the inotropic effects of numerous agents including α- and β-adrenergic stimuli are weakened.

The importance of cytokines in nonseptic heart disease remains to be established and may open new therapeutic horizons, which are desperately sought in view of the still very high mortality of sepsis and septic shock.

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# IN PURSUIT OF OPTIMAL CARE AND OUTCOMES FOR PATIENTS WITH CONGESTIVE HEART FAILURE: INSIGHTS FROM THE PAST DECADE

***TERRENCE MONTAGUE, KOON TEO, LAUREL TAYLOR, FINLAY MCALISTER, MARGARET ACKMAN, and ROSS TSUYUKI, FOR THE INVESTIGATORS AND STAFF OF THE HEART FUNCTION CLINIC,UNIVERSITY OF ALBERTA HOSPITALS, and THE CLINICAL QUALITY IMPROVEMENT NETWORK (CQIN)**

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***Summary.*** This chapter provides an integrated summary of current insights on the epidemiology and optimal management of patients with congestive heart failure (CHF) and reviews the authors' experience of CHF management in the last decade, including results from large clinical trials, demographic and practice pattern analyses, and related health outcomes studies.

CHF is a major public health burden, affecting one percent of the entire Canadian population. Moreover, its incidence and prevalence are increasing, and the mortality risk remains high. In the acute care setting, about one in five patients die during admission. Chronically, the one-year mortality risk for patients with systolic left ventricular dysfunction, who comprise more than three-quarters of all CHF patients, is 25 percent versus 16 percent for patients with predominantly diastolic dysfunction. The principal cause of death in CHF is progression of heart failure, although noncardiac causes, particularly cancer and pulmonary diseases, account for about 30 percent of all deaths. Therapy proven to reduce overall mortality in CHF is presently limited to angiotensin-converting enzyme inhibitors (ACEI), although angiotensin receptor antagonists, beta blockers, and cardiac allografts appear efficacious in small trials and/or major subsets of CHF patients. Diuretics and digoxin can decrease patient symptomatology and hospitalizations in some patients, but ACEI remain the only medication proven to decrease total hospitalizations. Medications, of themselves, do not improve quality of life in CHF, but medications, in combination with a global and seamless approach to care that includes an accent on patient education, are associated with increased patient satisfaction and improved life quality. Optimal care programs are also associated with favorable survival outcomes for CHF patients, which are in the range of that reported from recent CHF clinical trials and contemporary transplant data. However, the optimal effectiveness of therapy for the whole Canadian CHF population at risk is being limited by less than optimal prescription of, and suboptimal compliance with, proven therapy. As well, restrictive

reimbursement policies, which limit patient access to physician-prescribed drugs, threaten to add another impediment to population effectiveness of proven CHF therapy. As many as 50 to 75 percent of eligible CHF patients, particularly older patients and women, may not presently be receiving persistent beneficial therapy, which results in unnecessary adverse clinical and fiscal outcomes for themselves and for the nation. Moreover, in contrast to improvements in the acute cardiac diseases, the CHF practice patterns and outcomes appear more difficult to modify. In summary, CHF is an increasingly important and treatable disease in Canada, but its management and outcomes remain less than optimal. As the population ages, CHF will assume even greater relative importance as a chronic and terminal illness. More efficacious medications are needed for this future. In the interim, more effective application of proven therapies, particularly comprehensive and integrated disease management programs, would substantially improve duration and quality of life for patients with CHF.

In 1989, the Division of Cardiology at the University of Alberta Hospitals launched an initiative dedicated to the investigation and treatment of patients with congestive heart failure (CHF). The model, called the Heart Function Clinic, was stimulated because of a perception that an innovative outpatient-focused management plan was desirable, even necessary, to optimize care for the increasing numbers of patients with left ventricular dysfunction and CHF [1]. The primary goal of the Heart Function Clinic was, and is, the provision of appropriate CHF therapy [2]. This is defined as efficacious CHF therapy integrated with the management all of the patients' diseases, their functional and social status, and their inpatient care—that is, evidence-based, comprehensive and seamless care that can be effective in the whole CHF population at risk [2].

A major subsidiary goal of the Heart Function Clinic was both to investigate, alone and in partnership, the trials' efficacy and population effectiveness of CHF treatments and to propagate the findings through presentations, publications, and other continuing education programs [3–16]. One development of the latter activities was the formation of the Clinical Quality Improvement Network (CQIN), a group of like-minded Canadian stakeholders whose principal goal was to further define and improve treatment strategies and outcomes for important cardiac diseases, including CHF [17–23].

This review summarizes the principal insights gained from the investigation and management of CHF in the Heart Function Clinic and CQIN over the last decade.

## EFFICACY OF CHF THERAPY

In 1992, when we first reviewed the strategy and practices of the Heart Function Clinic, angiotensin-converting enzyme inhibitors (ACEI) drugs were identified as the only proven efficacious medical therapy for reduction of mortality and hospitalization among CHF patients [1,3,4]. By extrapolation, since coronary atherosclerosis was, and is, the preeminent etiology of left ventricular dysfunction and CHF, we postulated that beta-blockers, acetylsalicylic acid, other antiplatelet medications, and atherosclerosis prevention or reversal might also be efficacious therapy for the

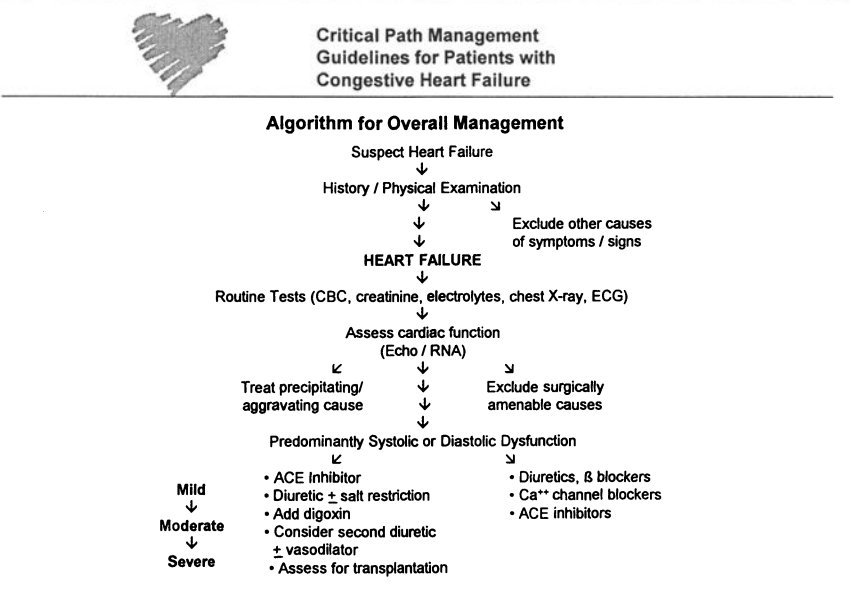


**Figure 1.** Critical path algorithm outlining the evidence-based consensus management of patients with congestive heart failure, as proposed by the Canadian Cardiovascular Society [5] and modified for hospital and community use in the Edmonton health region [23].

primary and secondary prevention of CHF [1]. Diuretics and digitalis therapy were recognized as frequently utilized, often symptomatically beneficial, and, on average, safe CHF therapies; although both remained untested, and hence unproven, in reducing death and hospitalization in randomized clinical trials [1]. Other, sometimes-used CHF therapies, including non-ACEI vasodilators and nondigitalis, positive inotropic agents, were judged as nonbeneficial or detrimental based on the available weight of trials' evidence [1]. In a subsequent analysis, cardiac transplantation was evaluated as being similar to diuretic therapy, which is almost certainly efficacious, but not trials proven and not likely to be the subject of a large randomized, controlled trial at any time in the near future [2].

Since 1992, the epidemiological burden of illness of CHF has remained high in Canada, presently affecting an estimated 300,000 citizens with the incidence and prevalence increasing [2,5,9]. Unfortunately, the number of available efficacious therapies has not greatly increased. A recent national consensus report of CHF therapeutic efficacy produced conclusions very similar to those of our 1992 analysis, summarized above [5]. Specifically, among all the recommended CHF management strategies of the Canadian Cardiovascular Society (figure 1), ACEI therapy remains the only intervention conclusively proven to reduce overall CHF mortality and hospitalization rates [3–5,9], although beta blockers and angiotensin receptor antagonists are promising.

An important ancillary insight has, however, emerged from the sequential trials of

ACEI therapy for left ventricular dysfunction and CHF [6–8]. In repeated subgroup analyses, ACEI therapy has been associated, not only with reductions in overall cardiovascular mortality and retardation of progression of heart failure, but also with a consistent decrease in acute ischemic events [6–8]. This apparent anti-ischemic effect has a different time course, and presumably different pathophysiological pathway, from the antifailure effect of ACEI [6–8]. In comparison to the antifailure effect of ACEI in CHF patients, the anti-ischemic impact does not become significant until several months to years after initiation of therapy [6–8]. In this regard, it is similar to the anti-ischemic impact of lipid lowering therapy in coronary heart disease and may, in fact, represent an antiatherosclerotic effect that is time-dependent at onset [6–8]. Proposed explanations for the possible antiatherosclerotic or cardioprotective effects of ACEI drugs include: enhanced coronary endothelial cell function; retarded smooth muscle cell infiltration and migration in arterial intima; and stabilization of the atherosclerotic plaque [6–8,19]. The anti-ischemic and antiatherosclerotic hypotheses are now being tested in at least two large, randomized clinical trials, with both clinical and coronary angiographic endpoints [24,25].

New data has come from the completion of the Digitalis Investigation Group (DIG) Trial, a large, simple, long-term, randomized trial of digoxin efficacy upon mortality in CHF [11–13]. Digoxin did not reduce overall mortality [12,13]. It did reduce, however, overall hospitalization rates and was also associated with prevention of heart failure events among sicker patient subgroups [12,13]. Overall, the routine clinical use of [1], and the consensus guideline recomendations (figure 1) [5] for, digitalis therapy in CHF are likely to remain unchanged by the DIG trial results.

## INSIGHTS FROM CHF EFFECTIVENESS STUDIES

### Utilization of proven and promising therapies

In a recent analysis of prescription medication use among 4606 CHF patients admitted to eight large Canadian acute care hospitals, the CQIN investigators found diuretics to be used in more than 80 percent of patients [21]. ACEI drugs were used in only about 55 percent of all patients, and digoxin use averaged 45 percent [21]. Beta blocker medication was used in 16 percent of patients [21].

In contrast, chronic utilization rates of the same medications in the Heart Function Clinic over its first three years of operation were as follows: diuretics, 66 percent; ACEI, 87 percent; digoxin, 61 percent; and beta blockers, 23 percent [1]. In a recent update of medication utilization patterns in the Heart Function Clinic, the respective rates were as follows: diuretics, 80 percent; ACEI, 80 percent; digoxin, 60 percent; and beta blockers, 28 percent [14]. These data reveal the chronic utilization patterns of CHF drugs in the Heart Function Clinic have remained qualitatively stable from 1989 to the present, with only small quanitative increases in the use of diuretics and beta blockers and small decreases in digoxin and ACEI [1,14].

However, the differences in CHF medication use between the unmanaged acute care setting and the managed care setting of the Heart Function Clinic illustrate a care gap between what may be usual care and what could be viewed as optimal care. This care gap may manifest as a large cost gap as well. For example, if 40 percent of all CHF patients that could be receiving ACEI are not receiving this efficacious medication, then they cannot hope to gain the clinical benefit of reduced hospitalizations, the single largest positive impact of the medication in the SOLVD trials [3,4]. Moreover, whoever is paying for these avoidable hospitalizations is paying unnecessarily. For the nation as a whole, with 40 percent of 300,000 CHF patients possibly not receiving ACEI, a feasible estimate of the total net costs of the avoidable hospitalizations, based on the SOLVD effect size differences and present costs of enalapril [3,4], is approximately 25 million dollars/year. Thus, at least for the management of CHF, there is a large fiscal stimulus to make best care, usual care!

**Nonprescription medications**

The use of nonprescription drugs among CHF patients was recently defined because of concern for possibly detrimental drug-drug interactions in this largely elderly, multiple comorbidity population. In a comparison analysis of a representative sample of 167 patients from the Heart Function Clinic and an age, sex, and socioeconomically matched control group without heart disease, it was found that both groups of older subjects (average age of CHF patients, 69 years) had very similar use patterns for nonprescription drugs [16]. The most commonly used drugs were vitamins, pain relievers, herbal products, and antacids; all were taken on a regular basis by 25 to 60 percent of both CHF patients and controls [16]. However, significantly fewer patients than controls used decongestants (one percent versus five percent), and more patients than controls used stool softeners (eleven percent versus two percent) [16]. These latter intergroup differences may be a reflection of the strong accent on direct patient education in the Heart Function Clinic [1,2].

**Survival**

When dealing with heart disease, perhaps the most important outcome variable is survival, or its converse, death. In the CQIN analysis of CHF in the acute care setting, the in-hospital mortality rate averaged 19 percent, with a range of 13 to 26 percent [21]. Mortality risk for CHF patients who were 70 years of age and older (21 percent) was significantly higher than that of patients who were less than 70 years old (13 percent), and logistic regression analyses confirmed older age to be associated with the greatest relative risk of death for both males and females [21]. ACEI use was associated with the least relative risk of dying in hospital, overall and in all patient subgroups, including males, females, older and, younger patients [21].

Chronic survival in the Heart Function Clinic has been assessed twice, with similar findings both times [2,14]. Long-term survival is strongly related to symp-

tomatic status [2,14], averaging about 80 percent at two years for patients with no or mild limitation of physical ability, but falling to about 35 percent survival at two years for patients with severe functional limitation [2,14]. Logistic regression analyses confirmed functional status as an important independent risk predictor, similar to the acute care setting, and also revealed older age to be associated with increased relative risk of dying with CHF.

One perspective that can be drawn from these chronic survival data of the Heart Function Clinic is that they compare favorably with the survival data in recent CHF clinic trials and with survival in the posttransplant setting [2–4,14]. Moreover, these outcomes were achieved with an average daily dose of the principal ACEI enalapril (11 mg) that was less than the average daily dose of the same medication used in the SOLVD trials (17 mg) [3,4]. This finding suggests that qualitative use of ACEI may be relatively more important than quantitative dose of ACEI to obtain desirable outcomes in CHF. Overall, these insights support the hypothesis that comprehensive, integrated and, evidence-based medical management of CHF patients is associated with beneficial clinical and fiscal outcomes [1,2,14].

### Cause specific mortality

Another clinical perspective was derived from a CQIN analysis of cause-specific mortality in CHF [22]. This study was done in three acute care hospitals and assessed the causal attribution of death among 2216 CHF patients admitted in 1992 and 1993 [22]. The overall mortality rate was 18 percent at an average of 76 years [22]. Cause of death was judged as cardiac in 72 percent of all deaths [22]. Among the nearly 30 percent of noncardiac deaths, cancer was the dominant cause, followed by pulmonary disease and cerebrovascular disease [22]. Overall, the noncardiac causes of death among CHF patients very much resembled the major causes of noncardiac death in the age-matched general population [22]. Relative to cardiac causes, the contribution of noncardiac causes of death in CHF patients may well be expected to increase in the future, if further success is gained in finding efficacious therapies for cardiac disease [22].

### Quality of life

Although the SOLVD studies were truly landmark achievements in the search for the most efficacious management of CHF, they were disappointing in terms of medication impact upon health-related quality of life [3,4]. The study drug, enalapril, was not associated with any consistent improvement in patients' perception of life quality, despite the marked improvements in survival and hospitalization rates [3,4]. Quality of life measures were, however, found to reflect a generally poor life quality and to be very significant predictors of mortality risk in CHF patients, independent of other traditional risk factors such as age [3,4]. Thus, the hypothesis remained, following the SOLVD studies, that if quality of life could be improved for CHF patients, their duration of life might also be improved.

We have evaluated CHF patients' perception of general quality of life on two

occasions and got the same answer both times [2]. Briefly, CHF patients in the Heart Function Clinic and the Heart Transplant Clinic were asked to evaluate their present all-inclusive life quality on a scale of one to ten, with ten representing the best possible life and one, the worst possible existence [2]. Moreover, they were also asked, using the same scale, to rate their quality of life prior to attendance at the clinics and to forecast it two years into the future [2]. In both settings there were perceptions of significant improvements in general quality of life associated with clinic attendance, and this improvement in quality of life status appeared to have a carry over effect manifest as hope for the future [2].

Repeated structural equation modeling analyses of what processes in the clinics drove the patients' improvement in quality of life suggested the drivers were complex, but the single most important factor identified was the transfer of knowledge regarding the CHF disease syndrome from the clinics' staff to the patients and their families (unpublished data). After determination of this finding, direct education to the patient became a very important facet of the integrated approach to CHF patient care in the Heart Function Clinic [2]. This approach remains, to our knowledge, the most promising process to produce enhanced duration and quality of life for CHF patients [2,14]. Obviously other promising therapies to improve life quality in CHF patients need to be tested, including the potentially beneficial effects of regular exercise [10].

**Systolic versus diastolic ventricular dysfunction**

Increasingly CHF patients are clinically classified as to whether they have predominantly systolic or diastolic left ventricular dysfunction (figure 1) [5,11–15]. Predominantly systolic dysfunction, defined as left ventricular ejection fraction equal to, or less than, 45 percent, is, by far, the more common of the two presentations. For example, in the DIG study population, CHF patients with left ventricular ejection fractions greater than 45 percent, which is predominantly diastolic dysfunction, accounted for only 13 percent of all patients [11]. In the Heart Function Clinic population, when using the same ejection fraction criterion, CHF patients with predominantly diastolic function accounted for 22 percent of all patients [14,15]. Moreover, there are important demographic and etiological differences between these two pathophysiological groups of CHF patients. Diastolic dysfunction patients are older (mean, 69 years) than CHF patients with systolic fault (mean, 65 years) [15]. Fifty percent of patients with predominantly diastolic dysfunction are females, whereas 28 percent of CHF patients with systolic dysfunction are female. Hypertension is a contributing etiology in 40 percent of diastolic patients versus only 28 percent prevalence among systolic dysfunction patients [15]. In contrast, ischemic heart disease etiology was present in 74 percent of patients with systolic dysfunction versus 43 percent prevalence among CHF patients with dominantly diastolic left ventricular dysfunction [15].

Perhaps the most important distinction between the diastolic and systolic dysfunction patient groups is found in the patterns of medical treatment and clinical outcomes in each of the groups [15]. Although there were no differences in

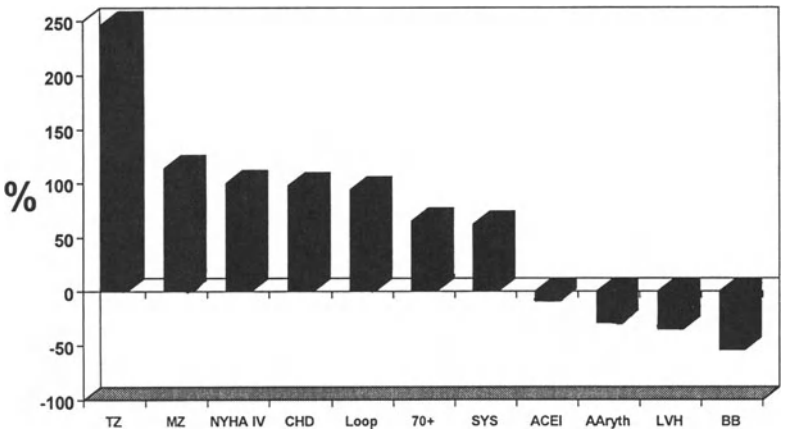


**Figure 2.** Logistic regression analysis of demographic and clinical variables relative to long-term mortality risk in 554 CHF patients managed in the Heart Function Clinic at the University of Alberta Hospitals, 1989 to 1996. ACEI, angiotensin-converting enzyme inhibitor therapy; AAryth, Atrial fibrillation and other atrial arrythmias; BB, beta blocker drugs; CHD, coronary heart disease; ≥70, 70 years of age and older; LVH, left ventricular hypertrophy; Loop, loop diuretic; TZ, thiazide diuretics; MZ, metalozone diuretic; NYHA, New York Heart Association functional classification of IV; SYS, systolic left ventricular dysfunction.

functional classification between the two groups at the time of entry into the Heart Function Clinic, the number of CHF patients with diastolic dysfunction who received ACEI (69 percent) and diuretics (72 percent) is less than the number of those with systolic dysfunction who received ACEI (86 percent) and diuretics (80 percent) [15]. Digoxin use was the same in both groups, and patients with diastolic dysfunction received more beta blockers (31 percent versus 22 percent) and calcium channel blockers (22 percent versus 13 percent) [15].

One-year mortality averaged 16 percent for Heart Function Clinic patients with diastolic dysfunction versus 24 percent for patients with systolic dysfunction [14,15]. Similarly, in the DIG trial, the overall mortality risk in patients with ejection fractions greater than 45 percent was lower, averaging 23 percent, than that of CHF patients with predominantly systolic dysfunction, who had an average mortality of 35 percent over the 37-month course of the study [12]. In the Heart Function Clinic population, logistic regression analysis of long-term mortality versus demographic and clinical variables revealed older age, CHD etiology, and systolic left ventricular dysfunction pathophysiology, as well as severity of clinical status, were the principal associations with excess risk of death (figure 2). Use of ACEI and beta blockers and presence of left ventricular hypertrophy and atrial arrythmias were associated with decreased relative risk (figure 2). There were similar associations with increased and decreased mortality risk in the CHF patient groups with systolic and diastolic left ventricular dysfunction (figure 2) [15].

### Age and sex biases

A consistent finding of medication utilization and mortality risk patterns in repeated analyses of cardiac patient groups in the CQIN studies has been the relatively lower use of proven medications and higher risk among females and patients 70 years of age and older [17,19–21]. This homogeneity of dichotomous treatment patterns and outcomes was true, as well, in CHF [21]. For example, in the recent CQIN analysis of 8 community and university tertiary care centers, in-hospital mortality for patients 70 years and older averaged 21 percent versus 13 percent for younger CHF patients [21]. Moreover, it is significant that ACEI and beta blockers were used in the older CHF patients less frequently than in their younger counterparts [21]. Logistic regression confirmed that (1) the independent nature of age is a factor conveying increased relative mortality risk, overall, and in both males and females, and (2) ACEI is associated with the least relative risk of in-hospital death in all age and sex strata of patients [21]. These latter findings underline the potential importance of optimally prescribing proven agents to all recognizable patient groups, particularly those at higher risk, like the elderly and women [21]. The age and sex differences remain, however, unexplained and unsolved at this time.

### Processes for outcomes improvement

An increasingly obvious reality arising from repeated practice pattern and outcomes analyses in several important cardiac diseases is the presence of a care gap, the difference between usual care and best care [2,14,17–21]. While Canada does not have a health care crisis, it seems evident that patient outcomes could be significantly improved if efficacious therapies for serious and widely prevalent diseases were made available to, and were persistently used by, the whole population at risk [2,14,17]. Closing the care gap is not easily accomplished [17,23]. It can, however, be done, and it should be remembered that closing the care gap in the management of important diseases produces an accompanying improvement in duration and quality of life [2,14,17]. In the cumulative CQIN experience, improved in-hospital care and outcomes have been more easily realized in the management of acute myocardial infarction [17] than in the management of CHF [23].

The reasons for the essentially unchanged utilization rates of ACEI among 2171 hospitalized CHF patients in Edmonton, in which 1040 patients were managed prior to the introduction of implicit management guidelines and 1131 patients were managed after introduction of the guidelines (figure 1), remains incompletely defined [23]. One obvious process difference and potential explanation for the differences in resulting practice changes between the disease management guidelines used for infarction therapy versus CHF was the very explicit nature of the infarction guidelines [17]. The use of comprehensive, specific, preprinted physicians' order sheets as the principal implementation tool for infarction management greatly focused the provided therapeutic guidance and it also minimized the chances of either a lack of timely knowledge or a failed memory as being causes of poor prescription of proven medications [17]. The factors of ubiquity and specificity of

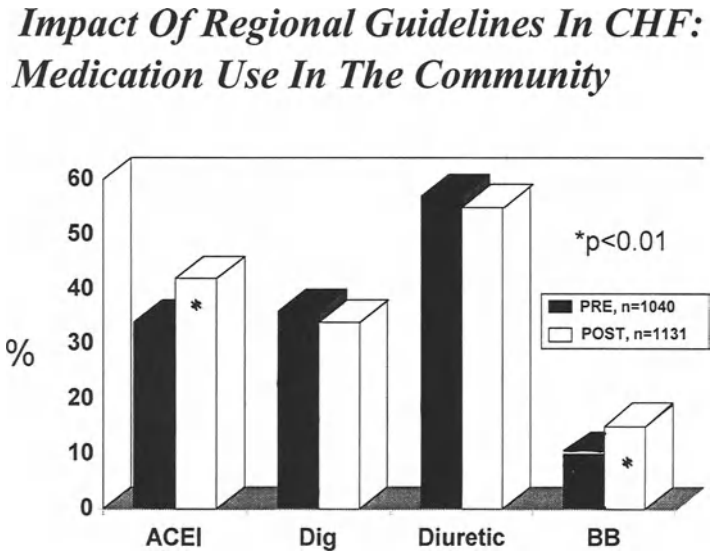


**Figure 3.** Comparative distribution of prehospital medication use among 2171 CHF patients, 1040 patients before the community introduction of implicit CHF management guidelines, outlined in figure 1, and 1131 patients postintroduction of the guidelines [23]. ACEI use in CHF outpatient community care increased significantly with introduction of the guidelines [23]. Abbreviations are the same as figure 2; Dig, digoxin.

guidelines were missing from the CHF management program [23]. As well, there was greater heterogeneity and, therefore, perhaps less intellectual and committed "buy-in" of the physician audience for the CHF program [23].

Interestingly, prehospital use of ACEI did significantly increase in the Edmonton region during the term of the CHF project, suggesting that the propagation of implicit guidelines was an effective intervention among communitybased physicians (figure 3) [23]. Nonetheless, at this time, it seems the more explicit the guidelines and the more they are accepted on a local physician basis, the greater the chances they will achieve the desired outcomes. It is equally obvious that better processes are required to manage the closure of the care gap. Hopefully, these processes will be forthcoming.

## CONCLUSIONS

The experiences and observations of the Heart Function Clinic and the CQIN investigators have produced many valuable insights into the epidemiology and management of CHF. CHF is an increasingly important and treatable disease in Canada, but its management and outcomes remain less than optimal. As the population ages, CHF will assume even greater relative importance as a chronic and terminal illness. More efficacious medications are needed for this future. In the interim, more effective application of proven therapies, particularly as comprehen-

sive and integrated disease management programs, would substantially improve duration and quality of life for patients with CHF. A particular challenge for the immediate future is the development of disease management or behavioral modification processes that will allow the accelerated closure of the gaps between the usual care and outcomes, and what they could optimally be.

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# ATHEROSCLEROSIS: IMPLICATIONS OF ANGIOTENSIN II AND THE AT-1 RECEPTOR

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***Summary.*** Atherosclerosis is a chronic inflammatory disease of the arterial intima. It is associated with the accumulation of monocytes, monocyte-derived macrophages, macrophage-derived foam cells, T-lymphocytes, and a sparse number of mast cells. Inflammation is the body's natural, protective response to injury. Paradoxically, this complex process of repair and healing can become destructive. This leads to fibrosis and scarring, with the eventual loss of the vital functions of the organs or tissues involved. In the atherosclerotic process, we see an extensive inflammatory infiltrate, which leads to plaque instability and its vulnerability to rupture.

Atherosclerosis is a fibroproliferative disease associated with the accumulation of collagen and extracellular matrix synthesized by the smooth muscle cell. The smooth muscle cell, so important and damaging in the early phase of atherosclerosis, paradoxically becomes the cell type so protective in stabilizing the vulnerable plaque in the later stages of this disease process. It may be referred to as the "sentinel" of the fibrous cap.

The driving force behind the atherosclerotic process is the retention of modified low-density lipoproteins.

As this inflammatory-fribroproliferative disease progresses, a custom delivery system of microvessels develops within the arterial wall, which supplies the necessary substrates to fuel this inflammatory, destructive process, called the "vasa vasorum".

In this chapter, the role of angiotensin (Ang) II and its receptor $AT_1$ is discussed. Their relation to the development, progression, and final fate of the atherosclerotic plaque and how they relate to cardiovascular disease cannot be overemphasized.

## INTRODUCTION

Atherosclerosis is the number one cause of death and disability in the United States. The cost to the nation's economy in 1990 was 144 billion dollars [1]. Coronary

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heart disease is the leading cause of premature permanent disability in the U.S. labor force [2] and causes 800,000 new myocardial infarctions and 450,000 recurrent myocardial infarctions each year. To put this in perspective, one person dies each minute from coronary atherosclerosis. These figures mentioned above look just at coronary disease. One can only imagine how the statistics would mount if stroke, peripheral vascular disease, and aneurysm were added.

With this disease, there are a number of associated diseases that cause an acceleration of the atherosclerotic process including hypertension, diabetes, dyslipidemias, age, male sex, postmenopausal females, smoking, obesity, sedentary life style, homocysteine, insulin resistance, and Lp(a).

Characteristically, there is a long asymptomatic phase lasting four to six decades while atherosclerotic plaques are developing within the arterial wall. When atherosclerosis becomes symptomatic, it may be gradual or acute depending on the changes of the atherosclerotic plaque including its growth and its vulnerability to rupture.

Lipid lowering therapy has been shown to have a statistically significant decrease in the number of cardiac events as well as all cause mortality in primary and secondary interventions studies [3–5]. The results of the 4S, Care, and WOS trials have been very encouraging to clinicians and researchers.

The study of Angiotensin (Ang) II inhibition by angiotensin-converting enzyme inhibitors (ACEIs) and Ang II receptor type 1 ($AT_1$) antagonists in coronary artery disease (CAD) is a promising area of basic science and clinical research. Completed trials in patients with left ventricular (LV) dysfunction such as the Studies of Left Ventricular Dysfunction (SOLVD) and the Survival and Ventricular Enlargement (SAVE) studies [6–11] demonstrated a 23%–24% reduction in risk of recurrent myocardial infarction regardless of ejection fraction, blood pressure response, or concomitant cardiac medication.

Clinical trials of ACEIs in patients with known CAD and preserved LV function are in progress, and results should be available in the near future.

There is exciting news available for all to review regarding endothelial cell dysfunction. The Trial on Reversing ENdothelial Dysfunction (TREND) was published this past year [12]. This trial demonstrated a significant reduction in vasoconstriction caused by acetylcholine, from 14.3% at baseline to 2.3% following 6 months of treatment with the ACEI Quinipril. About twice as many patients in the Quinipril group as in the placebo group exhibited dilation to acetylcholine at the time of follow up. The effect on endothelial cell dysfunction by Quinipril was substantial, as great as has been reported for lipid lowering [13]. The dose of Quinipril had no effect on blood pressures in these patients, and, therefore, it is unlikely that these results were due to any antihypertensive effect. This is why there is ever growing interest in this topic.

## BACKGROUND AND AUTHOR'S DEFINITION

Atherosclerosis is a complex multifactorial disease process of the arterial wall. The meaning is derived from the Greek words athero (gruel) and sclero (hardening).

## DEFINITION

Atherosclerosis is a focal, chronic inflammatory- fibroproliferative disease of the arterial intima caused by the retention of modified low-density lipoproteins.

It is focal in that the disease occurs at predictable anatomic sites of the arterial tree. It occurs at bifurcations, flow dividers, and side branches. It occurs predictably opposite the flow dividers, where there is low endothelial shear stress and turbulent blood flow. These predictable areas are where adaptive intimal thickening is seen in children before the atherosclerotic process has started.

There is an orderly cephalad progression starting in the iliacs and progressing to the aorta, coronaries, carotids, and the cerebral vessels. Because of this orderly cephalad progression, when patients have evidence of carotid atherosclerotic disease on ultrasound, there must be, by definition, coronary atherosclerotic disease, even if it is not symptomatic.

Atherosclerosis is a chronic inflammatory disease in that the monocyte, the macrophage, and the T-lymphocyte are the cell types involved throughout the disease process.

It is interesting to note that as early as 1815, London surgeon Joseph Hodgson [14] published an important monograph on vascular disease, claiming that inflammation was the underlying cause of atherosclerosis and not a natural degenerative occurrence of the aging process. He also identified that this disease process occurred in the intima, between the lumen and the media of the diseased vessels.

It is a fibroproliferative disease in that there is a tremendous amount of collagen synthesized by the smooth muscle cells of the arterial media in the response-to-injury mechanism described by Ross et al. [15].

Tabas et al. [16] shared with the community of atherosclerosis that the retention of modified low-density lipoproteins was an absolute requirement for lesion development and progression. He and others feel that lipoprotein retention is the key pathogenic event.

## IMPLICATIONS OF ANGIOTENSIN II (ANG II) AND THE ANGIOTENSIN TYPE I ($AT_1$) RECEPTOR

The renin-angiotensin system (RAS) is an enzymatic cascade [figure 1], resulting in the production of Ang-II. It begins in the liver with the synthesis of angiotensinogen, an alpha globulin, which is then cleaved by the aspartyl protease renin synthesized in the juxta-glomerular cells of the kidney to produce angiotensin I, a physiologically inactive decapeptide. Angiotensin I is then cleaved by the carboxypeptidase angiotensin-converting enzyme (ACE) that produces the physiologically active, omnipotent Ang II—an octapeptide.

The positive bradykinin effect is as follows: The kallikrein-kinin system results in the generation of bradykinin. ACE is also the same as kinase II, which breaks down bradykinin to inactive fragments. Bradykinin affects the endothelial cell by two separate mechanisms. The first activates the L-arginine nitric oxide synthase (NOS) pathway, and the second activates the arachidonic acid pathway, resulting in the

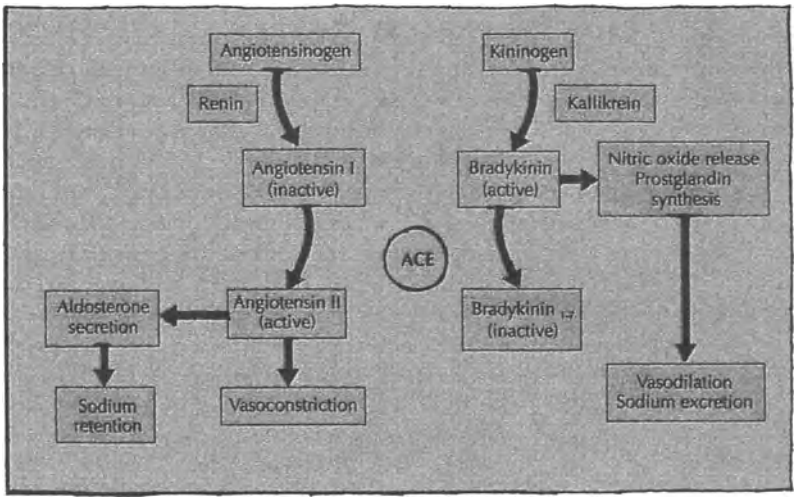


**Figure 1.** The RAS and kallikrein-kinin flow sheet.

production of nitric oxide (NO), cyclic guanosine monophosphate (cGMP), the prostaglandins PGI-2(Prostacyclin)/PGE2, and cyclic adenosine monophosphate (cAMP). Furthermore, bradykinin increases the endothelial-derived hyperpolarizing factor (EDHF), serves as an inhibitory-antiproliferative factor, suppresses platelet aggregation, is a potent stimulator of tissue plasminogen activator (t-PA), and increases the activity of the fibrinolytic system, providing an antithrombotic effect.

In summary, the antiatherosclerotic positive bardykinin effect causes:

1. Increased NO (L-arginine, NOS pathway)
2. Increased NO, prostacyclin (A.A. pathway) in the kallikrein-kinin system
3. Increased EDHF
4. Decreased platelet aggregation
5. Increased inhibitory-antiproliferative factors
6. Stimulation of t-PA production
7. Increased fibrinolysis

Ang II is a potent vasoconstrictor. When Ang II binds to its $AT_1$ receptor, there is an activation of a number of second messengers. It activates phospholipase C, which in turn forms metabolites that affect intracellular calcium and the calcium-sensitive protein kinase C. It opens calcium channels, which result in smooth muscle cell (SMC) contraction and increased vascular tone. These second messengers also activate nuclear elements that have effects on gene expression, transcription, protein synthesis, cellular hypertrophy, and mitogenesis, resulting in cell growth, proliferation, and migration. Thus, there seems to be two effects of Ang II in the atheroscle-

rotic process: a direct hypertensive effect of Ang II and a direct vascular cellular effect of Ang II.

Hypertension affects the structure and function of the arterial vessel wall and its contents. It modifies the endothelial cell by increasing permeability, impairing endothelial-dependent relaxation, and increasing the adherence of monocytes and their absorption. Hypertension increases SMCs and macrophages within the intima and causes an increase of extracellular matrix (ECM) including collagen, elastin, fibronectin, and glycosaminoglycans. Hypertension changes the phenotypic expression of SMCs from a resting state to a proliferative state. Elevation of blood pressure also affects plaque stability because of flow and shear stress.

Ang II increases the ECM directly, independent of any hypertensive affect. Ang II directly affects SMC growth and proliferation through its effect on fibroblast growth factor (FGF), transforming growth factor beta (TGFB-1), and, most importantly, platelet-derived growth factor (PDGF), as in Ross's article [15]. Dzau and Gibbons' data [17,18] show that TGFβ-1 seems to be the key determinant in the regulation of Ang II-induced hypertrophy. In the absence or inhibition of TGFβ-1, the principal effect of Ang II is mitogenesis or hyperplasia resulting from the activation of FGF and PDGF. The simultaneous activation of TGFβ-1 appears to override the proliferative activity of other growth factors and results in hypertrophy.

Dzau and Gibbons have shown convincingly the importance of tissue ACE with their study on in vivo gene transfer [19]. They transfected ACE cDNA into injured intact rat common corotid arteries in vivo and found a threefold increase in vascular ACE as well as local angiotensin-mediated hypertrophy in the transfected segments of the common carotid. Since the transfected segment is exposed to the same blood pressure and neurohormones as the control segment, these results are strong evidence for a local ACE effect in Ang II production and ultimately in function.

In summary, the direct cellular actions of Ang II on atherogenesis are activation of growth factors and cytokines, increased vascular SMC migration and hypertrophy, increased oxidative stress and oxidation of low-density lipoproteins, upregulation of cellular adhesion molecules, monocyte macrophage activation, and augmented ECM production.

## PLAQUE RUPTURE

In the past decade, there have been important advances in understanding the vascular biology of atherogenesis and the development of the atherosclerotic plaque. At the 69th Scientific Session of the American Heart Association in November 1996 in New Orleans, Louisiana Drs. Erling Falk, Peter Libby, James Muller, Frans Van de Werf, and Valentin Fuster spent the afternoon discussing plaque rupture and triggering of cardiovascular disease.

Plaque rupture has been implicated as the central feature of clinical events [20–27]. Clinical events are defined as cardiac death, myocardial infarction, and unstable or preinfarctional angina. Plaques that are unstable, vulnerable, or prone to rupture share four common features (figure 2):

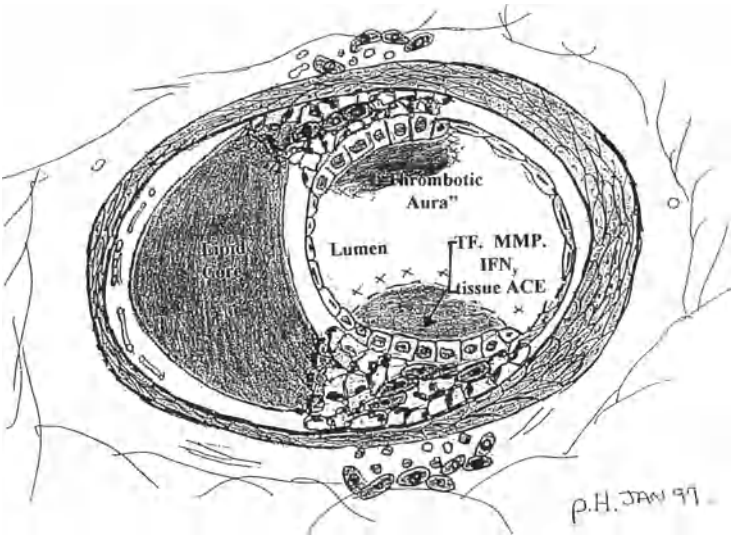


**Figure 2.** A representative cross-section of a vulnerable plaque demonstrating the (1) large lipid core; (2) thin fibrous cap; (3) decreased SMCs of the thin fibrous cap; and (4) inflammatory changes at the shoulders of the atherosclerotic plaque.

1. Large lipid core
2. Thin fibrous cap
3. Decrease in SMC's in the fibrous cap
4. Inflammatory changes at the vulnerable shoulders of the plaque

Lipid lowering trials have demonstrated a significant reduction in cardiac events [3–5]. The reduction in clinical events, while statistically significant, were not associated with regression of plaque size. It is currently felt that this reduction is due to plaque stabilization. It is the authors opinion that the reduction of recurrent events in the SAVE and SOLVD trials are also due to plaque stabilization as a result of inhibition of local tissue ACE and circulatory ACE, decreasing vascular tone and vasospasm, and the (7 point) positive bradykinin effect on the plaque, referred to earlier in this chapter.

### INFLAMMATORY CHANGES AT THE VULNERABLE SHOULDER REGION OF THE ATHEROSCLEROTIC PLAQUE

The periphery of atherosclerotic plaques is commonly referred to as the vulnerable shoulder region. This region is occupied by a heavy infiltration of monocyte-derived macrophages, macrophage-derived foam cells, T-lymphocytes, and a sparse number of mast cells. The primary cell type is the macrophage (figure 2).

These macrophages are unique in that they produce the four following sub-

stances: tissue factor (TF), matrix metalloproteinases (MMPs), cytokines -interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-a), and tissue ACE. TF is a potent thrombotic element of the extrinsic pathway of the coagulation system. MMPs are potent proteinases that break down collagen within the fibrous cap. IL-1 and TNF-a contribute synergistically to the thinning of the fibrous cap with interferon gamma (IFNg). Tissue ACE is found in large quantities in this shoulder location. In normal coronary arteries, local ACE expression is confined only to the endothelial cell.

The T-lymphocyte in the shoulder region produces the cytokine IFNg. IFNg causes the SMCs to decrease their production of collagen in the protective fibrous cap, causing the fibrous cap to thin. IFNg also promotes the breakdown of collagen by activating the macrophages and their MMPs. IFNg causes SMC apoptosis, thus causing fibrous cap thinning and decreased SMC content. It is interesting to note that the only thing that stands between a patient and an acute cardiac event is the integrity of the fibrous cap. The SMC is the sentinel of the plaque's fibrous cap. The T-lymphocyte has a heavy infiltration in the region of the adventita in advanced plaques.

The sparse mast cell contributes MMPs, which cause further breakdown of the collagen in the fibrous cap, resulting in continued thinning of the cap.

To summarize, there are four important elements in this vulnerable shoulder region of the atherosclerotic plaque:

1. Tissue factor
2. MMPs
3. Cytokines IFNg, IL-1, and TNF-a
4. Tissue ACE

All of the above four elements are a product of the inflammatory infiltrate of the vulnerable shoulder region of the atherosclerotic plaque that is prone to rupture. This is why the author placed in the definition; atherosclerosis is a chronic inflammatory disease.

## VASA VASORUM

The vulnerable shoulder region of these plaques are unique in that they are supplied with their individual microcirculation, the vasa vasorum (figure 3). The vasa vasorum (v.v.) are the microvessels that supply the larger musculo-elastic arteries, which include the epicardial vessels of the heart. Barger's classic work [28] confirmed the presence of the v.v., supplying the coronaries, and that the origin of these vessels were from the adventitial layer of the vessel wall. Zhang's data [29] revealed that these vessels delivered albumin, fibrinogen, and immunoglobulins to the vessel wall. He also found that these microvessels were leaky, as in the diabetic microvessels. As the intimal layer becomes more and more diseased by the atherosclerotic process, there is a relative ischemia of the vessel wall and it is felt (by

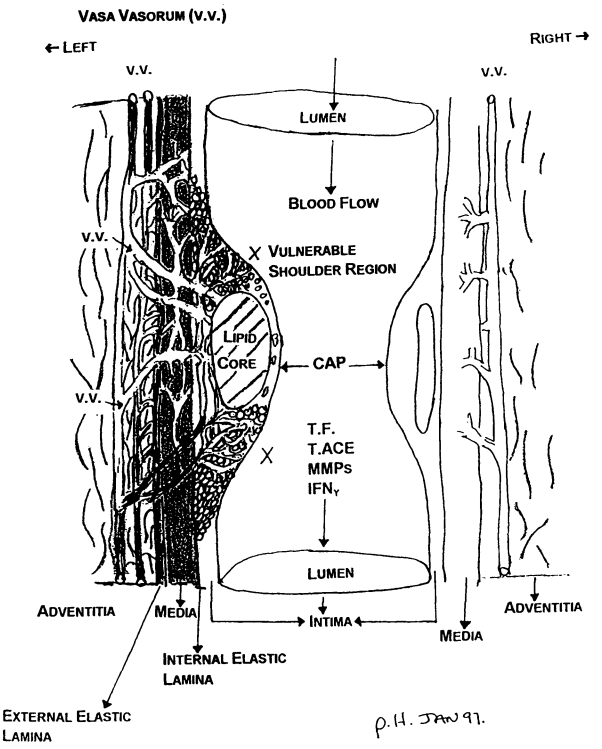


**Figure 3.** A longitudinal section of an atherosclerotic plaque. This figure demonstrates the malignant-like invasion by the vasa vasorum (v.v.) and inflammatory changes at the shoulders of the plaque (X). The plaque to the left demonstrates a vulnerable plaque while the plaque on the right demonstrates a stable plaque less prone to rupture.

author) that it is this vessel wall ischemia that induces the angiogenesis of the v.v. We know from Tyagi's work in this publication (See Chapter 40) that Ang II induces this angiogenesis in the heart.

In this vulnerable shoulder region of the atherosclerotic plaque, we have this marked inflammatory infiltrate loaded with tissue ACE and supplied with its own source of substrates for the local production of Ang II by the v.v. In addition, these microvessels are able to deliver the native LDL cholesterol, which serves as a substrate for the future oxidation and modification, which contributes to continued plaque growth. The v.v. also assumes the role of delivering the second wave of inflammatory cells to the atherosclerotic plaque. The adventitial T-lymphocytes may be delivered to the shoulders of the vunerable lesion by the v.v. The first wave is believed to arise from the endothelial luminal surface of the vessel, utilizing selectins and cellular adhesion molecules. The author feels that the v.v. is responsible for the autoamplification of the chronic inflammatory infiltrate within this vulnerable shoulder region.

In summary, the v.v. is a custom delivery system for the vessel walls' vulnerable shoulder region supplying:

1. Substrates of the RAS
2. Substrates of native LDL cholesterol
3. The second wave of inflammatory cells

The speakers at the 69th meeting of the American Heart Association frequently referred to these vulnerable shoulder regions as being hot and said that the future therapy of atherosclerosis would be to identify and cool off these hot chronically inflamed vulnerable regions. These areas of chronic inflammation are stabilized by lipid lowering drugs and ACEIs. The lipid lowering drugs (-statins) do this by decreasing the substrate of native LDL cholesterol, preventing the continued production of modified LDL cholesterol, which drives the inflammatory process. The ACEI (-prils) do this by inhibiting the circulatory ACE and tissue ACE of the macrophage and by providing the (7 point) positive bradykinin effect discussed earlier in this chapter.

When the $AT_1$ receptor antagonists (-sartans, losartan, valsartan irbesartan, and candesartan) block the $AT_1$ receptor, they effectively block the effects of Ang II on the atherosclerotic process, both the hypertensive effect and the nuclear elements explained earlier in this chapter. However, the -sartans would not have the positive bradykinin effect, which may be shown to be equally important in the atherosclerotic process. It will be a number of years before this question is answered. We can assume that the pharmaceutical industry and private investigators have only recently begun the appropriate studies to answer this question.

The-sartans have certainly been helpful to clinicians, who now have an alternate way to inhibit Ang II when patients develop the irritating cough and angioedema associated with the bradykinin effect of the ACEIs. These two side effects of ACEIs do not happen often (less than 3% in my experience), but when they do, it allows the clinician to have an alternate way to block the deleterious effects of Ang II. This is why molecular biology and laboratory research are so important.

At the conclusion of reviewing the literature and consulting colleagues, it is felt strongly by the author that most (if not all) patients with left ventricular dysfunction should be treated with ACEI or $AT_1$ receptor antagonists because of their inhibitory effect on myocardial remodeling, vessel wall remodeling, and the equal, if not greater, antiatherogenic, antithrombotic effects on the cardiac vessels and the vessel wall.

**ACKNOWLEDGMENTS**

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# INFLUENCE OF $AT_1$ RECEPTOR INHIBITION ON CARDIAC FUNCTION AND STRUCTURE OF DIABETIC RATS

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***Summary.*** It has been recently shown that inhibition of angiotensin-converting enzyme (ACE) is cardioprotective in diabetes [1,2]. This cardioprotection might be the consequence of reduced activation of the angiotensin receptor subtype $AT_1$, enhanced formation of bradykinin, or a combination of both. To specifically study the contribution of angiotensin (Ang) II in the development of cardiac dysfunction in diabetes, we treated streptozotocin diabetic rats with the specific $AT_1$ receptor antagonist ICI D8731 and determined the influence of the $AT_1$ blockade on myocardial structure and function.

Treatment with the $AT_1$ antagonist was cardioprotective in experimental diabetes. The impairment of contractility, as well as the development of interstitial and perivascular fibrosis, and cardiac hypertrophy typically seen after a diabetes duration of 6 months were prevented. In contrast to the functional and morphological data, $AT_1$ inhibition did not prevent changes in the expression of collagen I, $\alpha$- and $\beta$-MHC, or Glut4 induced by diabetes. Additionally, the expression of the $AT_1$ receptor was not enhanced after a diabetes duration of six months. These observations suggest that the protection of the heart in diabetes by $AT_1$ inhibition is preferentially achieved by changes in the translational or posttranslational signal pathways.

On the other hand, we did not observe a reduction in the diameter of myocardial capillaries enlarged or an increase in the reduced density of capillaries by $AT_1$ inhibition, as found in studies using the ACE inhibitors captopril and fosinopril [1,2]. These observations are consistent with the hypothesis that an enhanced formation of bradykinin represents an additional important factor for the therapeutical effects of ACE inhibitors.

We assume that the cross-talk between nitric oxide (NO) and the angiotensin ($AT_1$)-mediated signaling pathways is altered in diabetes and the impairment in the NO pathway reinforces the angiotensin actions without changes in the actual generation of Ang II or in the receptor expression. The vasculature in diabetes would be become more susceptible to the

deleterious effects of Ang II. Thus, it is intriguing to suggest that the enhanced generation of reactive oxygen species and the impairment of the NO-mediated effects on the vasculature are the primary causes for the reinforced action of Ang II on the diabetic heart. Treatment with ACE inhibitors or the $AT_1$ antagonist would not prevent the primary defect in myocardium in diabetes, but would inhibit some important consequences in the reaction cascade initiated by reactive oxygen species.

## INTRODUCTION

Recently we presented evidence that inhibition of the angiotensin-converting enzyme (ACE, Dipeptidyl carbocypeptidase EC 3.4.15.1) is cardioprotective in experimental diabetes. In insulin-dependent spontaneously diabetic, as well as in streptozotocin-diabetic rats, inhibition of ACE activity has been shown to increase the number of perfused capillaries and improve myocardial perfusion. Interstitial and perivascular fibrosis were partly prevented and the structure of myocardium was less damaged than in hearts of untreated diabetic rats [1,2]. These studies suggest that activation of the renin-angiotensin system plays an important role for the increased cardiac risk in diabetes.

Angiotensin (Ang) II has been described to activate the sympathetic nerve system and to promote cardiac and vascular fibrosis [3–5]. Additionally, coronary vasoconstriction and myocardial necrosis have been reported to be caused by an enhanced generation by Ang II in various forms of heart failure [3–5]. Thus, a reduced generation of Ang II might explain the cardioprotection observed by ACE inhibition in diabetes. However, the data regarding the activity of ACE in diabetes vary greatly, and we have no convincing evidence that the formation of Ang II is enhanced in diabetes. On the other site, ACE inhibition might also stimulate the formation of kinins. These compounds are potent stimulators of nitric oxide (NO), which has been shown to both play an important role in maintaining vascular tone and blood flow and act as an antiproliferative agent [6,7]. Recent data by Hou et al. [8] suggest a potentially important autocrine or paracrine role for NO in modulating the effects of Ang II. Thus, a diminished generation of NO, as observed in diabetes [9,10], would alter the fine tuning between NO and Ang II and make the diabetic heart more susceptible to the deleterious effects of Ang II without changing the absolute rate of Ang II formation. Stimulation of kinins by ACE inhibition could help balance this disturbed interrelationship and protect the heart in diabetes. A reduction of the sequelae of myocardial ischemia by kinins has been shown in various forms of heart failure [11].

Which effects and in which way the various actions of ACE inhibition contribute to cardioprotection in diabetes is not yet known. To specifically study the contribution of Ang II in the development of cardiac dysfunction in diabetes, we treated streptozotocin diabetic rats with the specific $AT_1$ receptor antagonist ICI D8731 and determined the influence of the $AT_1$ blockade on myocardial structure and function. By comparing these data with those obtained by ACE inhibition, it should be possible to distinguish whether and to which part the various mechanisms associated with ACE inhibition contribute to cardioprotection in experimental diabetes.

## MATERIALS AND METHODS

The $AT_1$ antagonist ICI D8731 was a kind gift from ICI Pharmaceuticals (Macclesfield, England). Intensifying screens and Hyperfilm MP were purchased from Amersham-Buchler (Braunschweig, Germany). All other reagents were of analytical grade.

### Animals and induction of diabetes

Diabetes was induced in male Wistar rats (250–300 g body weight) by an intraperitoneal injection of streptozotocin (60 mg/kg body weight) as previously described [1,9,12]. After verification of the diabetic state by determination of blood glucose (hexokinase method) and glucosuria, treatment of animals was started. The specific $AT_1$ receptor antagonist ICI D8731 was applied in the drinking water at a dose of 8 mg/kg body weight daily, respectively. The concentration in the drinking water was adjusted according to the daily consumption (12–15 ml/day for controls and 90–130 ml/day for diabetic rats). The blood pressure was only slightly reduced by $AT_1$ inhibition (data not shown). Untreated diabetic animals were used as controls. The diabetes duration was six months for the morphological studies and three months for the biochemical analysis.

### Preparation of the rat heart

Rat hearts were retrogradely perfused according to the Langendorff technique and fixed, as already described [1,9]. In brief, hearts were perfused at a constant pressure of 60 cm $H_2O$ with a modified Krebs Henseleit buffer (mmol/l: $CaCl_2$ 1.8, $MgCl_2$ 1.05, KCl 5.35, NaCl 136.9, $NaH_2PO_4$ 0.42, glucose 10.1, $NaHCO_3$ 23.8), prewarmed to 37°C, and equilibrated with 95% $O_2$/5% $CO_2$. Hearts were allowed to beat spontaneously. After an equilibration period of 25 min, coronary flow heart rate, maximal left ventricular systolic pressure (LVP), and maximal velocity of contraction (+dp/dt) were continuously measured over a period of 20 min as described previously. Following the pressure constant perfusion, the hearts were perfused with a 0.1 mol/l cacodylate buffered, 2% glutaraldehyde/2% paraformaldehyde fixative via the cannula located within the aorta near the coronary ostia.

Left papillary muscles were replaced and further fixed in 0.1 mol/l cacodylate buffered, 2% glutaraldehyde/2% paraformaldehyde followed by postfixation in 2% osmium tetroxide buffered at pH 7.3 with 0.1 mol/l sodium cacodylate for 2 hours at 40°C. The specimens were rinsed three times in cacodylate buffer, block stained in 1% uranyl acetate in 70% ethanol for 8 hours, dehydrated in a series of graded ethanol, and embedded in araldite. Semithin sections of plastic embedded papillary muscles were stained with methylene blue and investigated by aid of a computerized morphometrical analysis unit (Leica CBA8000). Ultrathin sections (30–60 nm) were obtained with a diamond knife on a Reichert ultramicrotome, placed on copper grids, and examined with a Zeiss EM 902A electron microscope. For light microscopical observations, 10 semithin sections of the left papillary muscle were studied

per heart. For electronmicroscopy, 5 thin sections of the left papillary muscle were studied per heart.

**Catecholamine fluorescence**

The right ventricles of isolated perfused hearts were rapidly cut off, freeze-clamped with a Wollenberger clamp cooled in liquid nitrogen, and stored at −80°C for histochemical examination of intraaxonal catecholamine stores. At a temperature of −30°C, the frozen myocardial tissue was 16.5 mm cryostats serial sections. Adrenergic nerve fibers of the right ventricle were made visible by means of glyoxylic acid-induced fluorescence of intraneuronal catecholamines using the method of De la Torre [13]. Quantitative assessment of individual tissue sections was performed by high resolution microfluorimetry. The system consisted of a Leitz orthoplan microscope equipped for fluorescence with epi-illumination and 3 mm BG 12 and Leitz K 490 primary and secondary filters, respectively. Using a residual light amplifying caesicon camera, tissues under study were focused in such a way that within the visible field of the individual preparation only sections with nerve fibers running in parallel were processed and sections containing sympathetic plexuses were excluded from measurement. After inversion of the primary image by means of a computerized image analyzing system (Artek 982, Fisher Sci.), fluorescing adrenergic nerves were easily detected as dark areas against a bright background. To eliminate background fluorescence, points below a given threshold intensity were filled up in brightness with the help of a specially designed measuring mask so that only fluorescing nerve fibers remained visible. For determination of the fluorescing area, one section per heart was taken from the right ventricle in a distance of 150 µm from the epicardial surface. The total observation area was 3 $mm^2$ per heart. The amount of the fluorescing area was estimated from 100 consecutive measurements per section and is given as percent of the total area. No correction for fading of the fluorescence image by photodecomposition was necessary as the whole processing was performed in 10 s. By using this technique, not only were the length and thickness of the adrenergic nerve fibers recorded, but the dimensions of their axonal dilatation, the varicosities, were recorded as well.

**Morphometric analysis**

Morphometric data were collected on the basis of randomly chosen transverse sections that consisted exclusively of muscle fibers and terminal exchange vessels (diameter 1–26 µm) from the left papillary muscles. Capillary diameter and capillary cross-section area were measured on a Leitz Medilux microscope connected to a Leitz CBA8000 image analyzing system. The capillary diameter [µm] was measured by recording the smallest profile diameter as the closest approximation of the true diameter. For each animal, 400 profiles were examined. The number of capillaries per field was counted according to the method of Gunderson [14].

The volume density of capillaries ($V_C$) was obtained from the areal density of capillaries ($A_C$) and the area of the containing space ($A_S$): $V_C = A_C/A_S$. The areal

density of capillaries ($A_C$) was calculated as the product of capillary density and capillary cross-sections area. The stereologic estimate of $V_C$ is independent of the sectioning area for anisotropic structures [15].

Additionally, the papillary muscle cross-sections were used for studies of myocyte hypertrophy. Sixty myocyte profiles per animal were drawn at the level of the nucleus. Their minimal diameter was measured automatically with the image analysis system mentioned above.

For trichrome staining, 1 mm blocs of myocardium containing parts of the left coronary artery were embedded in paraffin, and 5 mm thick slices were sectioned transversally to the longitudinal axis of the arteries. The slices were stained using a standard method described by Goldener [16] to distinguish connective tissue from myocytes, smooth muscle, and endothelial cells. The areas of connective tissue and the perimeter of coronary vessels were automatically detected and measured on a Leitz Medilux microscope connected with a Leitz CBA8000 image analyzing system for detection of real color. The area of connective tissue is given by the trichrome stained area (© in $\mu m^2$) related to the circumference of the vessel (v in $\mu m$) as c/v ($\mu m^2/\mu m$). For each heart, slices from five different parts of the left coronary artery were selected. The distance between the slices was set at more than 1 mm.

**Biochemical determinations**

For biochemical determinations, the hearts were washed in ice-cold phosphate buffer saline (PBS), frozen in liquid nitrogen, and stored at −70°C.

Hydroxyprolin was determined by the method of Prockop and Udenfriend [17]. The ACE activity (dipeptidylcarboxypeptidase I, EC 3.3.15.1) in serum and hearts was determined using hippuryl-histidyl-leucine as substrate, as described [18,19].

**Northern blot**

Total RNA was isolated from frozen hearts (−80°C) as described by Chomczynski and Sacchi [20].

20 µg RNA of each sample were submitted to formamide-formaldehyde agarose gel electrophoresis (40 mM MOPS, 10 mM Na-acetate, 1 mM EDTA, pH 7.0, overnight) and transferred to Hybond $N^+$ nylon membrane (Amersham, Braunschweig, Germany) by means of capillary blot (northern transfer). After UV fixation (5 min) and baking (2 h, 80°C), the membranes were prehybridized in 5× SSPE, 5× Denhardt's solution, 0.5% SDS, 50% (w/v) formamide, as well as 50 µl freshly denatured sheared salmon sperm DNA, for at least 2 h at 42°C [21].

Hybridization was carried out overnight at 42°C. Membranes were washed twice with 2 × SSPE/0.1% (w/v) SDS at room temperature, twice with 1 × SSPE/0.1% SDS at 50°C, air dried, and subjected to autoradiography. Quantification was done by use of the filmless radioactivity monitoring system FUJIX BAS 1000 (Raytest, Straubenhardt, Germany). Results are given as dpm per signal after having been compared with a known standard exposed simultaneously.

About 50 ng of 1.5 kb fragment of a rat Glut-4 cDNA cloned in pBluescript and

transfected in the *E.coli* strain JM 109 [22] were random primed labeled with ($-^{32}$P)-dATP (3000 Ci/mmol, random primed labeling kit, Boehringer, Mannheim, Germany). The labeled fragment was separated from unincorporated nucleotides by gel filtration on a Biogel P30 column (Biorad, Munich, Germany).

cDNA for collagen I ($\alpha$1) was a kind gift of D. Rowe, Australia [23], for GAPDH of J.M. Blanchard, France [24]. For hybridization of $\alpha$- and $\beta$-MHC, synthetic oligomers were used, which were complemented to the nucleotides 5855–5596 and 5825–5866, respectively [25,26] (EMBL Accession Nrs X15938 and 15939).

To determine the expression of the $AT_1$ receptor, 2 $\mu$g of total RNA were transcribed into cDNA by using oligo-(dT) priming and 2U of reverse transcriptase (Life Technologies, Eggenstein, Germany) in a 10 $\mu$l reaction (1 h, 37°C) under the recommended conditions. To 5 $\mu$l of cDNA preparation, the following solutions were added: 16.5 $\mu$l water, 5 $\mu$l 10× PCR buffer (Life Technologies), 3 $\mu$l MgCl2 (25 mM), 8 $\mu$l dNTPs (1.25 mM each), and 2.5 $\mu$l (4 $\mu$M) of the sense and antisense primers for the angiotensin receptor ($AT_1$) and GAPDH (as an internal standard). The primers for the angiotensin receptor were chosen to amplify a sequence of 607 bp corresponding to the 5′-noncoding end which is identical in both the $AT_1A$ and $AT_1B$ receptor subtype sequences (sense 5′-GGAAACAGCTTGGTGGTG-3′, antisense 5′-GCACAATCGCCATAATTATCC-3′) [27]. After 3 min at 94°C and 10 min at 72°C, the reaction was started at 72°C by adding 2.5 $\mu$l Taq-DNA-polymerase (1U/$\mu$l, USB). Thirty-five cycles were run: 30 s at 92°C, 60 s at 60°C, and 60 s at 72°C). After completing the last polymerase step for 10 min at 72°C, and aliquot of 10 $\mu$l was applied onto an agarose gel for separation of the products. Quantification was done by scanning densitometry. The intensity of the bands was measured; the value of the angiotensin receptor signal was standardized to the GAPDH signal of the same sample; and mean values were calculated for the different groups.

### Statistical analysis

Results are presented as the means ± SEM of n individual experiments. Statistical analysis was performed using the BMDP-PC 90 statistical program. Differences between group means were determined by calculation of one-way analysis of variance (ANOVA). Multiple comparisons were performed using adjusted t tests with p values corrected by the Bonferroni method ($\star p < 0.05$, $\star\star p < 0.01$).

## RESULTS

Injection of streptozotocin induced a severe hyperglycemic state. Only animals with a blood glucose concentration higher than 15 mM were taken as diabetic ones. The diabetic state was further characterized by glucosuria, loss of body weight, and polydipsia. The diabetic animals excreted ketone bodies but were not ketoacidotic. Thus, intraperitoneal injection of 60 mg/kg body weight streptozotocin caused a distinct hyperglycemia, but not an excessive catabolic state. A more extensive

characterization of the diabetic state induced by streptozotocin is given by Rösen et al. [9,12].

After a diabetes duration of months, heart performance was changed in diabetic rats similar to changes described previously [12,28]. The maximal rates of contraction and relaxation were diminished and the maximal left ventricular systolic pressure (LVP) and heart rate were slightly reduced. The spontaneous coronary flow was not significantly different in hearts of control and diabetic rats. The relative heart weight was increased by diabetes from 3.0 ± 0.1 g/kg body weight to 3.6 ± 0.2 g/kg body weight ($p < 0.01$). Treatment with the $AT_1$ antagonist did not influence heart performance of the controls. In diabetes, $AT_1$ inhibition did not alter the spontaneous coronary flow and had only a slight but insignificant effect on heart rate (228 ± 11 beats/min as compared to 202 ± 17 beats/min). The LVP was significantly reduced by treatment with the $AT_1$ antagonist. The reduction in the maximal rate of contraction (dp/dt), as well as the increase in the relative heart weight, was prevented (table 1).

In semithin sections of papillary muscles of control hearts, the capillaries were uniformly distributed and had comparable diameters in the control group. Furthermore, no signs of myocytolysis or deposits of collagen plaques could be detected (figure 1,a). In hearts of streptozotocin diabetic rats, on the other hand, capillaries were irregularly distributed and perivascular plaques of collagen were frequent. Distinct areas of myocytolysis were often observed (figure 1,b). By ultrastructural techniques, further abnormalities had clearly been identified: myofilament bundles were deteriorated and partly fragmented. Areas of focal necrosis and contraction bands can be found regularly [1,9]. The number of perinuclear vacuoles is usually increased. Mitochondria are, in part, severely damaged and are increased in diameter [9]. In hearts of diabetic rats treated with the $AT_1$ antagonist, structural abnormalities were not as obvious as in those of untreated diabetics. The diameters of capillaries were enlarged, and the distribution was not as uniform and regular as in the hearts of controls. However, areas of myocytolysis and deposits of collagen were rare (figure 1,c; table 1).

The ultrastructure of papillary myocardium of control, diabetic, and diabetic rats treated with the $AT_1$ antagonist is shown in figure 2. In controls, myocytes and

**Table 1.** Influence of treatment of diabetic rats with the $AT_1$ antagonist on myocardial function

| | Control | Diabetes | Diabetes treated with $AT_1$ |
|---|---|---|---|
| Coronary Flow (ml/min) | 13.7 ± 0.7 | 15.0 ± 1.1 | 14.7 ± 0.5 |
| Heart Rate (beats/min) | 246 ± 11 | 202 ± 17* | 228 ± 11 |
| LVP (mmHg) | 51 ± 7 | 50 ± 5 | 35 ± 4** |
| dp/dt (mmHg/s) | 1880 ± 158 | 1180 ± 163* | 2000 ± 102** |
| Relative Heart Weight | 3.0 ± 0.1 | 3.6 ± 0.1* | 3.3 ± 0.2** |

*$p < 0.05$ control vs diabetes, **$p < 0.05$ diabetes vs. treated diabetes.

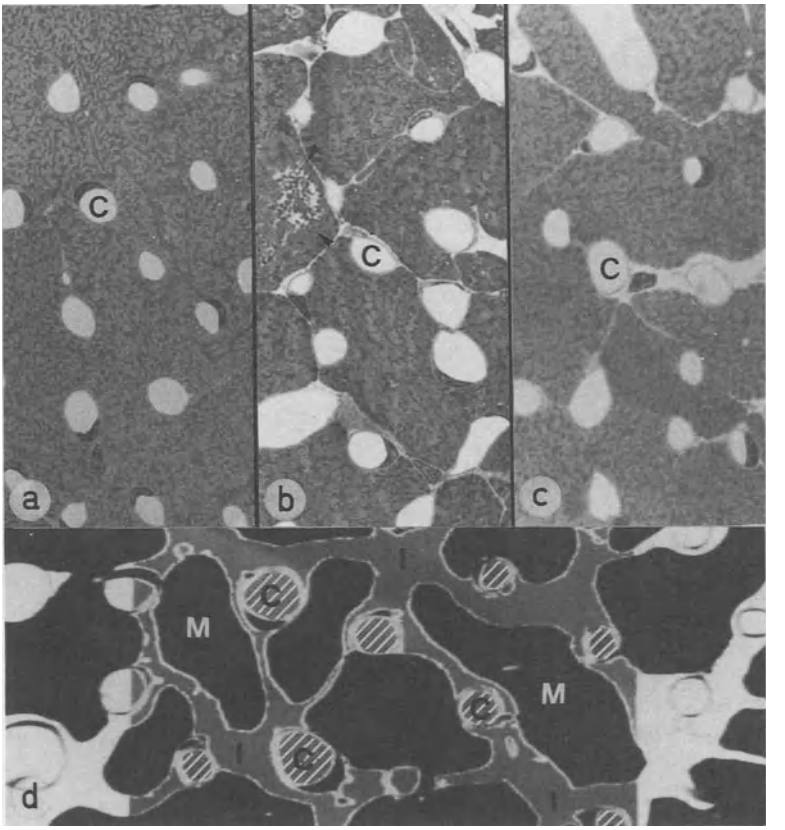


**Figure 1.** The AT$_1$ antagonist DI8731 protects the myocardium in diabetes. The samples were processed as described in Methods. For light microscopy, 1$\mu$ semithin sections of papillary muscles were taken from the left papillary muscle of controls (n = 6), diabetic (n = 8) and AT$_1$ antagonist-treated diabetic rats (n = 5) and cut transversally to the long axis of capillaries: *a*, Semithin sections of controls display an even distribution of capillaries (C) with comparable diameters in the control group. There are no signs of myocytolysis; *b*, The papillary muscle of untreated streptozotocin diabetic rats shows an irregular distribution and an increase in the diameter of capillaries. Perivascular collagen plaques (arrows) are frequent in the vicinity of capillaries (C). Distinct signs of myocytolysis (arrowheads) are regularly found in the myocardium (M) of diabetic rats; *c*, The myocardium (M) of treated Streptozotocin diabetic rats shows an increase of the capillary diameters but no sign of myocytolysis can be found; and *d*, Selective detection of capillaries (C), myocardium (M), and interstitium (I): the detection is performed automatically and followed by measurement of capillary diameter.

capillaries do not show any abnormalities. The capillary wall is formed by a single endothelial cell (figure 2A,a). There are no extracellular deposits, e.g., connective tissue, which restrict intercellular exchange or supply through the intercellular space. The basal lamina of the capillary endothelium can clearly be divided into the laminae densa and rara, which cover the outer surface of the vessel as a thin layer

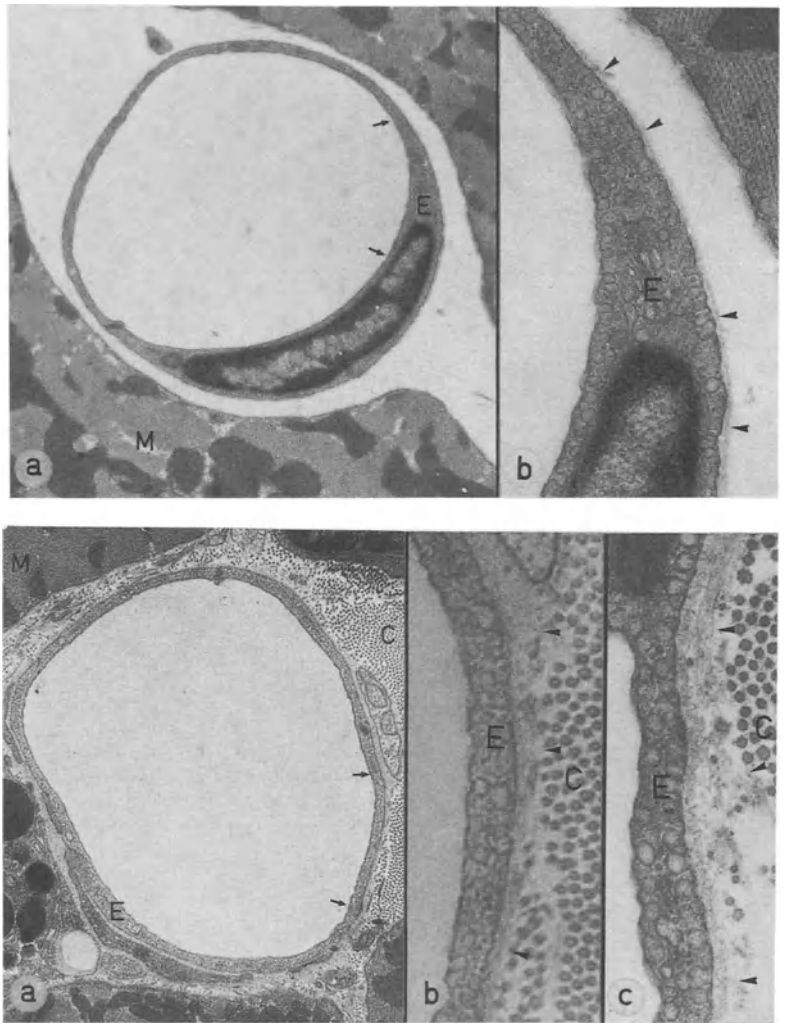


**Figure 2.** Ultrastructure of myocardial vessels in diabetic rats and the influence of $AT_1$ inhibition. The samples were processed as described in Methods. For electronmicroscopy 5 thin sections per heart were taken from the left papillary muscle of controls (n = 6), diabetic (n = 8), and $AT_1$ antagonist-treated, diabetic rats (n = 5). (*A*) Controls: *a*, Ultrastructure of cross-sectioned papillary muscle of control rats (12,000-fold): myocytes and capillaries are shown in good conditions. The capillary wall is formed by a single endothelial cell. There are no extracellular deposits, e.g., connective tissue, which restrict intercellular exchange or supply through the intercellular space; and *b*, Sector magnification (40,000-fold) of the basal lamina (arrowheads) of the capillary endothelium (E) can be divided into the laminae densa and rara, which cover the outer surface of the vessel as a thin layer, (*B*) Diabetes: *a*, There is distinct accumulation of extracellular deposits, sited between capillaries and myocytes, such as collagen fibrils (12,000-fold); *b*, Increase of thickness and concurrent loss of structure of basal lamina (arrowheads) is recognizable between capillary endothelium (E) and the surrounding collagen (C) (40,000-fold); and *c*, A section of a further capillary endothelium (E) shows a multilayered basal lamina (arrowheads) with a loss of structure (40,000-fold), (*C*) Diabetes treated with The $AT_1$ antagonist D8731: *a*, $AT_1$ inhibition leads to a diminished extracellular deposition of connective tissue (12,000-fold); and *b*, The basal lamina (arrowheads) shows a distinct increase of thickness in comparison to control and in several cases a twofold increase in the original thickness (40,000-fold).

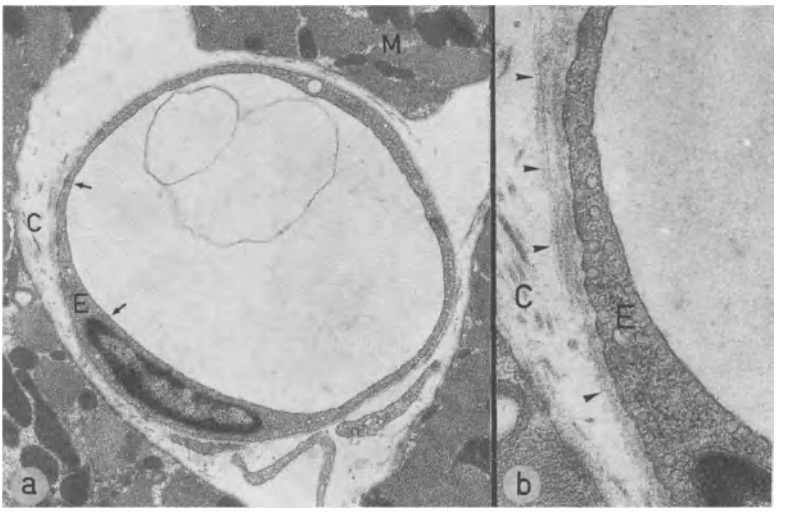


**Figure 2 (continued)**

**Table 2.** Influence of diabetes and the $AT_1$ antagonist D8731 on the size of capillaries and myocytes

| | Control | Diabetes | Diabetes treated with $AT_1$ |
|---|---|---|---|
| Capillary Diameter (μm) | 4.46 ± 0.19 | 4.94 ± 0.12* | 5.09 ± 0.13* |
| Capillary Density (number per $mm^2$) | 3201 ± 83 | 2807 ± 85* | 2601 ± 161* |
| Capillary Volume(%) | 7.85 ± 0.72 | 8.07 ± 0.38 | 7.95 ± 0.34 |
| Myocyte Diameter (μm) | 17.35 ± 0.28 | 19.27 ± 0.10* | 17.90 ± 0.51** |

*$p < 0.05$ control vs diabetes, **$p < 0.05$ diabetes vs. treated diabetes.

(figure 2A,b). In hearts of diabetic rats (figure 2B), distinct accumulation of extracellular deposits, sited between capillaries and myocytes—e.g., collagen fibrils—can be seen (figure 2B,a). The basal lamina is thickened (figure 2B,b). The typical structure of basal lamina between capillary endothelium and the surrounding collagen is lost. A section of a further capillary endothelium shows a multilayered basal lamina with a loss of structure (figure 2B,c). In hearts of diabetic rats treated with the $AT_1$ antagonist (figure 2C), the amount of extracellular deposits of connective tissue is diminished as compared to hearts of untreated diabetic rats (figure 2C,a). However, in comparison to controls, the basal lamina is thickened. In several cases a twofold increase is observed (figure 2C,c).

Morphometric data of the capillaries and the myocytes are given in table 2. In diabetes, the capillary density was reduced to 87% of the control, but the diameters of the capillaries were enlarged. Both diabetes induced changes were not signifi-

**Table 3.** Influence of diabetes and the $AT_1$ antagonist D8731 on the activity of the angiotensin-converting enzyme in serum and myocardium

| | Control | Diabetes | Diabetes treated with $AT_1$ |
|---|---|---|---|
| Myocardium (μU/mg protein) | 87.9 ± 5.2 | 66.9 ± 6.3* | 75.2 ± 9.7 |
| Serum (μU/l) | 46.2 ± 5.8 | 70.2 ± 8.9* | 60.1 ± 9.4 |
| Specific Activity (μU/mg protein) | 0.70 ± 0.08 | 1.16 ± 0.14* | 1.01 ± 0.17 |

*••.

cantly affected by treatment of the diabetic rats with the $AT_1$ antagonist. The diameter of myocytes and the relative heart weight were slightly, but significantly, increased in diabetes. Both parameters can be taken as indicators for the development of myocardial hypertrophy. Treatment with the $AT_1$ antagonist prevented the increase in relative heart weight and myocytes diameter.

In diabetic hearts, histochemical visualization and quantification of intraneuronal catecholamines revealed a progredient loss of histofluorescent nerve fibers in the myocardium of streptozotocin-diabetic rats similar to those recently described for hearts of spontaneously diabetic (B/B) rats [4]. These data indicate a loss of sympathetic nerve fibers and severe structural abnormalities that are very similar to alterations observed in peripheral nerves [29] (figures 3 and 4). Treatment of the diabetic rats with the $AT_1$ antagonist prevented this diabetic-specific loss of sympathetic nerve fibers and intraneuronal catecholamines. As in healthy myocardium, the autonomic nerve fibers were running parallel, and the intraneuronal amount of catecholamines was not different from that of healthy control rats.

ACE activity was increased in the serum of diabetic rats, but not in myocardium (table 3). Treatment with the $AT_1$ antagonist had no influence on ACE activity either in serum or in heart tissue. Diabetes reduced the mRNA encoding Glut4 to about 60% of controls [30] and diminished the mRNA encoding α-MHC (56.3 ± 10.4% to 29.0 ± 13.6%); however, it nearly doubled β-MHC mRNA (70.1 ± 13.6% vs 36.5 ± 11.7%). The mRNAs encoding GAPDH and collagen were not significantly affected by induction of diabetes. Treatment of the rats with the $AT_1$ antagonist had no significant effect on the expression of any of the studied proteins.

To examine whether diabetes affects the expression of the $AT_1$ receptor, the mRNA encoding $AT_1$ receptor was analyzed by RT-PCR. We did not observe that diabetes or the treatment with the $AT_1$ antagonist affected expression of this receptor (data not shown).

## DISCUSSION

The morphological and functional data reported in this study clearly show that treatment with the $AT_1$ antagonist protects the heart in experimental diabetes. The diminution of contractility typically observed in diabetic rats was completely prevented by treatment with the $AT_1$ antagonist. In addition, heart rate, which typically

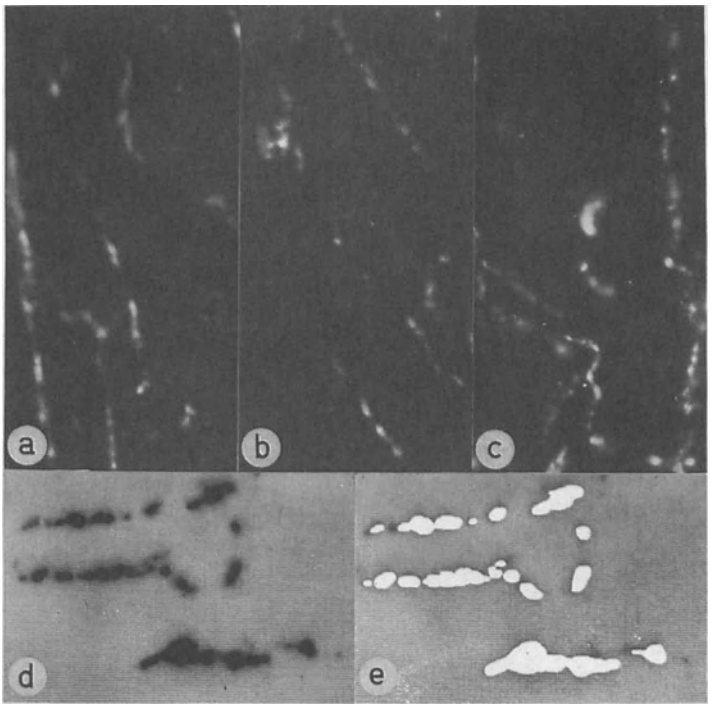


**Figure 3.** Autonomic neuropathy is prevented in the diabetic rat heart by $AT_1$ inhibition. As described in Methods and by De La Torre [27], the glyoxylic acid-induced fluorescence was determined in a section of the right ventricle taken in a distance of 150μm from the epicardial surface. Sections of controls (n = 6), diabetic (n = 8), and $AT_1$ antagonist-treated, diabetic rats (n = 5) were studied: *a*, In healthy myocardium, fluorescing nerve fibers run parallel to the longitudinal axis of cardiomyocytes and form chain-like varicosities; *b*, Distinct reduction of fluorescing nerve fibers in untreated streptozotocin diabetic rats; *c*, Treatment with The $AT_1$ antagonist D8731 halts the diminution of fluorescent nerve fibers; *d*, Appearance of the intraaxonal catecholamine content in cardiac adrenergic nerve fibers after acid-induced catecholamine histofluorescence. After inversion, nerve fibers are displayed as black areas; and *e*, After subsequent elimination of points below a given threshold intensity (background fluorescence), the area of the remaining points can be determined with the aid of a specifically designed mask. Only fluorescent nerve fibers are visible as white areas.

is severely reduced in the streptozotocin-diabetic rat, was slightly increased. Morphological determinations revealed that myocytes become enlarged in diabetes and the relative heart weight increases, which indicates the development of myocardial hypertrophy. In addition, thickening of the basement membrane and an increase in the number of extracellular deposits of matrix material were observed as expected in diabetes [1,31]. Both cardiac hypertrophy and deposition of matrix material in the extracellular space of the coronaries were prevented or largely reduced by treatment with the $AT_1$ antagonist. Lastly, we have shown previously that diabetes severely affects the cardiac sympathetic nerves [29], an alteration which was also largely

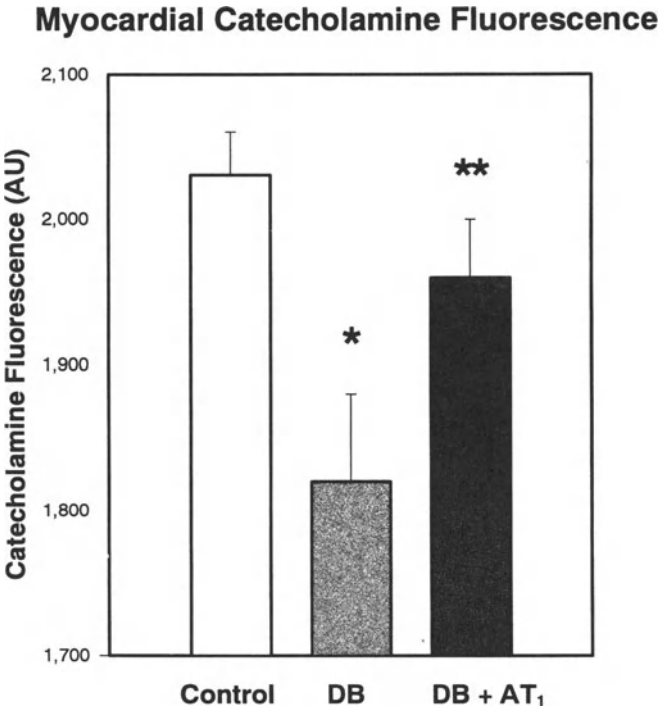


**Figure 4.** Changes in catecholamine-related fluorescence. As described in Methods and by De La Torre [13], the glyoxylic acid-induced fluorescence was determined by microfluorimetry. In a total area of 3 $mm^2$ per heart, the fluorescing area was measured by 100 consecutive measurements using a section of the right ventricle taken in a distance of 150 µm from the epicardial surface. The fluorescent area is given as percent of the total area: mean ± SEM of control (n = 6), diabetic (DB, n = 8), $AT_1$ antagonist-treated, diabetic rats (DB + $AT_1$, n = 5). * $p < 0.05$ control as compared to diabetes.

prevented by the $AT_1$ antagonist. Similar cardioprotective effects by inhibition of ACE by captopril and fosinopril in different types of diabetic rats have been previously observed [1,2]. Taken together, these findings suggest that the cardiac hypertrophy and the cardiac fibrosis that is very often found in the diabetic heart is mediated by Ang II and the activation of the $AT_1$ receptor. This receptor is dominant for the induction of hypertrophy and fibrosis [32] and is specifically inhibited in this study by the $AT_1$ receptor antagonist ICI D8731. This conclusion is largely compatible with the spectrum of actions expected by Ang II [3–5]. It has been shown previously that Ang II is one of the most powerful factors to induce cardiac fibrosis and myocardial hypertrophy [3–5] and to stimulate the release of catecholamines from the sympathetic nerve fibers of the heart, which may reinforce the action of Ang II [33,34].

Cardiac hypertrophy and fibrosis are slowly developing processes in diabetes when compared with other pathophysiological situations, such as heart failure [11].

Severe changes in the deposition of collagen have only been seen after a diabetes duration of six months. After three months, we observed only a tendency for hydroxyproline to increase as a biochemical marker of cardiac fibrosis. Additionally, we did not find significant changes in the expression of collagen I ($\alpha 1$). In agreement with already published data, the amounts of mRNA encoding for Glut4 and $\alpha$-MHC were decreased, whereas that of $\beta$-MHC was nearly doubled [31,35]. We found no evidence that the expression of the various genes which have clearly been shown to be modulated in diabetes is affected by the $AT_1$ antagonist. Even in the case of Glut4, we could only demonstrate an increase in the Glut4 protein, not in the mRNA [30]. The influence of $AT_1$ receptor inhibition on the protein level and on the stability and the translation of mRNA seems to be of major importance for cardioprotection of the heart in diabetes.

It is not yet clear why the action of Ang II is reinforced in diabetes. We observed only a small increase in the activity of ACE in serum, whereas the activity of ACE in myocardium was not altered at all. This is in contrast to various types of heart failure in which the activity of the ACE was strongly increased [36]. That all components of the Ang II generation are locally expressed in the rat heart has already been shown [37]. The expression of the $AT_1$ receptor is increased in rat heart several days after induction of diabetes [38], but not in the chronically diabetic state that has been used in this study. It might be speculated that $AT_1$ receptor expression is associated with the very early phase of the metabolic state after insulin deficiency, but is not typical for the metabolically more stable chronic state. Thus, there presently is no evidence that either an increased generation of Ang II or changes in the expression of the $AT_1$ receptor are involved or necessary for the impairment of cardiac function in experimental diabetes.

On the other hand, Hou et al. [8] have recently documented that chronic NO blockade enhances the Ang II-dependent development of cardiac fibrosis and hypertrophy by altering the fine tuning between the opposing effects of NO and Ang II on vasomotion and on cellular proliferation and regulation of catecholamine release by the sympathetic cardiac nerve fibers. A similar mechanism might work in diabetes, too. We and others have recently shown that NO-mediated vasomotion is disturbed in the diabetic heart [9,10] because NO is quenched by superoxide anions permanently released by the diabetic heart [9]. If there is a cross-talk between the NO and the angiotensin ($AT_1$)-mediated signaling pathways, an impairment in the NO pathway would reinforce the angiotensin actions without changing the actual generation of Ang II or the receptor expression. The vasculature in diabetes would become more susceptible to the deleterious effects of Ang II. Thus, it is intriguing to suggest that the enhanced generation of reactive oxygen species and the impairment of the NO-mediated effects on the vasculature are the primary causes for the reinforced action of Ang II on the diabetic heart. Treatment with ACE inhibitors or the $AT_1$ antagonist would not prevent the primary defect in myocardium in diabetes but would inhibit some important consequences in the reaction cascade initiated by reactive oxygen species.

On the other hand, we did not observe a reduction in the diameter of myocardial capillaries enlarged in diabetes or an increase in the reduced density of capillaries as found in studies using the ACE inhibitors captopril and fosinopril [1,2]. These observations are consistent with the hypothesis that an enhanced formation of bradykinin represents an additional important factor for the therapeutical effects of ACE inhibitors [11,39,40]. Thus, the stimulation of NO and prostacyclin by bradykinin seems to be of special importance for the protection of myocardial autoregulation and the functional state of coronary vessels.

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# ROLE OF MYOCARDIAL TISSUE ANGIOTENSIN (ANG) II IN CARDIAC PATHOLOGY

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***Summary.*** During the past couple of decades, the understanding of the tissue renin-angiotensin system (RAS) has increased especially in its role in compensatory hypertrophy and remodeling of the myocardium. Angiotensin (Ang) II-induced growth and proliferation of vascular smooth muscle cells are important elements in hypertension and atherosclerosis. Thus, treatment with angiotensin-converting enzyme (ACE) inhibitors has proved efficacious in preventing both atherosclerosis and hypertension. Left ventricular remodeling following myocardial injury is another area of interest, where recent research has been focused. Increased contractile recovery in low-flow ischemia in hearts treated with ACE inhibitors was demonstrated recently in our laboratory. The contribution of RAS in other pathological conditions such as diabetes-induced cardiomyopathy has also been studied recently. These studies only provide preliminary results, and further studies are needed to completely delineate the role of myocardial RAS in disease-related cardiomyopathy. The development of ACE inhibitors of higher specific binding and high-lipid solubility has recently been the goal of many pharmaceutical companies, and many new ACE inhibitors have emerged in the market. Ang II receptor subtypes, $AT_1$ and $AT_2$, have also been the focus of recent research conducted by selective modulation of these receptors rather than by using ACE inhibitors to reduce Ang II levels. Future research in this area will provide tools to benefit from increased Ang II, yet will prevent the maladaptive deleterious effects of Ang II by antagonizing the specific subtype of receptor.

## INTRODUCTION

The role of plasma renin-angiotensin system (RAS) in cardiovascular disease has long been recognized. More recent evidence for the existence of tissue-based RAS

*N.S. Dhalla, P. Zahradka, I. Dixon and R. Beamish (eds.). Angiotensin II Receptor Blockade: Physiological and Clinical Implications.* 

[1] has established that the RAS can operate as both an endocrine (circulating) and an autocrine/paracrine (tissue) system. The active peptide angiotensin (Ang) II from the circulation may bind the target organ receptors. The tissue components of RAS may act locally or be released into the circulation to act at a distant target [2]. The circulating Ang II binds to receptors at multiple organ sites and plays only a short-term role in cardiac homeostasis, which includes vasoconstriction, adrenal aldosterone release, renal sodium reabsorption and possibly act as cardiac positive inotrope. The tissue RAS, which has long-term regulation effect, may cause vascular and cardiac hypertrophy and remodeling. In addition, the cardiac RAS may act as a mediator of the inflammatory response through its effects on bradykinin concentrations. Thus, both the circulating and tissue RAS systems play a role in the pathophysiological cycle of maladaptive structural remodeling. Inhibition of the RAS and the tissue RAS are likely to mediate beneficial effects in cardiovascular disease. The purpose of the present treatise is to review the current evidence which supports this contention.

## EVIDENCE FOR THE EXISTENCE OF TISSUE RAS

The evidence for the existence of a tissue (autocrine/paracrine) RAS has been obtained through the extraction of various components of RAS from the isolated tissue. These components include angiotensinogen, renin, and angiotensin-converting enzyme (ACE). In addition, local synthesis of angiotensin has been demonstrated in isolated perfused organ preparations [3]. Northern blot analysis demonstrated the expression of angiotensinogen in RNA in various rat and mouse tissues, such as kidney, adrenal gland, and heart [1]. ACE has also been identified in almost all parts of the heart tissue by using histological techniques. High density of ACE has been found in the right and the left atrium, the ascending and the descending aorta, and the valvular structures [4]. An autoradiographic study of radiolabeled ACE inhibitor binding showed a wide distribution of ACE in kidney, adrenal, aorta, and heart tissue. Oral administration of ACE inhibitors to rat before autoradiography substantially decreased the ACE radiolabel concentration binding in these tissues [5].

## TISSUE RENIN-ANGIOTENSIN SYSTEM IN CARDIOVASCULAR FUNCTION

Activation of tissue and circulating RAS during the course of congestive heart failure (CHF) demonstrated that the tissue RAS regulates long-term effect while the circulating RAS exerts short-term influences [6]. Hirsch et al. [6] found that the circulating RAS is activated during the acute phase of cardiac decompensation. In the compensated phase of heart failure, the tissue RAS is activated while the circulating RAS remains normal. In the end-stage of decompensation, both tissue and circulating RAS are activated. The circulating Ang II mediates systemic vasoconstriction, aldosterone release, and renal sodium reabsorption. These changes will affect both preload and afterload. In the heart, Ang II has a direct effect on contractility, as well as cardiac metabolism. Evidence also points to a role for Ang

II in the modulation of vascular structure and left ventricular hypertrophy in hypertension. Cardiac RAS may also mediate inflammatory response through its effects on bradykinin concentration, as well as its interactions with macrophages and fibroblasts [2].

## RENIN-ANGIOTENSIN SYSTEM IN CARDIOVASCULAR DISEASE

Both the circulating and the tissue RAS play significant roles in the pathophysiological cycle of the remodeling and maladaptive structural changes.

### Contribution of cardiac tissue RAS to ventricular remodeling in myocardial ischemia

After myocardial injury, the noninfarcted part of the heart tissue must compensate for the infarcted segments. This leads to cardiac hypertrophy in the normal segments and ventricular dilation in the infarcted segments. This cardiac remodeling will eventually become maladaptive and result in CHF and end-stage heart disease [7]. Ang II exerts direct cell growth and stimulates expression of photooncogene [8] and synthesis of platelet-derived growth factor (PDGF). Multiple lines of evidence indicate that ACE inhibitors and Ang II receptor antagonists reduce or prevent ventricular dilation and heart failure and increase survival rate after myocardial infarction [9–12]. In one study in rat, when the left ventricle was infarcted, a twofold rise in the right ventricular ACE activity was observed. A comparable increase in ACE activity was observed in the intraventricular septum of rat with experimental heart failure [13]. In studies on the growth of cell culture consisting of mouse fibroblasts [14,15], saralasin (as Ang II receptor antagonist) was found to have a stimulatory effect on intracellular renin concentration and an inhibitory effect on cell growth. Ang II had the opposite effect; it stimulated cell growth and inhibited intracellular concentration of renin. Angiotensinogen mRNA expression is increased in the atria and ventricles of rat in the early phase after experimental myocardial infarction [16]. Enhanced production of Ang II in the infarcted cardiac tissue has also been reported [17]. Marked increases in the ACE activity in the scar tissue have been shown recently [18]. Evidence also suggests that Ang II in rat causes fibroblast proliferation and scar formation in the heart [19–20]. Treatment with ACE inhibitors after the infarction was shown to inhibit the cardiac ACE, including the high level found in the scar tissue [18].

In a recent study in our laboratory, we investigated the cardioprotective effects of structurally different ACE inhibitors on low flow (25%) ischemia produced in isolated rat heart. A significant protection from the ischemic damage was observed when the ACE inhibitors were present in the perfusion medium (table 1). The hearts treated with analapril demonstrated the greatest protection and showed significant (35–40%) recovery of contractile function during low flow ischemic perfusion. The protection by analapril is higher than that by captopril and fosinopril. This higher protection by analapril may be due to its higher specific binding to the receptor sites and its high-lipid solubility.

**Table 1.** Effects of ACE inhibitors on LV function during ischemia and reperfusion in isolated perfused rat heart

| | % Contractile Function | | | |
|---|---|---|---|---|
| | | ACE Inhibitors (400 uM) | | |
| Heart Perfusion | Control | Analapril | Captopril | Fosinopril |
| Normal (8 ml/min) | 100 | 100 | 100 | 100 |
| Global Ischemia (5 min) | 3 ± 0.9 | 7 ± 2.0 | 5 ± 0.5 | 4 ± 0.5 |
| Low Flow Ischemia (2 ml/min for 15 min) | 19 ± 2 | 43 ± 3* | 32 ± 1.5 | 22 ± 1.0 |
| Normal Reperfusion (8 ml/min for 30 min) | 70 ± 8 | 85 ± 3* | 72 ± 5 | 75 ± 4 |

Note: The values represent mean ± S.E. of 5–6 experiments in each group.

**Table 2.** Diabetes-induced alterations in myocardial ang II receptors

| Treatment | % Change in Myocardial Ang II Receptors |
|---|---|
| Control (nondiabetic) | 00.00 |
| Diabetic (6 weeks, no treatment) | 206.4 |
| Diabetic (6 weeks, insulin treatment) | 78.1 |
| Diabetic (6 weeks, captopril treatment) | 56.3 |

Note: The values represent the average of 4 experiments in each group.

### Tissue RAS in diabetes-induced cardiomyopathy

Diabetes-induced cardiomyopathy has been documented both in humans and in animal species [21,22]. In streptozotocin (STZ)-induced diabetic rat model, abnormalities in myocardial function are seen as early as twelve weeks after the induction of diabetes [23]. Both systolic and diastolic functions of the heart have been shown to be impaired in these animals [23]. The impairment of the left ventricular function in the diabetic rat has been attributed to the defects in $Ca^{2+}$ transport at subcellular level [24–26].

Ang II has been implicated in the regulation of cellular growth and cardiocyte hypertrophy [15,27]. There is evidence suggesting downregulation of the plasma RAS in STZ and alloxan-induced diabetic rats [28,29]. Others have reported that plasma renin concentration does not change in STZ-induced diabetic rats [27]. On the other hand, an increased expression of $AT_1$, Ang II receptor subtype in cardiac tissue has recently been reported [30]. $AT_1$ receptor mRNA and increased receptor density has been reported in two-week-old diabetic rats [31]. Large increases in Ang II receptors were demonstrated recently in the hearts of rats with STZ-induced chronic diabetes [32]. Treatment with insulin (5–6 U/day) normalized plasma glucose levels but only partially prevented the increase in Ang II receptors (table 2). Treatment with the ACE inhibitor captopril also partially prevented the increase in Ang II receptors. Although we did not measure plasma angiotensin levels, others

have reported [27] that plasma renin concentration does not change in STZ-induced diabetic rats. This would indicate that the substantial increases in Ang II receptor density in diabetic rat heart are due to local changes in the expression of these receptors and may be related to the alterations in cardiac function, a consequence of diabetes-induced cardiomyopathy.

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## C. ANGIOTENSIN BLOCKADE AND CARDIAC HYPERTROPHY AND HEART FAILURE

# MECHANICAL STRESS, LOCAL RENIN-ANGIOTENSIN SYSTEM AND CARDIAC HYPERTROPHY: AN OVERVIEW

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***Summary.*** Hypertrophy is a fundamental adaptive process employed by postmitotic cardiac and skeletal muscles in response to mechanical load. External load also plays a critical role in determining muscle mass and its phenotype in cardiac myocytes. Interestingly, cardiac myocytes have the intrinsic ability to sense mechanical stretch and convert it into intracellular growth signals, which finally culminate in hypertrophic growth. Mechanical stretch of cardiac myocytes in vitro causes activation of multiple messenger systems, upregulation of many immediate early genes (e.g., c-fos, c-myc, c-jun, etc.), and re-expression of fetal-type genes (e.g., atrial natriuretic factor, skeletal α-actin, β-myosin heavy chain), reminiscent of cardiac hypertrophy in vivo. Stretch of neonatal rat cardiac myocytes stimulates a rapid secretion of angiotensin (Ang) II and an upregulation of all major components of cardiac renin-angiotensin system (RAS) genes. Ang II, along with other (secreted) growth factors, mediates many, if not all, stretch-induced hypertrophic responses. In this review, the relationship between mechanical loading, cardiac RAS, and cardiac hypertrophy is discussed. In addition, various cell signaling mechanisms initiated by mechanical stress on cardiac myocytes are briefly summarized.

## INTRODUCTION

An increasing body of molecular biological, biochemical, and physiological evidence now suggests that RAS plays a predominant role in regulating cardiovascular homeostasis. As is well recognized, the principal mediator of RAS is the octapeptide Ang II produced from its precursor angiotensinogen by the successive action of two proteases, renin and angiotensin-converting enzyme (ACE). In the past 20 years or so, the contribution of RAS to human pathophysiology, its role in human cardio-

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vascular diseases, and its therapeutic modulation have been extensively investigated. The findings have provided a number of valuable insights into mechanisms of disease and have resulted in the development of the ACE inhibitors and the angiotensin receptor antagonists. Until recently, RAS, as far as its effect on the heart, has been considered to be a classical endocrine system, with the circulating Ang II solely mediating the biological actions of RAS on the cardiac tissue. However, in the past decade or so, several local RAS have been described in various tissues including adrenal, blood vessels, brain, kidney, and the heart [1–5]. These studies have defined a whole new scenario for cardiac (local) RAS and provide direct evidence for autocrine and paracrine pathways for the actions of Ang II. Of paramount importance has been the demonstration that mechanical stress on cardiac myocytes in vitro causes a concomitant secretion of Ang II, which then acts as a critical mediator of stretch–induced hypertrophic responses [6]. Numerous in vitro and in vivo studies have also shown that myocyte hypertrophy is accompanied by an upregulation of the local (cardiac) RAS and have shown the importance of the (secreted) Ang II in this response [7–9].

Mechanical stimuli also cause a rapid change in gene expression [10,11]. The phenotypic feature of stretched myocytes is very similar to that of pressure overload-induced hypertrophy in vivo [12]. Myocardial stretch and RAS have been implicated in the development of cardiac hypertrophy through the activation of specific target genes. In this review, the role of the cardiac RAS in load-induced cardiac hypertrophy is discussed. In addition, various cell signaling mechanisms initiated by mechanical stress and Ang II on cardiac myocytes are also summarized. The role of the Ang II receptors (both $AT_1$ and $AT_2$), which play a crucial role in the pathophysiology of RAS, is also discussed, with special reference to the regulation of cardiac hypertrophy.

## CARDIAC RENIN, ANGIOTENSINOGEN, ACE, AND ANGIOTENSIN RECEPTORS

The identification of an independent cardiac RAS requires the demonstration of tissue synthesis of the components necessary for the synthesis of Ang II. Renin and angiotensinogen mRNA have been detected in the rat and mouse heart by reverse transcription-polymerase chain reaction (RT-PCR), Northern hybridization analysis, S1 nuclease protection assay, and in situ hybridization analysis [13–16]. Renin activity has also been documented in isolated rat cardiomyocytes and is inhibitable by a renin-specific antibody [13]. Renin and angiotensinogen protein and mRNA have been localized to cultured cardiomyocytes and fibroblasts isolated from the ventricles of neonatal rats [5,17]. However, the significance of renin and angiotensinogen in nonmyocytes needs to be explored further. ACE has been shown to be associated with vascular smooth muscle [18], adult rat cardiomyocytes [19], and cultured ventricular myocytes and fibroblasts from neonatal rat hearts [20].

Thus, the complete RAS cascade has been reported in cultured neonatal rat ventricular myocytes and fibroblasts [5,20]. The presence of nanomolar concentra-

tions of Ang I and Ang II in media from cultured cardiomyocytes and fibroblasts suggests that sufficient levels of peptide may be produced by these cells to be physiologically relevant [20]. Very recently, the concentrations of Ang I and Ang II in cardiac interstitial fluid, reaching a nanomolar range, were reported to be 100-fold greater in magnitude than those of plasma level [21]. Thus, physiologically significant levels of Ang II seem to be present in the heart in vivo. Even though intracellular localization of renin, angiotensinogen, and ACE in cardiomyocytes and fibroblasts is documented, which may reflect synthesis and cellular processing of these components, it is concievable that, following processing in the Golgi apparatus, RAS components may be copackaged in intracellular vesicles where they could interact to form Ang II. The immunoreactive staining patterns of Ang I and Ang II are very similar to that of renin, angiotensinogen, and ACE, which supports this hypothesis [23]. Intracellularly generated Ang II may be exported and have paracrine or autocrine functions or may remain within the cell and exert intracellular effects. The latter possibility is supported by evidence that internalized Ang II localizes in mitochondria and nuclei [22].

The functioning of cardiac RAS requires the presence of appropriate angiotensin receptors coupled to signal transduction pathways. Cardiac angiotensin receptors have been demonstrated in sarcolemmal membrane preparations from avian [23], bovine [24], guinea pig [25], rat [7], and human myocardium [26]. The actions of Ang II, the main agonist of RAS, in the cardiovascular system are transmitted by two known ($AT_1$ and $AT_2$), and possibly some unknown, angiotensin receptor subtypes. $AT_1$ and $AT_2$ both correspond to G protein-coupled receptors with seven hydrophobic transmembrane domains, several N-glycosylation sites, and a potential G protein binding site [27]. The classification of Ang receptor subtypes is based on different binding affinities of $AT_1$ and $AT_2$ for the subtype-especific compounds losartan ($AT_1$ binding affinity) and CGP 421121A and PD 123177 ($AT_2$ binding affinity) [28]. In the rat, two isoforms of $AT_1$ exist, $AT_{1A}$ and $AT_{1B}$, which exhibit 95% homology at the protein level [29,30].

## MECHANICAL LOADING AND CARDIAC HYPERTROPHY

Hypertrophy is a fundamental adaptive process employed by postmitotic cardiac and skeletal muscles in response to mechanical or hemodynamic overload. Since most cardiac myocytes, if not all, are terminally differentiated and lose their ability to replicate soon after birth, they respond to increased workload only by an increase in cell size (hypertrophy) and not by an increase in cell number (hyperplasia). An intriguing and unresolved aspect of this process has been the ability of muscle cells to sense the stimulus of mechanical stress (presumably on the membrane) and convert it into intracellular growth signals. Mechanical forces have been known to cause a variety of effects on the structure and function of cells, but how mechanical stimuli regulate cell function and gene expression is poorly understood [31,32]. For example, it has been demonstrated that cultured skeletal and cardiac myocytes, grown on an elastic substrate, undergo hypertrophy in response to static stretch of

the substrate [10,11,32,33]. Since this phenomenon of stretch-induced hypertrophy can be observed in a serum-free environment, muscle cells clearly have the inherent ability to sense external load even in the absence of neuronal and hormonal factors.

One experimental model which has yielded tremendous knowledge of the underlying mechanisms and the intracellular signal transduction pathways associated with cardiac hypertrophy and its link with local RAS is the in vitro stretch model using cultured neonatal rat cardiac myocytes [11,34]. By using this model, Ang II has been demonstrated to act as a critical mediator of stretch-induced cardiac hypertrophy. Several investigators examined the intracellular signaling mechanisms of the stretch-induced hypertrophic response in cardiac myocytes using this in vitro stretch model [10,34,35]. Culture medium conditioned by stretched cardiac myocytes was demonstrated to contain a factor or factors that cause induction of immediate early genes and activation of second messenger systems, such as mitogen-actiuated protein kinases (MAPKs), in nonstretched myocytes [6]. This factor was later identified to be Ang II, and mechanical stretch of neonatal rat cardiac myocytes in serum-free culture caused a more than 100-fold increase in Ang II concentrations in culture media ($\approx$500 pmol), peaking at 10 mins [6]. This acute release seems to be from intracellular stores rather than an increase in synthesis or production. The phenomena of stretch-induced secretion of Ang II from neonatal rat cardiac myocytes has been confirmed by three other independent groups [36–38], thereby establishing a link between mechanical stress, local (cardiac) RAS, and cardiac growth (discussed below). Nonetheless, the mechanism of Ang II release from cardiac myocytes in response to stress and the associated signal transduction pathways remains elusive.

Mechanical stretch-induced growth factor secretion in other cell systems, e.g., endothelin-1 release from endothelial cells [39], shows a transient secretion peaking at about 10–120 minutes, indicating the presence of growth factor stores that can be released in response to acute mechanical stretch. In atrial myocytes, stretch causes secretion of ANF from stretch-sensitive, rapidly depletable pools that consist of newly synthesized ANF from its precursors [40]. Thus, there are several different possibilities of Ang II release, but a unifying hypothesis needs to be established. First, mechanical stress may transiently alter sarcolemmal membrane permeability, which may allow the release of cytosolic growth factors. Recently altered sarcolemmal permeability was shown to account for pacing-induced basic fibroblast growth factor (FGF) release from adult rat ventricular cardiac myocytes [41]. Secondly, membrane tension may directly regulate membrane traffic and stimulate exocytosis/secretion [42].

Besides the release of Ang II, how the stretch message is communicated to hypertrophy-related genes remains elusive. A number of candidates have been hypothesized as couplers of physical stretch to growth initiation, including neural and hormonal factors, stretch–activated and stretch-inactivated ion channels, microtubules, microfilaments, and contractile activity. Recently a desmin-lamin intermediate-filament network and nuclear envelope-associated chromatin was

shown to undergo spatial rearrangement in stretched cardiac myocytes, and this interaction was hypothesized to activate hypertrophy-related genes [43]. Mechanical forces have also been shown to have a stabilizing effect on the cellular levels of beta 1-integrin and vinculin, and thereby regulate their association with the formation of focal adhesions and costameres [44].

## ANGIOTENSIN II

There are several lines of evidence suggesting that local synthesis of angiotensins occurs within the myocardium. Angiotensins detected in tissue could conceivably result from local synthesis or from sequestration of peptide or percursors from the circulatory system. However, a variety of experimental evidence suggests that cardiac RAS may function separately from the circulatory system [45], and, as already discussed, this is supported by multiple findings from biochemical, immunohistochemical, and molecular biological demonstrations of all components of RAS in the heart [5,46,47]. Accumulating evidence suggests that Ang II (locally produced or circulating), among the various growth factors, may be the critical factor mediating load-induced hypertrophy in vivo. First, treatment of rats having aortic coarctation with an ACE inhibitor or Ang II type 1 receptor antagonist (losartan and TCV 116) prevented or caused regression of left ventricular hypertrophy by pressure overload [7,48,49]. An ACE inhibitor also prolonged survival of rats having pressure overload [50]. Similarly, cardiac hypertrophy that occurs in rats with aortocaval shunt and volume overload can be prevented with losartan or an ACE inhibitor (quinapril) with high affinity for cardiac ACE [51,52]. Treament of patients suffering from myocardial infarction with ACE inhibitors prevented cardiac dilatation and reduced mortality [53]. These results are consistent with the involvement of RAS and its activation by a variety of hemodynamic loading in vivo and subsequent pathogenesis of cardiac hypertrophy and failure.

Further, evidence supporting the predominant role of Ang II as a growth and hypertrophic factor has come from studies using Ang II receptor antagonists, such as [$Sar^1$, $Ile^8$] Ang II (antagonists for the Ang II type I and II receptors) and losartan and TCV 11974 (antagonist for the Ang II type I receptor). These Ang II receptor antagonists inhibit major markers of stretch-induced hypertrophy, such as c-fos gene expression, MAPK activation, an increase in the rate of protein synthesis, and induction of fetal type genes (e.g., atrial natriuretic factor and skeletal $\alpha$-actin), which suggests that Ang II plays a critical role in stretch-induced hypertrophy in the neonatal rat myocyte culture system [6,38]. However, even in this model, studies show some components of the hypertrophic response, such as increased rate of protein synthesis [54] and MAPK activation [38], are Ang II antagonist uninhibitable, thereby suggesting that part of the hypertrophic response may be mediated by some other autocrine/paracrine growth factors.

Recently it was reported that the vasoactive peptide endothelin-1 (produced by endothelial and epithelial cells, macrophages, fibroblasts, as well as cardiac myocytes) also plays an important role in mechanical stress-induced cardiac hypertrophy [55].

Tumor necrosis factor α (TNFα), a proinflammatory cytokine with a broad range of pleiotropic effects, is expressed de novo by cardiac myocytes after certain forms of stress [56]. Recently TNFα was shown to provoke a hypertrophic response (in cultured adult feline cardiac myocytes), suggesting that this cytokine may play an important role in myocardial homeostasis after environmental stresses [57]. In isolated adult rat ventricular myocytes, release of basic fibroblast growth factor (bFGF) has been shown to play a critical role in pacing-induced cardiac hypertrophy [41]. In a somewhat different scenario, adult cardiac myocytes have been shown to respond to elevation in wall and myocyte stress (induced by nonocclusive coronary artery narrowing) by activating an insulin-like growth factor-1 and insulin-like growth factor-1 receptors ($IGF_1$, $IGF_1$-R) autocrine system, which may modulate the induction of late growth-related genes (proliferating cell nuclear antigen, PCNA, and histone $H_3$ genes), essential for DNA replication and myocyte cellular hyperplasia [58]. It is concievable, therefore, that mechanical stretch itself may activate certain other signalling mechanisms, and the resulting hypertrophic response (and in an exceptional situation, hyperplasia) may be variable, depending on the autocrine and paracrine factors secreted, the cell density, nature of the extracellular matrix (e.g., interactions between cell integrins and the extracellular matrix), type and degree of mechanical stress, and lastly the age and species of the experimental animals [6,38,54].

## SIGNAL TRANSDUCTION PATHWAYS ACTIVATED BY MECHANICAL STRETCH

Over the last several years, many genes that are responsive to increased workload have been identified in the heart. These genes can be divided into two classes. One class is called immediate early genes (IEG), a group of genes whose transcription is activated rapidly and transiently within minutes of extracellular stimulation [59]. The tightly controlled expression of IEG suggests a regulatory role in the cellular response to external stimuli. The IEG activated by mechanical stretch include c-fos, c-jun, Egr-1, and c-myc [10,11]. The second class of genes is called the late response genes or the so-called stable late markers of myocardial hypertrophy, whose expression is induced more slowly, over hours, and which also needs new protein synthesis. Cardiac hypertrophy is associated with upregulation of the fetal program; that is, this response is characterized by re-expression of protein isoforms that are ordinarily expressed in the embryonic heart, but not in the adult heart. These late response genes include skeletal α-actin, β-MHC, and ANF [12,60–62]. Mechanical stretch of cultured cardiac myocytes also induces the expression of skeletal α-actin, β-MHC, and ANF [11]. Recently, stimulation of RAS (in response to abdoiminal aorta banding) was shown to be crucial for the activation of the β-MHC [63], thus defining an important interaction between phenotypic reprogramming, mechanical stress, and upregulation of RAS.

In cultured neonatal cardiac myocytes, mechanical stretch causes activation of multiple messenger systems including phospholipases C, D, and $A_2$; tyrosine kinases; $p21^{ras}$; Raf-1; mitogen-activated protein kinases (MAPKs) such as ERKs (ERK1 and

ERK2), JNKs (c-Jun N-terminal kinases), and p38/RK, activators of MAPKs such as MEK1 (MAPK kinase) and MEKK1 (MEK kinase); 90-kDa S6 kinase ($pp90^{RSK}$); protein kinase C (PKC); and probably other molecules [32, 64–68]. A comprehensive description of the various intracellular signalling molecules activated by mechanical stretch and the possible mechanosensors involved in the process have been reviewed recently [69,70].

## RENIN-ANGIOTENSIN SYSTEM AND CARDIAC HYPERTROPHY

Several lines of in vivo evidence also suggest that the cardiac RAS is upregulated chronically in load-induced hypertrophy. These studies have demonstrated that mRNA expression of angiotensinogen, ACE, and Ang II type 1 ($AT_1$) and Ang II type 2 ($AT_2$) are all upregulated in response to pressure overload cardiac hypertrophy and myocardial infarction [7, 71–75]. At the protein level, upregulation of ACE activity and Ang II receptor binding was demonstrated [74,76], and the percent of myocytes containing renin, Ang I, and Ang II was significantly increased in hypertrophied hearts [77]. In a canine model of right ventricular hypertrophy and failure, upregulation of ACE and $AT_2$ receptor was demonstrated recently [78]. Similarly, cardiac renin activity, as well as renin mRNA, is increased in experimental animals with volume overload-induced cardiac hypertrophy [79,80].

The upregulation of cardiac RAS genes, including renin, angiotensinogen, ACE, and $AT_{1A}$ receptor, has also been observed in mechanical stretch of neonatal rat cardiac myocytes in vitro [81–83]. Interestingly, treatment of cultured cardiac myocytes with exogenous Ang II also upregulates mRNA expression of angiotensinogen, renin, and ACE, but not Ang II receptor [35,81]. This suggests that mechanical stretch initially causes acute secretion of preformed Ang II and that secreted Ang II may initiate positive feedback mechanisms, thereby upregulating the local RAS over time. Upregulation of all components of RAS in cardiac hypertrophy has been also shown in adult rats with ischemic cardiomyopathy [77].

Ang II-induced hypertrophy of neonatal cardiac myocytes has a phenotype indistinguishable from stretch-induced hypertrophy. This phenotype includes an increase in the rate of protein synthesis with a modest increase in total protein content; expression of various immediate early genes; induction of fetal-type genes including ANF, skeletal α-actin, and β-myosin heavy chain; and reorganization of the actin cytoskeleton [11]. Ang II stimulates angiotensinogen gene expression and transforming growth factor β expression and release and thus may initiate a positive feedback mechanism in cardiac hypertrophy [35,84]. Ang II through $AT_1$ stimulates ANF release in isolated rabbit hearts independently of hemodynamics [85] and thus activates counter-regulatory processes in hypertension. Ang II acts as a mitogen on neonatal rat cardiac fibroblasts and stimulates cellular proliferation and collagen synthesis [86–88]. The $AT_1$ receptor modulates fibronectin expression and cardiac fibrosis. In isolated adult human cardiac fibroblasts, Ang II stimulates proliferation and collagen production [89,90].

Ang II activates multiple second messenger systems via the $AT_1$ receptor-dependent mechanism. These signaling events include the stimulation of

phospholipase C, A2, D, and PKC; activation of the mitogen-activated protein kinases (MAPKs); the 90-kDa and 70-kDa S6 kinases; the proto-oncogenes ras and raf; and the activation of the immediate early genes c-fos, c-jun, c-myc, and egr-1 [91–94]. Recent findings also associate Ang II with the tyrosine phosphorylation of proteins, thus suggesting a similar signaling pathway as found in cytokine receptors, including the activation of Src family kinases, $pp^{60c\text{-}src}$, and janus kinases, JAK2 and TYK2. Ang II-mediated phosphorylation of JAK2 and TYK2 leads to tyrosine phosphorylation and nuclear translocation of the JAK substrates STAT1 and STAT2 (signal transducers and activators of transcription) proteins [95–97]. This indicates that the JAK-STAT pathway may also be involved in the mediation of the angiotensin-related trophic response.

On the other hand, the signal transduction mechanisms associated with the $AT_2$ receptor remain elusive. So far no influence of the $AT_2$ receptor on the generation of phospholipid-derived second messengers, intracellular calcium release, or cAMP production has been demonstrated [98]. The role of $AT_2$ in the cardiovascular system is not as well established as that of $AT_1$. Based on interaction between $AT_1$ and $AT_2$ signaling pathways, $AT_2$ could potentially counteract the growth-promoting effects of Ang II that is mediated via the $AT_1$ receptor by dephosphorylation of proteins phosphorylated after $AT_1$ receptor stimulation [99]. An unopposed antigrowth effect of $AT_2$ receptor on Ang II-induced cardiomyocyte hypertrophy in culture has also been demonstrated recently [100]. This proposed antigrowth effect of $AT_2$ is supported by the observations that overexpression of $AT_2$ attenuates neointima formation in the rat carotid arteries [101] and that $AT_2$ receptor inhibits proliferation of endothelial cells [102]. Further, the antigrowth effects of $AT_2$ are highlighted by its participation in the induction of apoptosis [103–105].

## TRANSGENIC ANIMAL MODELS

The function of the angiotensin receptors ($AT_1$ and $AT_2$) and other genes of the RAS has been investigated in several transgenic and knockout animal models. Targeted disruption of the $AT_{1A}$ receptor gene in mice confirms its crucial role in the maintenance of blood pressure. Pressor responses to infused Ang II were virtually absent in homozygous animals [106].

Recently $AT_2$ receptor knockout mice have been created by two experimental groups. In both cases, these animals exhibit significant increase in blood pressure, increased sensitivity to the pressor action of Ang II, and altered exploratory behavior which suggests a role of $AT_2$ in brain function and blood pressure control [107,108].

Ang II receptor regulation has also been assessed in Tsukuba hypertensive mice which carry the human genes for renin and angiotensinogen [109]. In these mice, the plasma RAS is activated, and the mice develop cardiac hypertrophy. Ang II receptor is upregulated at the mRNA and protein level, suggesting that expression of the $AT_1$ gene increases in response to Ang II-induced cardiac hypertrophic changes.

The primary function of RAS in maintaining blood pressure has also been confirmed by disruption of the angiotensinogen gene by homologous recombination

in embryonic stem cells in mice [110]. Very recently, mechanical stretch-induced activation of MAPK (Erk kinase) was analysed in cardiomyocytes of angiotensinogen gene-deficient mice (Agt−/−). Mechanical stretch resulted in activation of MAPKs in Agt−/− cardiac myocytes that was significantly greater than that in wild type myocytes (Agt+/+), and CV-11974, the $AT_1$ receptor antagonist, suppressed stretch-induced activation of MAPKs in only wild-type cardiac myocytes, but not in Agt−/− cells, which suggests that Ang II is dispensable for mechanical stretch-induced activation of MAPKs in Agt−/−cardiac myocytes and that unknown compensatory mechanisms exist in the absence of Ang II. Endothelin receptor blocker BQ123 had no effect in stretch-induced MAPK activation in wild type and Agt−/− cells. [111]. In other transgenic models, for example, the transgenic rat TGR(mRen2) 27, which carries the mouse Ren-2 gene, experiments have shown that the cardiac RAS is responsible for cardiac hypertrophy, phenotypic modulation, and remodeling [112,113].

## CONCLUSION

RAS plays an important role in the hypertrophic responses in cardiac myocytes through the activation of signal transduction pathways and expression of proto-oncogenes. Numerous studies have also demonstrated that Ang II acts directly on cardiac myocytes as a growth-promoting factor and that ACE inhibitors and Ang II receptor blockers induce regression of hypertrophied hearts in experimental animals and humans. Although in vitro studies using mechanical stretch of neonatal rat cardiac myocytes have clearly shown that autocrine secretion of Ang II plays an essential role in stretch-induced cardiac hypertrophy in vitro, many questions remain unanswered. First, whether Ang II functions as a primary mediator of stretch-induced hypertrophic responses in the hearts of different ages and species, as well as those in vivo, needs further investigation. In addition to Ang II, many other growth factors (endothelin-1, bFGF, etc.) have been shown to cause cardiac hypertrophy when exogenously applied in vitro. At the present time, whether these factors are physiological mediators of load-induced cardiac hypertrophy in vivo is unclear. Second, the molecular identity of the initial "trigger" or "mechanosensor" of cardiac myocytes remains an enigma. Also, the signal transduction mechanisms initiated by stretch itself, besides Ang II, and their role in hypertrophic responses need to be defined. Finally, the mechanism of intracardiac generation and release of Ang II remains to be identified, despite the demonstration of angiotensinogen, renin, and ACE mRNAs in the heart. This would require further identification and localization, within the heart, of individual cell types that are responsible for producing components of the RAS; regulation of the synthesis, storage and secretory pathways for the individual components and integration of the cardiac RAS with other effector pathways in the heart. Thus, the relative role of the cardiac RAS compared with the systemic RAS needs to be determined.

One can envisage that several new physiological and pathophysiological facets of the cardiac RAS will be discovered in the coming years and that they will finally

lead to the development of therapeutical strategies for the treatment and prevention of cardiac hypertrophy, remodeling, and heart failure.

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# ROLE OF RENIN-ANGIOTENSIN SYSTEM IN CARDIAC HYPERTROPHY AND FAILURE

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***Summary.*** Although the circulatory renin-angiotensin system (RAS) was discovered a century ago, it is only recently that the presence of tissue RAS has become evident. Angiotensin (Ang) II has been demonstrated to influence heart function by effecting cardiac contraction, myocyte growth, cardiac matrix, and cardiac metabolism. These actions are initiated by the binding of Ang II to plasma membrane receptors, namely, $AT_1$ and possibly $AT_2$, which stimulates phospholipase C (PLC) to produce phosphatidylinositol 4,5-bisphosphate, thus forming diacylglycerol (DAG) and inositol 1,4,5-triphosphate ($IP_3$). An increase in the intracellular $Ca^{2+}$ appears to result from $IP_3$-mediated release of $Ca^{2+}$ from the intracellular stores, and this effect may be associated with an increase in cardiac force development. On the other hand, DAG activates protein kinase C (PKC) which stimulates cardiac growth and other actions of Ang II. Intracellular signaling of Ang II-stimulated cardiomyocyte growth may include the activation of tyrosine kinase and mitogen-activated protein kinase (MAPK) cascade. Alterations in different components of RAS, such as renin, angiotensinogen, and angiotensin-converting enzyme (ACE), as well as Ang II receptors ($AT_1$ and $AT_2$), have been shown to occur in different pathological conditions of cardiac hypertrophy and heart failure. On the other hand, ACE genotype has been shown to exhibit a close relationship with myocardial infarction. In this article, we have attempted to review the influence of ACE inhibitors on different types of cardiac hypertrophy and heart failure. Although existing results are controversial, ACE inhibitors, in general, have been shown to exert beneficial effects on cardiac function in myocardial infarction, cardiac hypertrophy from volume or pressure overload, as well as heart failure because of pacing and cardiomyopathy. The possible mechanisms of the effects of ACE inhibitors may include reduction in both circulating and local RAS, scavenging of free radicals, improvement of energy metabolism, modification of the autonomic nervous system, and increase of bradykinin concentration. More importantly,

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ACE inhibitors may improve cardiac function by remodeling the cell membranes, mobilizing $Ca^{2+}$, and attenuating the shift in myosin isozymes. Although Ang II receptor antagonists have also been shown to have protective effects on contractile function in cardiac hypertrophy and heart failure, the mechanisms remain to be fully understood.

## RENIN-ANGIOTENSIN SYSTEM AND HEART FUNCTION

Although RAS was discovered in 1898 by Tigerstedt and Bergman [1], attention was not given to this major cardiovascular control mechanism until 1934 when Goldblatt et al. [2] developed a reproducible model which showed that the renal pressor substance is an enzyme. The term "angiotensin" was coined in 1958 for the active end-product of RAS, whereas the importance of this vital neuroendocrine system was only recognized in hypertension and heart failure upon the availability of angiotensin-converting enzyme (ACE) inhibitors in the late 1970s [3,4]. The classic view of the RAS is based on the premise that various components are derived from different organs and are in turn delivered to their site of action via the circulatory system. The primary components are (1) angiotensinogen, a large globular protein that is secreted as the substrate for renin; (2) renin, an enzyme that catalyzes the proteolytic conversion of angiotensinogen to the decapeptide Ang I; (3) angiotensin-converting enzyme, a dipeptidyl carboxypeptidase that converts Ang I to Ang II by cleavage of the two carboxyterminal amino acids; (4) Ang II, a highly active octapeptide; and (5) Ang II receptors, specific receptors in the cell membrane upon which Ang II acts to produce physiological actions.

Renin, the rate-limiting enzyme of the cascade that leads to Ang II formation, is an aspartyl protease with a molecular mass between 37,000 and 40,000. Its primary structure contains double domains; the amino- and carboxyl termini contain areas of similar sequence [5]. Renin is widely distributed and mRNA repression of renin can be found in kidney, adrenal, heart, ovary, testis, lung, and adipose tissue [6]; however, the main source of renin is the kidney [7]. Human renin gene is a 12.5 Kb DNA sequence. On the other hand, angiotensinogen is an $\alpha_2$ globulin with a molecular weight of 54,000 to 60,000. It is the only known substrate for renin and is the only known precursor for angiotensin peptides in vivo. There is only a single gene of angiotensinogen with a 13 Kb sequence [8], and the majority of the circulating angiotensinogen is from the liver. It is pointed out that ACE is a zinc metallopeptidase [9] that catalyzes the conversion of Ang I to Ang II and the breakdown of bradykinin to kinin, in addition to catalyzing a broad range of substrates [10]. This enzyme has two active catalytic sites that are encoded by two different mRNAs from a single gene, which exists in atria, ventricles, and conduction system. Two isoforms of ACE exist, namely, somatic ACE and germinal ACE. Both isoforms exhibit similar enzyme activities, but differ in molecular size and immunological properties [11]. The ACE gene has been cloned in animals and humans with a molecular weight ranging from 90 to 160 Kd in different tissues [12,13]. This gene has been shown as an insertion/deletion polymorphism based on the presence of insertion (I) or deletion (D) in intron 16 of the ACE gene. This structure results in three genotypes: *DD* homozygous, *II* homozygous, and *ID*

heterozygous [14]. The *DD* allele is associated with higher levels of ACE in plasma [15] and is considered to increase the risk of cardiac disease [16–18]. Ang II is considered to be an important factor for the regulation of vascular tone, blood flow, and cardiac function. There is evidence that there are multiple biochemical pathways for the formation of Ang II [19,20]. These pathways may include the direct synthesis of Ang II from angiotensinogen [21] or another non-ACE enzyme such as chymase [22–24], which has been demonstrated to be present in the heart. The existence of non-ACE pathways can suggest that long-term therapy with ACE inhibitors may not lower the plasma and tissue Ang II levels appreciably [25] despite effective normalization of blood pressure and significantly suppressed ACE activity [26]. The distribution of ACE and chymase in the heart differs; ACE is in the cardiac luminal surface, whereas chymase is in endothelial cells and cardiac interstitium [27,28].

By using specific nonpeptide antagonists, two Ang II receptors have been identified as $AT_1$ and $AT_2$ [29,30], and cDNAs encoding each type of Ang II receptor have been identified [31]. The location of genes for $AT_1$ and $AT_2$ is different; the gene for $AT_1$ receptor is located on chromosome 3, whereas the $AT_2$ receptor gene is on the X chromosome [32]. The Ang II receptor gene structure, distribution, and regulation in different pathophysiological conditions has been fully reviewed [31,33]. Ang II receptors are up- and downregulated by some biophysical mechanisms, such as internalization and phosphorylation, as well as disease conditions [34–36]. The structure specificity of Ang II receptor is high; the affinity for binding to Ang II is similar to Ang II circulating concentration ($10^{-10}$M). The $AT_1$ receptor is a seven-transmembrane receptor with two subtype receptors, $AT_{1\alpha}$ and $AT_{1\beta}$; these subtypes have similar polypeptides, containing about 360 amino acids, but they have different tissue distribution [19,37]. This class of Ang II receptors is associated with $G_q$ protein [38] and is responsible for almost all the physiological actions of Ang II and selective antagonists. The $AT_1$ receptor can initiate either a rapid or a slow signal transduction event. In the rapid signal transduction event, the phosphoinositide message system is involved [39]. Ang II through the type 1 receptors activates $Ca^{2+}$ channels through $G_q$ proteins to allow more $Ca^{2+}$ into the cells. In turn, PLC is activated to generate inositol bisphosphate, which activates PKC, and finally regulates cell function [40]. The slow signal transduction event involves the phosphorylation of tyrosine and activation of MAPK, which stimulates cell growth and causes hypertrophy [41–43]. All the effects induced by Ang II can be blocked by losartan, indicating that functional activity is mediated mainly by the $AT_1$ receptor. On the other hand, the $AT_2$ receptor is quite different from the $AT_1$ receptor; the $AT_2$ receptor is blocked by the compound PD123319, a selective $AT_2$ receptor antagonist [33]. The function of the $AT_2$ receptor is not yet clear. Recently, growing evidence has shown that the $AT_2$ receptor is also involved in functional activity. PD123177 has been shown to delay and attenuate the $Ca^{2+}$ spike induced by Ang II in cultured bovine adrenal medullary cells. By using PC12w cells, which express high levels of $AT_2$, but not $AT_1$ receptor, Yamada et al. [44] recently reported that the $AT_2$ receptor involves dephosphorylation of MAP kinase

and results in apoptosis. This $AT_2$-mediated MAPK dephosphorylation and apoptosis can be blocked by vanadate and antisense oligonucleotide to MAPK. Another study indicates that the $AT_2$ receptor is regulated by PKC-calcium pathway; the increase of Ang II receptor gene expression was inhibited with cycloheximide, a PKC inhibitor [45].

## CARDIAC RENIN-ANGIOTENSIN SYSTEM

Multiple lines of biochemical and molecular evidence support the existence of a local RAS [46–52]. The most convincing evidence for a cardiac RAS is the expression of renin, angiotensinogen, and ACE genes in cardiac tissues [53–56]. The renin and angiotensinogen mRNA have been shown to exist in all four chambers of the heart, with a different distribution depending on species and pathophysiological conditions [57]. In contrast, von Lutterotti et al. [58] indicated that renin is not synthesized by cardiac tissue and that the local RAS is accumulating the renin from the bloodstream. Ang I and Ang II can be measured in the isolated rat heart when renin is added to a perfusion buffer; this means that angiotensinogen and ACE, but not renin, exist in isolated heart tissues [59]. Experiments have indicated that ACE is not uniformly distributed in the heart. By using $^{125}$I-351A as a radioligand, Yamada et al. [60] demonstrated that in rat heart, the highest density of ACE is in valve leaflets and the lowest is in endocardium. Upon combining in vitro autoradiography with examination of tissue morphology, Sun et al. [61,62] showed that low density ACE was found throughout the ventricular myocardium, whereas high density of ACE exists at the site of high collagen turnover, including heart valve leaflets. The distribution of ACE in the heart indicates that some Ang II is possibly generated in the heart. In fact, cardiac Ang II production has been demonstrated [22,63]. Receptors that are related to the function of Ang II have been characterized in the cardiovascular system [34,64]. It is thus likely that local Ang II plays an important role in cardiovascular homeostasis in autocrine and paracrine fashions and may be involved in cardiac remodeling.

## EFFECT OF ANG II ON CARDIAC FUNCTION

Ang II influences cardiac function by effecting cardiac contraction, myocytes, cardiac matrix growth, and cardiac metabolism. These actions are initiated by the binding of Ang II to a plasma membrane receptor that stimulates PLC to produce hydroxyl phosphatidylinositol 4,5-bisphosphate and thus forming diacylglycerol (DAG) and inositol 1,4,5-triphosphate ($IP_3$). The subsequent rise in intracellular $Ca^{2+}$ that results from $IP_3$-mediated release of $Ca^{2+}$ from intracellular stores, together with DAG, activate PKC. In cultured rat ventricular myocytes, Ang II not only activates the phosphoinositide pathway, but also activates the phospholipase D and $A_2$ pathways [41,65]. Ang II has long been recognized to influence cardiac contractility [66–68]. A positive inotropic effect of Ang II was reported on isolated neonatal rat cardiomyocytes [69,70], pithed rabbit preparations [71], as well as perfused rabbit and rat hearts [71,72]. Under in vivo conditions in rabbits, Zhang et al. [73] have

shown that Ang II elicited a dose-dependent increase in blood pressure, left ventricular pressure, rate of contraction (+dP/dt), and rate of relaxation (−dP/dt), as well as heart rate. The increase in both +dP/dt and −dP/dt by Ang II was confirmed in isolated rabbit hearts [74] and rat myocytes [75].

It may be noted that Ang II was found to increase interleukin-1-induced nitric oxide synthesis; this effect was blocked by a PKC inhibitor, calphostin [76]. Unlike beta blocker or diuretics, the reduced levels of cholesterol and lipoprotein by an ACE inhibitor were increased by Ang II [77,78]. Ang II interacts with the sympathetic nervous system through presynaptic transmitter release and with an improvement of the baroreceptor reflex function [79,80]. Interactions between the RAS and parasympathetic nervous system in heart failure are also observed. Heart failure patients show a reduction in vagal tone [81], and baroreflex sensitivity, associated with an increased plasma renin activity [82,83]. Thus, it appears that the action of Ang II in cardiovascular system is both of direct and indirect nature.

## EFFECT OF ANG II ON $Ca^{2+}$ MOBILIZATION

Ang II has been reported to be involved in $Ca^{2+}$ mobilization in ventricular myocytes through the activation of slow calcium channels in the sarcolemmal membrane [70]. Allen et al. [69] have observed that Ang II can stimulate contractile frequency and calcium sensitive calcium current. Arnaudeau et al. [84] indicated that angiotensin $AT_1$ receptor stimulates $Ca^{2+}$ sparks through activation of L-type $Ca^{2+}$ channels without involving $IP_3$-induced $Ca^{2+}$ release; this stimulatory effect was blocked by PKC inhibitor but not by propranolol [85]. Ang II induced the cytosolic free calcium increase in chick myocytes in a dose-dependent manner [86]. Unpublished data from our laboratory have revealed that Ang II can increase intracellular $Ca^{2+}$ in isolated adult rat myocytes in a dose-dependent manner; this effect was abolished by both Ang II receptor antagonist losartan and PD123319. Although Ang II can be seen to cause an increase in free $Ca^{2+}$ in the myocytes, the results are controversial. Ang II ($10^{-8}$M) induced a significant increase of fractional shortening that was not associated with an increase of calcium transient or any effect on L-type calcium inward current [87]. In isolated rabbit myocytes, Ang II stimulated the rate of contraction (+dP/dt) and relaxation (−dP/dt) but failed to show any increase in intracellular $Ca^{2+}$. On the other hand, in neonatal rat heart myocyte cultures, the frequency of contraction and $Ca^{2+}$ current were increased when a PKC activator, phorbol ester 12-0-tetradecanoylphorbol-13-acetate (TPA), was added to the buffer containing Ang II; this effect was not seen with 4-α-phorbol-12,13-didecanoate (α-PDD), which does not activate PKC [88].

## ANG II-INDUCED CARDIAC HYPERTROPHY AND HEART FAILURE

Cardiac growth is affected by mechanical load and neurohumoral substances, such as Ang II, which acts as an endogenous growth factor. Ang II stimulates cardiac growth that is involved with myocyte hypertrophy, as well as growth of nonmyocytes, such as collagen and fibronectin. During hypertrophy, Ang II has

been shown to stimulate synthesis of protein and DNA, secretion of growth factors, and formation of cardiac matrix [89–94]. Ang II, at 10 μM concentration, increased collagen and fibronectin synthesis and their mRNA expression in cultured rat vascular smooth muscle cells [94]. In neonatal rat cardiac fibroblasts, 24-hour exposure to 1 μM Ang II increased the rates of phenylalanine, thymidine, and uridine by 58%, 103% and 118%, respectively [95]. Intracellular signalling pathways of Ang II may include: (1) the phosphatidylinositol message pathway, (2) the tyrosine kinase pathway via the Ras/Raf pathway to activate the protein kinase, and (3) a cascade to activate MAP kinase. These events in sequence may cause protein transcription by stimulation of the growth factor-dependent c-fos, c-jun, and Egr-1. Although Ang II stimulates these intracellular mechanisms by its interaction with angiotensin receptors present in the cell membrane, the contribution of each receptor type ($AT_1$ and $AT_2$) is far from understood. It should be mentioned that cardiac hypertrophy is an adaptive response to an increased load on the myocytes. This response allows the heart to increase work in the presence of normal systolic fiber shortening [96]. On the other hand, heart failure is a complex syndrome in which a number of subcellular biochemical alterations have been identified [97,98]. However, understanding events that are associated during the transition of cardiac hypertrophy to heart failure is a real challenge.

By using chick heart cells, Baker and Aceto [90] found that Ang II significantly stimulated protein synthesis through the participation of $AT_1$ receptors. Under the same experimental conditions, it was shown that Ang II-induced protein synthesis was time- and dose-dependent [90]. Likewise, Greenen et al. [91] demonstrated that Ang II increased cardiac protein synthesis in adult rat heart. The work from Schunkert et al. [99] not only confirmed that Ang II stimulated protein synthesis in adult rat hearts directly but also explained that this stimulation was mediated through $AT_1$ receptors and PKC activation. The Ang II-induced ventricular hypertrophy was not a consequence of high blood pressure because lowering the blood pressure or vasodilator therapy did not regress the cardiac hypertrophy [100]. Conversion of intracardiac Ang I to Ang II was fourfold higher in hypertrophied rats because of an increase of the ACE activity; these changes were reversed by an ACE inhibitor, which suggests that ACE is a key enzyme involved in cardiac hypertrophy [99]. It should be pointed out that administration of an ACE inhibitor not only prevented cardiac hypertrophy but also caused a regression of the previously developed left ventricular hypertrophy. The recently established new transgenic (TGR9nRen2) rat was an ideal model to demonstrate the direct effect of RAS on cardiac hypertrophy [101]. This transgenic rat has been shown to develop hypertension, which can be normalized by an $AT_1$ receptor antagonist [102,103].

The formation of cardiac extracellular matrix has been demonstrated to be increased by the RAS [104,105] and appears to play an important role in the transition from hypertrophy to heart failure [106]. A marked ACE binding in rats after coronary ligation was associated with fibrillar collagen formation in the infarcted and remote areas [62]. In cultured cardiac fibroblasts, Ang II induced an early growth response (Egr-1) gene as well as increased mRNA levels for c-fos,

fibronectin, and laminin, from two- to fourfold [107]. Not only is collagen a major component of the extracellular matrix but also the accumulation of fibrillar collagen in the cardiac interstitium is the major morphological feature of ventricular hypertrophy [108]. The increase in collagen I and III contents in the myocardium was attenuated in the presence of $AT_1$ and $AT_2$ receptor antagonist [109]. Infusion of Ang II was found to stimulate fibronectin gene expression accompanied with an increase in collagen I and IV gene expression in rat hearts [110,111]. Compared with other growth factors, Ang II showed a strong effect on early oncogene, Egr-1, and extracellular matrix gene, such as fibronectin and laminin [107]. On the other hand, some investigators did not observe any change in collagen gene expression by Ang II [107]. The pathway for Ang II-mediated nonmyocyte hypertrophy has been reviewed recently by Dostal et al. [112].

## MECHANISMS OF CARDIAC REMODELING BY ANG II

By stimulating the formation of extracellular matrix, Ang II is considered to alter the size and shape of cardiomyocytes and thus results in remodeling of the heart. In cultured rat cardiac cells, the c-fos mRNA expression was significantly induced by Ang II [112]. The pressure overload-induced c-fos expression was also found to be Ang II dependent. In stretched adult failing cardiomyocytes, c-fos mRNA was increased three- to fourfold after Ang II treatment. This increase and the early gene, c-fos expression were blocked by $AT_1$ receptor antagonist [41]. Late hypertrophy response, skeletal $\alpha$-actin, atria natriuretic factor, and protein synthesis were suppressed by Ang II receptor antagonist [113]. In myocardial infarction-induced hypertrophy, c-myc, c-jun, and Ang II receptor mRNA were increased significantly [114]. In heart failure due to coronary occlusion, Ang II receptors, c-myc, and c-jun were also increased in myocytes [115]. Ang II has been shown to stimulate growth factor in adult cardiac fibroblasts [116]. Ang II also caused a marked increase of insulin-like growth factor-1 receptor gene expression and gene transcription in rat aortic smooth muscle cells [117]. In rat cardiac hypertrophy induced by abdominal coarctation, an increase in $AT_1$ receptor mRNA was associated with a twofold increase of the transforming growth factor-$\beta_1$ mRNA. This increase can be blocked by an Ang II receptor antagonist, DuP 753 [118]. Ang II stimulated MAPK by PKC dependent [119] or independent [120] pathways with an increase of intracellular $Ca^{2+}$ and thus stimulating myocyte growth [121].

An increase in the activity and expression of the local RAS gene was evident in cardiac hypertrophy and heart failure. This increase was associated with an increase in plasma renin activity by 45% and total RNA by 68% in rats with heart failure [122]. The increased ACE mRNA level was accompanied by a decrease of $AT_1$ receptor mRNA to 46% without any change in $AT_2$ receptor mRNA in myocardium of decompensated rats [122]. The cardiac renin, angiotensinogen, ACE, and $AT_1$ and $AT_2$ receptors were expressed in volume-overloaded rat heart in which increases in renin, angiotensinogen, as well as ACE mRNA, unlike mRNA for Ang II receptors, were evident [123]. The level of ACE mRNA increased in the

ventricles during cardiac hypertrophy by aortic banding and increased in the model of low-output cardiac failure by coronary ligation [124,125]. In the hypertrophied rat heart, ACE mRNA was twofold more than that in the normal ventricle. Infusion of Ang I into the hypertrophied heart for 15 min caused intracardiac conversion of Ang I to Ang II; this increase was fourfold compared to the sham control [99]. The increase of Ang II receptor was not only evident at the site of myocardial infarction, but also in fibrous tissues involved in myocardial infarction and pericardial fibrosis [126]. In hypertrophied heart from myocardial infarction, gene expression and protein content of renin, angiotensinogen, ACE, as well as the angiotensin receptors increased significantly [127]. On the other hand, a decrease in Ang II receptor mRNA was seen in patients with heart failure, and this reduction was attenuated by losartan and PD123319 [128]. Both $AT_1$ and $AT_2$ receptors were found to increase in injured and noninfected tissues, but only $AT_1$ antagonist attenuated this receptor change in rat after coronary artery ligation [129]. Ang II receptors were downregulated in pressure overload myocardial hypertrophy and heart failure in rats but upregulated in postinfarcted cardiac hypertrophy and heart failure [130–133]. Reduction of both Ang II receptors, with loss of $AT_1$ receptor mRNA, is noticeable in patients with end stage heart failure [134]. In severe heart failure, plasma renin and plasma Ang II concentrations increased threefold. In parallel, renal renin and angiotensinogen mRNA expression also increased. These observations suggest that RAS is activated in heart failure, but the changes in its different components seem to depend on the type and stage of the disease [135].

From the foregoing discussion, it is clear that ACE genotype has a close relationship with cardiac hypertrophy and heart failure and has also been implicated in cardiac dilatation and myocardial infarction [18,136,137]. Since Cambine et al. [17] first reported a deletion polymorphism in the ACE gene (*DD*), which was associated with an increased risk of myocardial infarction, the relationship between ACE genotype and cardiac hypertrophy or heart failure has been studied more extensively [138–140]. This relationship is demonstrated by (1) a higher occurrence of cardiac hypertrophy and heart failure in patients with a *DD* genotype; (2) a greater amount of *DD* ACE genotype gene in hypertrophy or heart failure patients; (3) an influence of the *DD* genotype on survival following heart failure. Schunkert et al. [141] suggested that the *DD* genotype may act as a marker associated with an elevated risk of left ventricular hypertrophy in men, since men have a stronger association of the *DD* genotype than women. In contrast, no relationship of ACE genotype with cardiac hypertrophy was reported [142].

## EFFECTS OF ACE INHIBITORS ON CARDIAC HYPERTROPHY AND HEART FAILURE

Since the synthesis of the first oral ACE inhibitor, captopril, in 1977 [3,4,143], several other ACE inhibitors have been synthesized, and their effects on hypertension and heart failure have been fully investigated [144,145]. Although it is generally accepted that ACE inhibitors have a beneficial effect in heart failure, the mechanism of such a protective effect is still far from being fully understood [146,147]. The

following discussion is devoted to analysis of the actions of some ACE inhibitors in different types of hypertrophied and failing hearts.

**Effect of ACE inhibitors on heart failure induced by myocardial infarction**

Left ventricular infarction in rat has been used as an ideal model of cardiac hypertrophy and heart failure. The RAS is known to be activated during postinfarction and is thought to play an important role during the remodeling period. Therefore, this model has also been used to demonstrate the benefits of ACE inhibitors. Myocardial infarction has been characterized as a combination of pressure and volume overload in which the myocardium faces an excessive workload [148]. Marked changes in ventricular hemodynamics, volume, and mass are related to infarct size. Cardiac function is lowered, which is characterized by lower output, reduced ejection fraction, elevated end-diastolic pressure, ventricular dilatation, and ventricular hypertrophy, which finally leads to heart failure.

Because infarct size is an important factor that influences the postinfarction process and the heart failure occurrence, attention has been paid to the fact that ACE inhibitors may reduce infarct size. Although several studies have examined the effect of ACE inhibitors on infarct size, the results are controversial. Reduction of the infarct size by ACE inhibitors has been reported in dog [149–151], rat [152,153] and cat [154]. When the ACE inhibitor is administered from 15 min to 6 hours after the coronary occlusion, infarct size reduction was evident in dogs, and it was suggested that ACE inhibitors can reduce infarct size by increasing collateral flow to the areas of infarction as well as areas at risk [155]. Treatment with captopril for 3 weeks starting 3 weeks after coronary artery occlusion reduced the infarct size only by 9% in rats [152]. On the other hand, various studies failed to observe any change in infarct size upon ACE inhibitor therapy. In a dog model of coronary artery occlusion, captopril improved cardiac output significantly but did not decrease the infarct size [156]. In addition, ACE inhibitors failed to reduce infarct size in conscious dogs [157,158]. Both reduction [159] and no change in the infarct size have been reported upon occluding coronary artery in rats [160]. Such a discrepancy in results seems to be due to the time of administration and dose of ACE inhibitor.

Improved systolic and diastolic function in both experimental animals and patients with heart failure have been well documented by the use of various ACE inhibitors, such as captopril [153,161], enalapril [162], trandolapril [163], idrapril [160], and ramipril [164]. Pfeffer et al. [165] were first to report that captopril significantly prevented the ventricular dysfunction. Captopril, given to infarcted rats for 3 weeks, showed a shortening of peak time tension, an increase in $\pm dP/dt$ and developed tension, and a particular reduction of myocardial stiffness [152]. In general, ACE inhibitors improved ventricular hemodynamics, attenuated ventricular dilatation, and reduced wall stress and stiffness. ACE inhibitors also increased the baseline and maximum stroke volume index, cardiac output, and coronary circulation. Some investigators have failed to show the protective effect or even partial protective

effects of ACE inhibitors. Although ACE inhibitors significantly improved cardiac function in heart failure, their effects on cardiac systolic and diastolic function are different. A recent study has demonstrated that long-term ACE inhibitor treatment improved diastolic function more than systolic function. These data indicate that the diastolic filling abnormalities are almost completely normalized but diastolic dimensions and posterior thickening are left unchanged after long term treatment with captopril [166].

Compared to other drugs such as vasodilators, β-adrenergic blockers, and $Ca^{2+}$ antagonists, ACE inhibitors demonstrate the greatest advantage in delaying the development of heart failure and increasing the survival rate. The effect of chronic ACE inhibition on long-term survival after myocardial infarction was first demonstrated in rats with myocardial infarction [153]. Subsequently, ACE inhibitors were used in clinical trials and showed their beneficial effect on mortality and morbidity in heart failure [167–169]. Some of the studies, however, have shown a negative action of ACE inhibitors [170]. In a one-year survival study, an ACE inhibitor, trandolapril, showed improved survival rate in myocardial-infarcted rats only during the initial 6 month period [171]. Our experiments with a new, long-acting ACE inhibitor, imidapril, have shown that ACE inhibitors may produce beneficial effects irrespective of the time of treatment following coronary occlusion. Both early (1 hour after coronary artery occlusion) or late (3 weeks after coronary artery occlusion) treatments of rats reduced the mortality compared to that in the respective untreated infarcted group. The mechanism by which ACE inhibitors improve survival following early or late myocardial infarction may, however, be different (unpublished data).

Remodeling of the heart subsequent to myocardial infarction is characterized by progressive left ventricular dilatation and enlargement of the chamber size. At the cellular level, remodeling of the heart may include changes in membranes, contractile proteins, and cardiac matrix. It is currently well reviewed that ACE inhibitors appear to prevent the occurrence of cardiac remodeling in heart failure [172–174]. Early treatment with ACE inhibitor was found to prevent the progression of left ventricular remodeling in dogs with left ventricular dysfunction caused by sequential intracoronary embolizations with polystyrene latex microspheres [175]. In addition, the beneficial effects of ACE inhibitors on cardiac remodeling were also seen in noninfarcted regions of the myocardium [176] and large arteries [177]. Ramipril, when given to sheep for eight weeks starting two days after coronary occlusion, limited the decline in function in noninfarcted regions and prevented the percent circumferential shortening in the subendocardium [176]. Captopril significantly reduced collagen levels and reduced the artery media thickness, which was accompanied by improved hemodynamic functions in the coronary occluded rats [178]. The multiple factors that may contribute to the action of ACE inhibitors caused by remodeling of the heart are as follows: a decrease of hemodynamic load, an increase in bradykinin levels, a regression of myocyte hypertrophy, and a decrease in collagen accumulation [174]. Although ACE inhibitors are considered to prevent cardiac remodeling by affecting the size and shape of hypertrophied myocytes by decreasing

the formation of cardiac matrix, ramipril did not normalize the elevated collagen content in rats with myocardial infarction [178].

In spite of the evidence that ACE inhibitors improve cardiac function, delay the occurrence of heart failure, and prolong the survival, the time of administration and doses of these agents are still in discussion [179,180]. Treatment of rats with idrapril before coronary occlusion showed a marked improvement in left ventricular function and prevention of cardiac remodelling [160]. A recent report stressed the importance of early ACE inhibitor treatment [180]. Early administration of lisinopril to patients with acute myocardial infarction showed a long-term benefit of survival rate [171]. On the other hand, an earlier study demonstrated that late, but not immediate, treatment with captopril improved cardiac function following heart failure subsequent to coronary occlusion [181]. The doses used for treatment also affected the beneficial influence of ACE inhibitors [182]. In rats with heart failure after myocardial infarction, one-year survival rate improved with high doses, but not with low doses, of lisinopril [183]. In view of the differences in the molecular structure of the two binding sites of ACE [184], it is possible that different ACE inhibitors may interact with ACE at one or both sites. This may explain the differences in the time- and dose-dependent effects of these agents.

### Effects of ACE inhibitors on cardiac hypertrophy and heart failure from volume overload

Heart failure from volume overload is different from other types of heart failure as it is characterized by an eccentric pattern of hypertrophy and dilation of the ventricular cavities. The effects of ACE inhibitors on heart failure from volume overload have not been studied extensively; however, a recent review has discussed the status of volume overload hypertrophy and heart failure [185]. There is a characteristic elevation of plasma atrial natriuretic factor (ANF) because of increased release and synthesis of ANF in volume-induced heart failure [186]. Winkins et al. [187] suggested that ANF could be used as a good indicator of cardiac volume overload in aortocaval fistula because the level of plasma ANF correlates with the degree of cardiac hypertrophy and urinary excretion of cGMP. Arnal et al. [188] observed that perindopril exerted a beneficial effect on cardiac hypertrophy and suggested that ACE inhibitors may regress cardiac hypertrophy mainly via their effect on the pressure load, rather than the volume load. By combining the pressure and volume overloads, Takeda et al. [189] showed that captopril significantly increased the tension ($dT/dt_{max}$) and attenuated the shifts of myosin isozyme. Furthermore, the fact that ACE inhibitors significantly attenuated the ventricular ACE mRNA expression, as well as the mRNA of $AT_{1\alpha}$ and $AT_{1\beta}$, supports the idea that RAS is involved in volume overload heart failure [125]. Treatment of rats with enalapril for 7 weeks significantly reduced the increased LVEDP from volume overload [190,191]. Similar results were also seen with captopril treatment for 3 weeks [192]. The improved hemodynamics associated with a regression of cardiac hypertrophy indicated that RAS may exert some direct effects on volume overload cardiac hypertrophy [193]. Since the changes in extracellular matrix are invariably

observed in hypertrophied hearts, the effects of ACE inhibitors on collagen and elastin have been investigated in volume overload cardiac hypertrophy. In contrast to other forms of cardiac hypertrophy, collagen was reduced in volume-overloaded hypertrophied left ventricle, and this reduction was attenuated by enalapril treatment. Enalapril also blocked the initial increase of elastin in the same model [193]. Although ACE inhibitors reduced the increased level of ANF in heart failure from coronary occlusion, no such reduction was seen in the volume overload heart failure in spite of the beneficial effects on hemodynamics [192]. Differential effects of ACE inhibitors on circulating versus cardiac ANG II appear to explain the differences in the beneficial effects of various agents on cardiac hypertrophy and hemodynamic changes from volume overload [194,195].

### Effect of ACE inhibitors on cardiac hypertrophy and heart failure from pressure overload

Cardiac hypertrophy from pressure overload (a concentric hypertrophy) is characterized by a concentric increase in wall thickness without increase in chamber radius or volume. The left ventricle has been shown to increase by about 50% within 6 to 12 weeks of aortic banding in rats [196]. Significant prevention of cardiac hypertrophy by the use of different ACE inhibitors has been demonstrated in the rat aortic stenosis model [197–199]. ACE inhibitors not only produced a regression of cardiac hypertrophy but also prolonged the survival of rats with aortic stenosis [200]. Hemodynamic measurements showed that although the left ventricular systolic pressure was still high after treatment of pressure-overloaded rats with fosinopril, the left ventricular diastolic pressure was markedly reduced [201]. Assessment of the left ventricular geometry and function in rats with aorta banding revealed that fosinopril prevented the increase in left ventricular cavity size, increased the left ventricular wall stress, and attenuated the systolic and diastolic functions from pressure overload [201]. Both ramipril and enalapril were beneficial in regressing cardiac hypertrophy from constriction of the abdominal aorta in rats regardless of whether the administration was immediate or 3 weeks after the operation. Ang II receptor antagonists also reduced cardiac hypertrophy but to a much lesser degree than the reduction following surgical removal of aorta banding [202].

### ACE inhibitors on heart failure from pacing or dilated cardiomyopathy

The direct benefit of ACE inhibitor on cardiac tissue was determined by using different models of cardiomyopathy. In pacing-induced cardiomyopathy, fosinopril not only improved cardiac function but also improved the myocytes velocity of shortening after β-adrenergic receptor stimulation resulting from increased β-adrenergic receptor density [203]. Captopril treatment maintained normal cardiac output and pulmonary capillary wedge pressure following heart failure from rapid right ventricular pacing [204]. The beneficial effect of ACE inhibitors on cardiomyopathy may be through the elevation of circulating Ang I [205]. ACE inhibitors decreased cardiac collagen accumulation differently in various strains of cardiomyopathic hamsters. Masutomo et al. [206] demonstrated that enalapril sig-

nificantly decreased collagen concentration, the ratio of collagen (1:3), as well as collagen 3 mRNA expression, in the BIO14.6 strain of cardiomyopathic Syrian hamsters, but not in the BIO53.58 strains.

## POSSIBLE MECHANISMS OF THE BENEFICIAL EFFECTS OF ACE INHIBITORS

### Free radical scavenging properties

Some experiments have provided evidence regarding the radical scavenging properties of ACE inhibitors containing a sulfhydryl group. In an early study, Chopra et al. [207] suggested that captopril may act as a powerful free radical scavenger. In their study, free radicals were generated by photo-oxidation of dianisidine sensitized by riboflavin, and captopril was shown to possess a scavenging ability in a dose-dependent manner. Captopril also demonstrated a powerful effect in scavenging superoxide anion radicals, hydroxyl radicals, and hypochloride radicals [208]. Labeling ACE inhibitors as free radical scavengers is limited because not all ACE inhibitors contain the sulfhydryl group, yet these agents have a similar protective effect on cardiac functions.

### Cellular mechanisms

Change in intracellular calcium handling is shown to occur in heart failure, and the beneficial effect of ACE inhibitors in improving cardiac function is possibly associated with improvements of the sarcoplasmic reticulum $Ca^{2+}$ ATPase gene expression in renal hypertensive rats [209]. Unpublished data from our laboratory have indicated that the ACE inhibitor, imidapril, not only prevents the remodeling of the sarcoplasmic reticulum membrane but also affects the remodeling of sarcolemmal membrane during the development of heart failure from myocardial infarction. We have demonstrated that imidapril benefited gene expression and protein content in both sarcoplasmic reticulum and sarcolemma in heart failure from coronary occlusion. ACE inhibitors have also been shown to improve the response to $Ca^{2+}$ stimulation in hypertrophied myocytes which may be important for preventing the transition from compensated hypertrophy to heart failure [210]. In addition, we have shown that ACE inhibitors improved β-adrenergic receptor transduction by preventing the depression in $\beta_1$-adrenergic receptor density and decreased adenylyl cyclase activity, and attenuating G protein changes in the failing hearts. Sanshi and Takeo [211] have also reported that long-term trandolapril treatment significantly attenuated the cardiac β-adrenoceptor response in rat with heart failure following coronary occlusion.

### Effect on bradykinin

Besides the inhibition of RAS, the inactivation of bradykinin might be responsible for some beneficial effects of ACE inhibitors. This view is supported by the fact that the protective role of ACE inhibitors in ischemic heart [212] and the regression of cardiac hypertrophy by ramipril in hypertensive rats are abolished upon administrating a bradykinin antagonist [213]. The effect of ACE inhibitors involves bradykinin-

mediated actions which include increasing the coronary blood flow, improving the left ventricular pressure, decreasing the arterial ventilation, and reducing the proliferating properties [214,215]. It should be noted that bradykinin is a vasodilator which acts by increasing the release of endothelium-derived factors, such as nitric oxide and prostacyclin. Bradykinin may also improve the status of high-energy phosphates in ischemic myocardium [215,216]. Although the protective effect on cardiac function and regression of cardiac hypertrophy by ACE inhibitors can be considered to be part of the function of bradykinin, it is not related to the equally effective Ang II antagonists.

**Effect on myosin heavy chain**

A positive relationship exists between myosin heavy chain and cardiac muscle contractility. Reduced myosin heavy chain content and the isoform shift in heart failure can be attenuated by ACE inhibitor treatment. In our laboratory, rats with heart failure induced by coronary occlusion showed lower myosin heavy chain content and shifted, altered myosin isoform gene expression. By using the ACE inhibitor imidapril for four weeks, a significant improvement of myosin heavy chain content and normalization of the myosin isoform shift were observed (unpublished data). Lambert et al. [217] also showed that perindopril significantly limited the shift of isomyosin in the cardiomyopathic Syrian hamster. Michel et al. [218] showed that the treatment of myocardial infarcted rats with an ACE inhibitor over a two month period significantly attenuated the isoform shift.

**Effect on neuroactivity**

Diminution of parasympathetic tone associated with enhanced arrhythmogenesis and sudden cardiac death is a feature of congestive heart failure [81]. ACE inhibitors have been shown to exert vagomimetic action in congestive heart failure [219]. ACE inhibitors significantly increased the baroreflex sensitivity in patients with idiopathic dilated cardiomyopathy and coronary artery disease [219]. Captopril significantly attenuated the depressed baroreflex sensitivity in patients with acute myocardial infarction [220]. Although ACE inhibitors were known to affect sympathetic activity, no action of ACE inhibitors on healthy subjects was observed [221].

**Effect on energy metabolism**

Lactate dehydrogenase (LDH) and its isoenzymes are closely related to aerobic and anaerobic metabolism. Shifts of LDH isoenzymes are dependent on the state of the oxygen supply and may serve as a marker for the energy state of the myocyte; LDH1 acts as a marker for the aerobic state whereas LDH5 is a marker for the anaerobic state [77,222]. In cardiac hypertrophy and heart failure, reduction of available energy is evidenced by changing the isoforms of LDH, or decreasing the ADP/ATP ratio; this shift in the LDH isoenzyme and alteration of the ADP/ATP ratio can be interpreted to reflect the beneficial effects of the ACE inhibitor therapy.

Treatment with enalapril for six months shifted LDH towards LDH1, and the ADP/ATP carrier concentration increased to normal levels. A significant effect on the hemodynamic index was also observed. Although this study indicated the protective ACE inhibitor effect on metabolism, it is unclear whether the change in energy metabolism is a cause or a consequence of the hemodynamic alterations [219]. Enalapril significantly increased LDH1 concentration, preserved myocardial creatine kinase, and improved the survival of rats with heart failure [223]. The study from Zhu et al. [216] indicated that the possible mechanism by which ACE inhibitors improve metabolism is via bradykinin instead of Ang II inhibition. On the other hand, ACE inhibitors, such as captopril, enalapril, and ramipril, significantly improved the myocardial oxygen consumption in dogs by increasing the nitric oxide accumulation [224].

## EFFECTS OF ANG II RECEPTOR ANTAGONIST ON CARDIAC HYPERTROPHY AND HEART FAILURE

In view of the indirect evidence that the RAS is at least partially responsible for the progression of heart failure, this system is considered to influence the prognosis in heart failure. The discovery of the Ang II receptor antagonists has provided an adequate tool for studying the role of Ang II receptors and RAS in heart function. By using Ang II receptor antagonists, one can avoid, to some extent, the side effects caused by ACE inhibitors. Furthermore, by blocking Ang II with nonpeptides that lack agonist activity, it is now possible to confirm that the efficacy of ACE inhibitors is due to a decrease in the Ang II level instead of an increase in the bradykinin level. The earliest Ang II antagonist was reported to block Ang II receptors and reduce blood pressure but was found to exhibit some Ang II agonist activity [225]. The first nonpeptide $AT_1$ receptor antagonist, losartan, was discovered to possess agonist activity [226,227]. Losartan was found to affect the Ang II receptors by interacting with amino acid in the transmembrane domains of $AT_1$ receptors, occupying space among seven helixes, and thus preventing the binding of Ang II [228].

Losartan is a novel, orally active, nonpeptide Ang II receptor antagonist that blocks the Ang II $AT_1$ receptor specifically. In an early study using rats with heart failure following coronary occlusion, Raya et al. [229] found the beneficial effects of both Ang II blocker and ACE inhibitor with respect to changes in LVEDP, left ventricular end-diastolic volume, and the venous compliance. Smits et al. [230] later showed that both early and late treatments with losartan following myocardial infarction were beneficial in modifying the changes in the central venous pressure as well as in inhibiting the collagen deposition and regressing the cardiac hypertrophy. However, losartan failed to show any beneficial effect with respect to changes in cardiac output and inhibition of the DNA synthesis in the failing ventricle. Nonetheless, losartan has been used in heart failure patients, and clinical data confirm the beneficial effect for lowering the systemic vascular resistance and increasing the cardiac output [231,232]. Furthermore, short-term administration of losartan has been shown to significantly improve impaired cardiac function, reduce systemic vascular resistance as well as pulmonary capillary wedge pressure, and increase

cardiac index [233]. In addition to being beneficial in heart failure that is due to myocardial infarction, Ang II antagonist blockers have been shown to exert beneficial actions in volume overload-induced [190], pressure overload-induced [202], and pacing-induced heart failure [234]. The Ang II receptor antagonist TCV-116 at a dose of 10 mg/kg/day significantly reduced the increased left ventricular weight and left ventricular thickness caused by pacing of the heart [235]. As well, this treatment attenuated the shift of the beta myosin heavy-chain isoforms and inhibited cardiac hypertrophy by inhibiting the [$^3$H]phenylalanine incorporation, MAPK activity, and the c-fos expression induced by stretch of cardiomyocytes. Losartan has also been shown to attenuate the altered response of myocytes to Ang II in heart failure caused by pacing [236]. Since cardiac remodeling after myocardial infarction has a close relationship with the expression of the phenotype genes, the modulation of cardiac phenotype gene expression by TCV-116 revealed beneficial effects on remodeling of cardiac tissue [236]. Attenuation of ventricular dilatation after myocardial infarction by TCV-116 [237] indicates that Ang II antagonists are capable of delaying the development of heart failure after myocardial infarction.

Although there are many similar effects of ACE inhibitors and Ang II receptor blockers, some differences exist between the two classes of drugs. ACE inhibitors inactivate the metabolism of bradykinin. ACE inhibitors do not completely inhibit the production of Ang II pathways, whereas Ang II receptor antagonists directly block the action of Ang II by suppressing its receptors [238]. Losartan has tissue specific effects on endogenous levels of angiotensin and bradykinin, but the increase of bradykinin does not contribute to the action of losartan [239]. Losartan (10 mg/kg) was found to increase plasma renin and cardiac Ang II and decrease plasma angiotensinogen and increase plasma ACE, but it does not increase the tissue ACE levels [240]. It should be pointed out that some discrepancies exist concerning the effects of ACE inhibitors and Ang II antagonists. Losartan at a dose of 15 mg/kg had no effect on cardiac hypertrophy induced by coronary artery ligation, whereas in the same model, captopril significantly regressed the hypertrophied heart [240]. On the other hand, the increased left and right ventricular weights were significantly depressed by losartan but only moderately attenuated by enalapril in the volume overload model [202]. A clinical study showed that losartan and enalapril were of comparable efficacy and tolerability in moderate or severe congestive heart failure [241]. Losartan had significant advantages, with respect to its long duration of action, oral absorption, and absence of Ang II agonist activity. The improved tolerability of losartan in heart failure is not seen with ACE inhibitors because of cough that develops from the effects of bradykinin and prostaglandin. Some reports have indicated that about 10 to 15% of patients on ACE therapy must have it discontinued because of bradykinin-related cough [242]. On the other hand, ACE inhibitors also show some advantage over the Ang II blockade. One of the advantages of therapy with ACE inhibitors compared to that with Ang II receptor blockers is the increased circulating Ang II level that is known to exert a positive inotropic effect on the myocardium [205]. The most important advantage is that ACE inhibitors significantly reduce mortality and delay the development of heart

**Table 1.** Use of various ACE inhibitors for the treatment of experimentally-induced myocardial infarction

| Authors & Reference# | Animal | ACE Inhibitor | Start Treatment | Duration of Treatment | Changes of Infarct Size |
|---|---|---|---|---|---|
| van Wijingaarden et al. [243] | rat | spirapril | immediately | 6 weeks | ↔ |
| Fornes et al. [163] | rat | trandolapril | 7 days after surgery | 1 year | ↔ |
| Ertl et al. [155] | dog | SQ14225 | 30 min-6 hr after surgery | 6 hr | ↓ |
| van Wijingaarden et al. [244] | rat | captopril | before surgery | 8 weeks | ↔ |
| Litwin et al. [152] | rat | captopril | immediately after surgery | 21 days | ↔ |
| van Krimpen et al. [92] | rat | captopril | immediately | 7 days and 21 days | ↔ |
| Wollert et al. [183] | rat | lisinopril | 6–8 days after surgery | 7 days, 6 weeks, and 1 year | ↔ |
| Hock et al. [160] | rat | enalapril | 1 min after surgery | 24 hr | ↔ |
| Sweet et al. [245] | rat | enalapril | 7 days after surgery | 1 year | ↔ |
| Liang et al. [157] | dog | teprotide | 40 min after surgery | 10–40 min | ↔ |

failure; however, no such evidence for Ang II blockers is available yet in the literature. Likewise, in contrast of different types of ACE inhibitors (table I) [92,152,155,157,160,183,243–245], a great deal concerning the time and duration of treatment of myocardial infarction with Ang II antagonists needs to be discovered.

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# EFFECT OF ANGIOTENSIN-CONVERTING ENZYME INHIBITION AND OF ANGIOTENSION II RECEPTOR BLOCKADE ON THE DEVELOPMENT OF CARDIAC HYPERTROPHY IN RATS

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***Summary.*** Comparison of the results of three experimental models of cardiac hypertrophy in rats reveals that angiotensin-converting enzyme (ACE) inhibition and angiotensin (Ang) II receptor blockade have different effects. In the model of pressure-induced left ventricular (LV) overload induced by constriction of the aortic arch, ACE inhibition with ramipril had no effect whatsoever on the development of cardiac hypertrophy both at the gross and the cellular morphological level. This may be because in this particular model, ACE inhibition was prevented from having an effect on blood pressure since the site of aortic constriction was quite near to the left ventricle. It may therefore be hypothesized that the clinical effects of this treatment may be related to the blood pressure-lowering effect. The local renin-angiotensin system (RAS) seems not to play a major role in this experimental model for the hypertrophy process. This is somewhat different in the model of right ventricular (RV) pressure overload induced by pulmonary artery stenosis. The development of RV hypertrophy was not influenced by treatment with ramipril; however, the cell morphology revealed a reduction in cell volume and cross-sectional area. Thus, the typical morphological feature of concentric hypertrophy was attenuated.

While the effects of ACE inhibition are somewhat variable in different experimental models, the effects of Ang II receptor blockade appear to be more consistent and promising, as judged from our own experimental results obtained so far. In the model of long-term noradrenaline administration, the development of LV hypertrophy was attenuated by losartan. Very recent studies on hypoxia-induced RV hypertrophy have also shown that losartan had an effect [1]. It seems therefore appropriate to continue along this line and include more refined parameters, in particular those related to molecular biology, in further studies.

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## INTRODUCTION

Cardiac hypertrophy is an independent risk factor for a number of severe dysfunctions of the heart [2,3]. The identification and description of factors that interfere with the initiation and development of cardiac hypertrophy is therefore of utmost clinical significance. The mechanical load imposed on the heart is one important determinant of myocardial structure and function. This has been shown in several experimental in vivo and in vitro studies [4–6]. However, experimental and clinical observations indicate that cardiac hypertrophy is not only load-dependent but also may be related to several hormonal, humoral, paracrine, or autocrine mechanisms. Among these, catecholamines have been demonstrated to be particularly involved [7–11].

In this respect, the renin-angiotensin system (RAS) has also been considered as a relevant humoral hormone system that has been shown to play a role in several organ systems, including the heart [12]. Locally generated angiotensin (Ang) II appears to be involved in the regulation of cell growth and protein synthesis in rat aortic smooth muscle cells [13–16]. In the heart, functional Ang II receptors have been shown to exist on bovine ventricular sarcolemma and on cultured cardiac myocytes [17,18]. Moreover, clinical and experimental studies have shown regression of left ventricular (LV) hypertrophy after angiotensin-converting enzyme (ACE) inhibition [19,20]. Finally, Ang II stimulates protein synthesis cell growth in cultured cardiac myocytes [21].

Data will be presented to show how ACE inhibition by ramipril affects the development of pressure-induced left and right ventricular (RV) hypertrophy in rats. RV hypertrophy has been included since it has become possible to measure RV function in small laboratory animals with special ultraminiature catheter pressure transducers and thus to characterize the effects of pressure overload [22]. In these two experimental models, cardiac myocytes were isolated to characterize cardiac hypertrophy at the cellular level also. On the other hand, Ang II receptor blockade by losartan was performed in rats with continuous noradrenaline infusion, which has likewise been shown to induce LV hypertrophy [10] without affecting the right heart [23]. To make these experimental approaches comparable in different model systems, the study period throughout all protocols was 2 weeks.

## CONVERTING ENZYME INHIBITION IN PRESSURE-INDUCED LEFT VENTRICULAR HYPERTROPHY

In this part of the study, the experiments were done on female Sprague-Dawley rats of about 200–240 g body weight. The rats were divided into four groups: sham operation without and with ramipril treatment and aortic stenosis without and with ACE inhibition with ramipril. Ramipril was dissolved in distilled water and administered orally in a single daily dose of 1 mg/kg body weight over 14 days. The treatment was started 1 hour before the surgical procedure. The rats were anesthetized with 100 mg/kg thiopental sodium intraperitoneally, 24 hours after the last ramipril administration. The hemodynamic parameters were measured by

catheterization of the left ventricle [24] with the ultraminiature catheter pressure transducer, model PR 249 (Millar Instruments, Houston, Texas). Cardiac output was determined using the thermodilution method. After the functional assessment [25], the hearts were quickly excised, the aorta was cannulated, and the hearts were perfused with collagenase to isolate cardiac myocytes for the measurement of cell parameters.

To induce pressure-induced cardiac hypertrophy, the chest of the ether-anesthetized and artifically ventilated rats was opened by midsternal incision, and the aortic arch was exposed. Stenosis was produced by placing a wire of 1.0 mm diameter alongside the aortic arch with a thread around it. This was tightly fixed with a knot; the wire was removed leaving the aortic arch constricted to an outer diameter corresponding to the diameter of the wire. This experimental stenosis of the aortic arch was placed between the branching points of the common carotid arteries so that LV function and pressure in the ascending aorta could be measured with an ultraminiature catheter pressure transducer introduced into the right carotid artery, and poststenotic aortic pressure could be measured by placing the catheter in the left carotid artery. The transstenotic pressure gradient was 80 mm Hg in all experiments [25]. Sham-operated animals were treated in the same manner except that aortic constriction was not performed.

The morphological parameters of isolated cardiac myocytes were measured after perfusion of the Langendorff hearts with Joklik media containing collagenase. The tissue was minced in calcium-free media, and isolated cells were poured through a nylon mesh into a fixation solution containing 1.5% glutaraldehyde in 0.08 M phosphate buffer. Isolated myocytes were centrifuged through 4% Ficoll in 0.15 M phosphate buffer. A channelyzer (model 256, Coulter Corp., Hialeah, Florida) was used to determine the volume of fixed, isolated myocytes. The length of the cells was measured by using a microscope (Axioskop, Zeiss, Oberkochem, Germany) equipped with phase optics. Mean cross-sectional area was obtained by dividing cell volume by cell length [25].

None of the experimental animals with aortic arch stenosis, whether treated with ramipril or not, showed signs of heart failure. All animals did gain weight during the 14 days after surgery. The main results of this study are summarized in figures 1 and 2. LV systolic pressure and mean arterial pressure were elevated after aortic arch constriction in all animals, independent of whether they had received ACE inhibition. Cardiac output was not changed markedly. The transstenotic pressure gradient was also similar in all rats with constriction of the aortic arch (figure 1). The LV weight/body weight ratio was increased 14 days after aortic constriction to about the same extent (between 30 and 35% compared with the sham-operated controls) in both the untreated and treated group. Likewise, cell volume was higher by about 20% during pressure overload. ACE inhibition with ramipril had no effect (figure 2). Since the cells were elongated slightly, but to a similar extent in both experimental groups, the increase in cross-sectional area indicating concentric pressure-induced hypertrophy was modest and again similar in untreated and treated experimental animals.

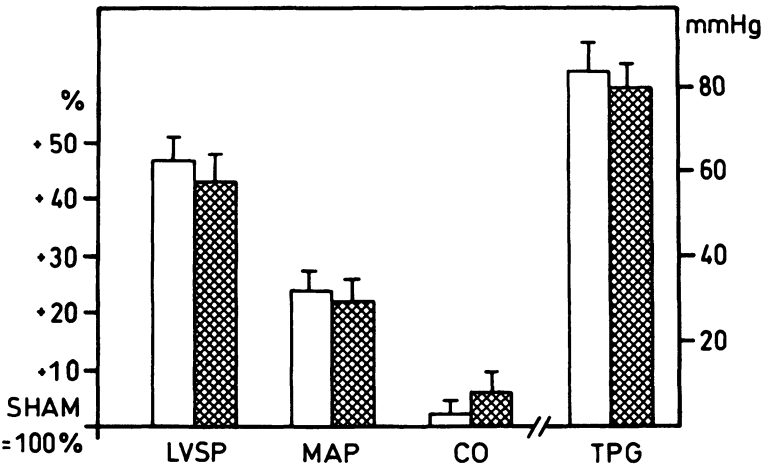


**Figure 1.** Effects of the aortic arch stenosis in rats without (open bars) and with (crossed bars) ramipril treatment for 14 days on left ventricular systolic pressure (LVSP), mean prestenotic aortic pressure (MAP), cardiac output (CO), and transstenotic pressure gradient (TPG) in rats. The data are expressed as percent changes and are compared to sham-operated controls. The number of experiments ranged from 10 to 18.

The results of this portion of the study are not in agreement with the conclusions obtained in earlier investigations. In the study of Linz et al. [20] the abdominal aorta was constricted above the left renal artery. This may have resulted in a different activation of the systemic or local RAS. The increase in blood pressure was relatively small in this study, and the time course was different. In another study [26] in which the abdominal aorta of rats was constricted, the increase in cardiac load was mild as indicated by the mean carotid artery pressure of around 140 mm Hg. However, this was measured only in a portion of experimental animals and not in sham-operated controls. An experimental model that was similar to our preparation consisted of constriction of the ascending aorta in rats [27]. In accordance with our results, ACE inhibition applied immediately at the onset of pressure overload was not effective in preventing cardiac hypertrophy. However, delayed onset of ACE inhibition reduced cardiac hypertrophy. Unfortunately, there are not data presented in this study as to the hemodynamic effects of drug treatment in animals with aortic constriction.

Our results on the effect of ACE inhibition in LV pressure overload are similar to those of the study of Clozel et al. [28] in which ACE inhibition with cilazapril was applied in the model of hypoxia-induced pulmonary hypertension. Cilazapril completely prevented the remodeling of the pulmonary arteries, while the hypoxia-induced elevation of pulmonary artery pressure and the development of RV hypertrophy were not significantly affected. Based on and encouraged by these findings,

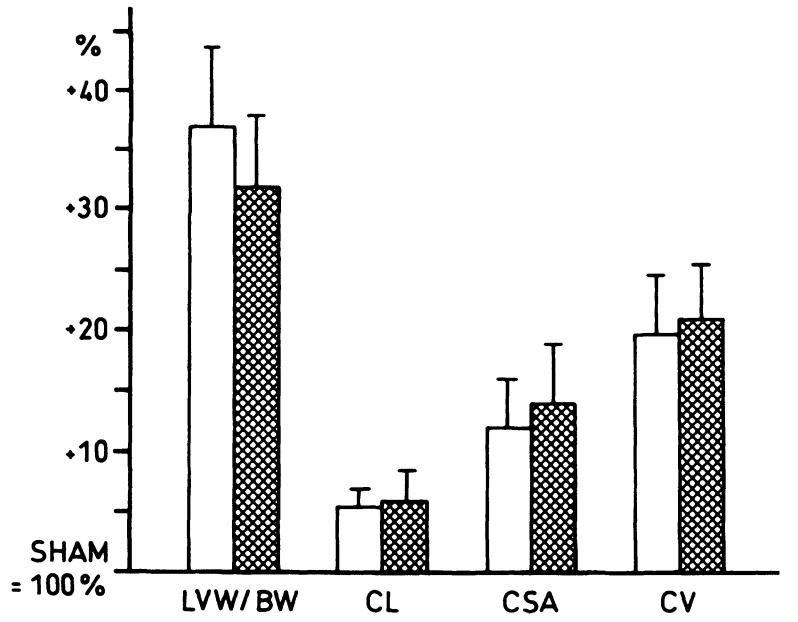


**Figure 2.** Effects of the aortic arch stenosis in rats without (open bars) and with (crossed bars) ramipril treatment for 14 days on the left ventricular weight/body weight ratio, and on cell length (CL), cross-sectional area (CSA), and cell volume (CV) of cardiac myocytes isolated from the left ventricle. The values are expressed as percent changes and are compared to sham-operated controls. The number of experiments ranged from 4 to 8.

it was of interest to us to investigate converting enzyme inhibition in a model of RV pressure overload.

### CONVERTING ENZYME INHIBITION IN PRESSURE-INDUCED RIGHT VENTRICULAR HYPERTROPHY

These experiments were also performed on female Sprague Dawley rats with a body weight similar to that in the previous study. The animals were divided into four main groups: sham operation without and with ramipril treatment and pulmonary artery stenosis without and with ramipril treatment. Ramipril was dissolved in distilled water and administered orally in a single daily dose of 1 mg/kg body weight for the entire study period (14 days). This dose has been shown to cause sufficient inhibition of ACE [29]. Twenty-four hours after the last treatment, the animals were anesthetized intraperitoneally (thiopental sodium 100 mg/kg i.p.), and the hemodynamic parameters were obtained using Millar ultraminiature catheter tipmanometers (model PR 249 and SPR 392 for left and right heart catheterization, respectively; Millar Instruments, Houston, TX, U.S.A.). After the hemodynamic measurements had been completed, the hearts were rapidly excised. In one series of experiments, the hearts were removed, the aorta was cannulated, and the hearts

were perfused with collagenase in a modified Langendorff apparatus for isolation of cardiac myocytes.

To produce RV afterload elevation, the thorax was opened in ether-anesthetized, ventilated animals by a midsternal incision, and the pulmonary artery was exposed. Stenosis of the pulmonary artery was induced with a wire of 1.7 mm diameter placed alongside the pulmonary artery, tightly fixed with a thread and then removed, leaving the pulmonary artery constricted to an outer diameter equivalent to the diameter of the wire. In sham-operated animals, the procedure was the same except that the thread was fixed. The chest was closed, and the animals recovered rapidly [30]. The average weight gain of the four groups during the subsequent 2 weeks was similar (between 16 and 24 g).

Fourteen days after surgery, LV functional parameters as well as cardiac output were essentially the same in sham-operated animals and animals with pulmonary stenosis. Treatment with ramipril resulted in a reduction of left ventricular systolic pressure (LVSP) and LV$dP/dt_{max}$; however, the differences were significant only in the operated groups without and with ramipril treatment. Constriction of the pulmonary artery elicited a considerable increase in all RV functional parameters (figure 3). Concomitant ramipril treatment did not influence these changes. There were no significant differences in LV weight among the four groups. The marked elevation of right ventricular systolic pressure (RVSP) (+74%) in the animals with pulmonary artery constriction was reflected in a highly significant increase in the RV weight/body weight ratio. Ramipril did not affect the RV weight/body weight ratio in sham-operated animals (figure 4). The isolated hypertrophy of the RV was demonstrated by the increase in the RV weight/LV weight ratio. The change of this parameter induced by pulmonary artery constriction was not affected by ramipril (figure 4).

As to the morphological parameters, the length of cardiac myocytes isolated from the RV was similar in all groups (figure 5). Constriction of the pulmonary artery without treatment was associated with a marked increase in cell volume (+58%) and cross-sectional area. These results are typical for concentric hypertrophy that has developed as a result of pressure overload. The cell morphological data also correlate very well with the increase in RV weight/body weight ratio in this group (+57%). Administration of ramipril to animals with pulmonary artery constriction attenuated the increase in cell volume (+27%) and cross-sectional area significantly, although the hemodynamic load imposed on the RV was similar in both groups. Thus, there was a discrepancy between the gross morphological and cellular parameters under the influence of ACE treatment in that the increase in pressure-induced RV weight was not affected, but the increase in cell volume and cross-sectional area was attenuated. One is therefore inclined to suggest that ramipril treatment in this particular experimental model affects the cardiac myocyte compartment. Why the same ramipril treatment for the same period of time in an equivalent pressure overload model of the LV has no effect remains unclear. One may speculate or anticipate that the RV free wall, which is much thinner than the LV free wall, may developed failure earlier because of the decrease in cellular morphology.

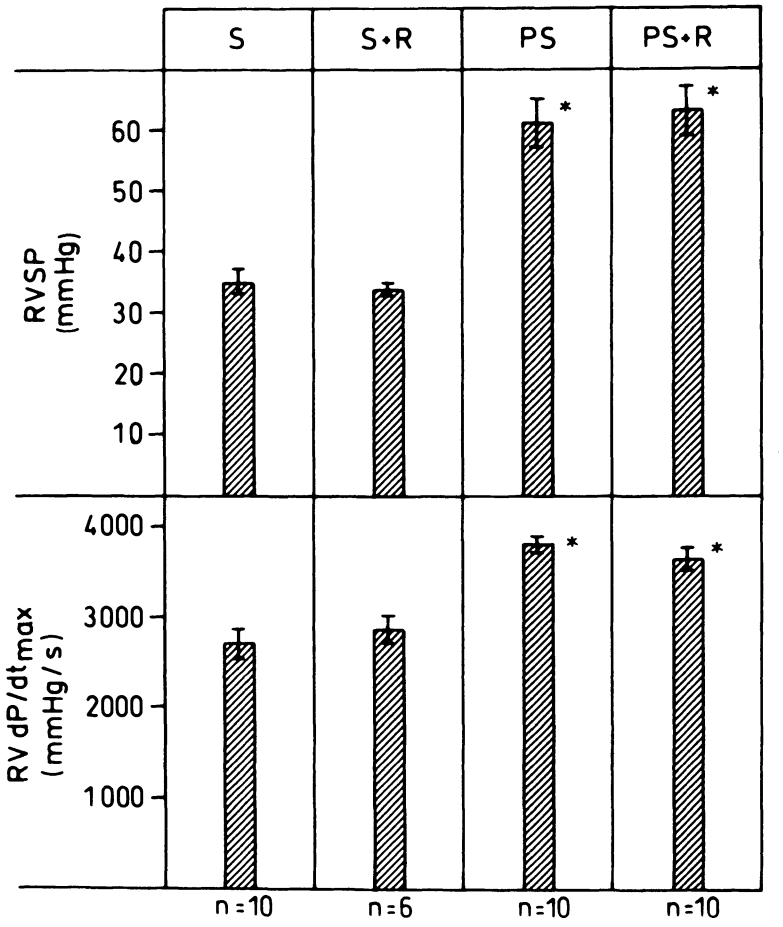


**Figure 3.** Right ventricular systolic pressure (RVSP) and maximal rate of rise in RV pressure (RV $dp/dt_{max}$) in rats 14 days after sham operation (S) and pulmonary artery stenosis (PS) without and with ramipril treatment (R). The data are mean values ± SEM; n = number of experiments. *$p < 0.05$ vs. controls.

## ANGIOTENSIN II RECEPTOR BLOCKADE IN NORADRENALINE-INDUCED CARDIAC HYPERTROPHY

In this experimental series, female Sprague-Dawley rats (253 ± 6g), obtained from Charles River (Sulzfeld, Germany), were again used. All drugs were administered intravenously and continuously for 14 days with Alzet osmotic pumps (model 2ML2, Alza, California, USA). The pumps were implanted subcutaneously and connected to catheters (Cardioflex 0.6 × 1.0mm, Vygon, Aachen, Germany) inserted into the left jugular or left femoral vein. A piece of silicone tubing the size of the osmotic pumps was implanted subcutaneously in the control animals, and the left jugular vein was ligated instead of the catheter implantation.

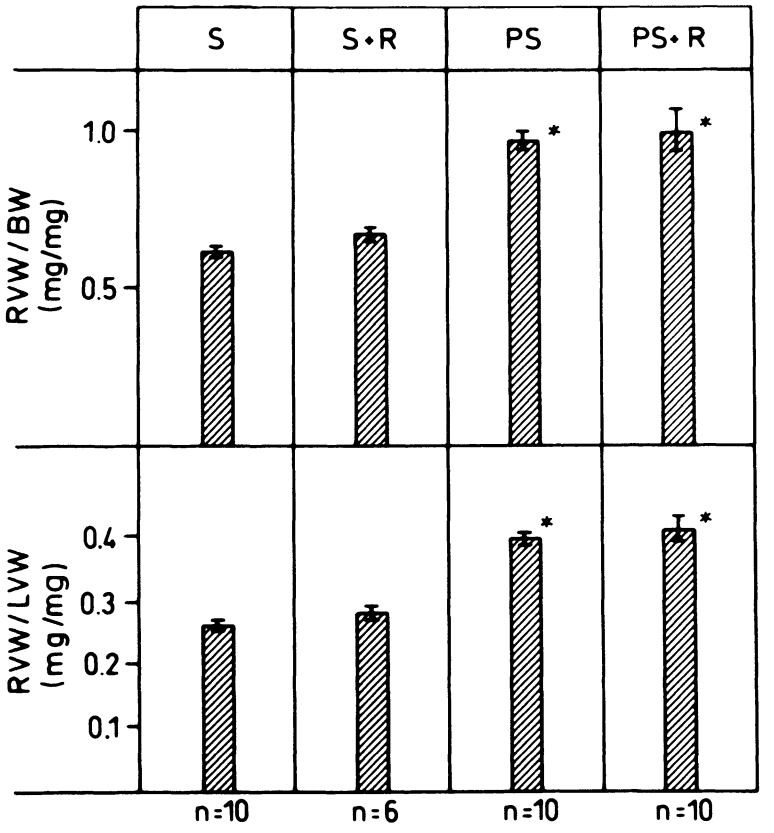


**Figure 4.** Right ventricular weight (RWV) related to body weight (BW) and to left ventricular weight (LVW) in rats 14 days after sham operation (S) and pulmonary artery stenosis (PS) without and with ramipril treatment (R). The data are mean values ± SEM; n = number of experiments. *$p < 0.05$ vs. controls.

The animals were anesthetized with ether for insertion of the infusion catheters and the infusion pumps at the beginning of every experimental series. After the surgical procedure and for the 14 days of the infusion, the animals were able to move around freely in their cages with access to tap water and control rat chow diet (Altromin C 100, Altromin GmbH, Lage, Germany). All substances were dissolved in 0.9% sodium chloride, except where otherwise noted. To prevent oxidation of the catecholamines, 200 mg/L L-(+)-ascorbic acid (Merck, Darmstadt, Germany) was added to the solutions. The infusion rate of the osmotic pumps was 5 µl/h. (−)-Noradrenaline-HCLr (NA) was purchased from Sigma Chemical (Deisenhofen, Germany) and administered at a dosage of 100 µg/kg/h. Metoprolol-tartrate was obtained from Ciba-Geigy (Wehr, Germany) and administered at a rate of 1 mg/kg/h in combination with NA. The angiotensin II receptor antagonist losartan was

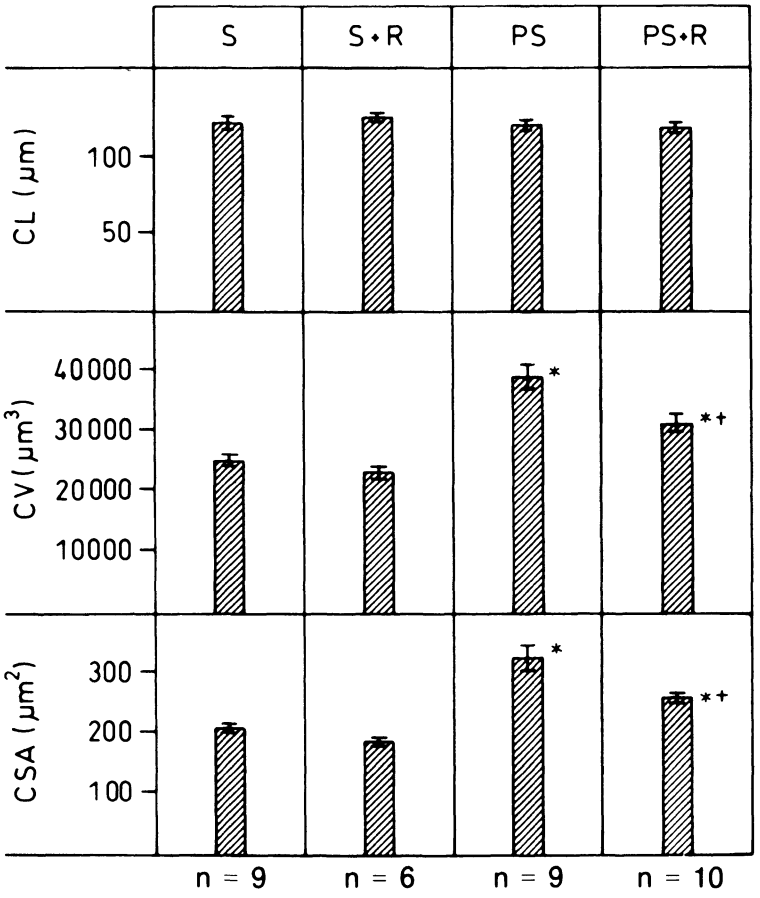


**Figure 5.** Cell size data obtained in myocytes isolated from the right ventricle of rats 14 days after sham operation (S) and pulmonary artery stenosis (PS) without and with ramipril treatment (R): CL, cell length; CV, cell volume; CSA, cross-sectional area. The data are mean values ± SEM; n = number of experiments. $*p < 0.05$ vs. S, $^{\dagger}p < 0.05$ vs. PS.

donated by MSD Sharp & Dohme (München, Germany) and infused alone and in combination with NA at a dosage of 12 mg/kg/day. Losartan was dissolved in water.

After 14 days of continuous NA infusion, heart rate was increased significantly. The parallel administration of metoprolol and NA resulted in a heart rate that was below the control values. Losartan alone had no effect on heart rate but prevented the NA-induced increase. NA induced an elevation of LVSP compared to the control values after 14 days of infusion (figure 6). This NA-induced increase in pressure was not influenced by the additional administration of metoprolol. When losartan alone was given for two weeks, LVSP was not changed from the baseline condition. As opposed to metoprolol, losartan did prevent the NA-induced rise in

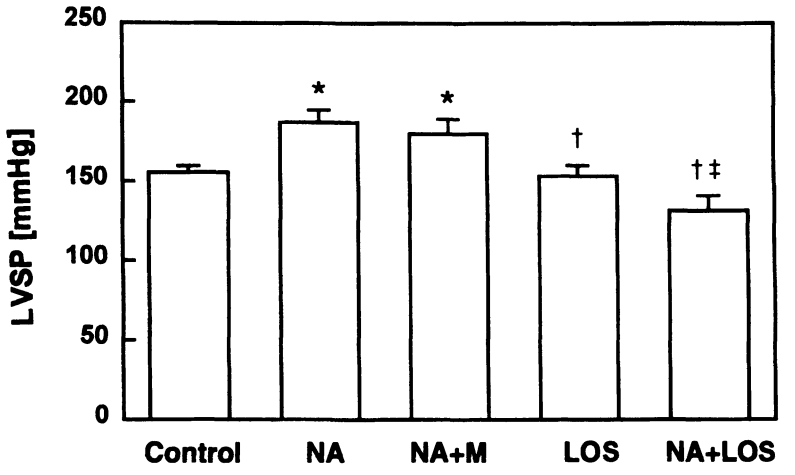


**Figure 6.** Effect of noradrenaline (NA) at 100μg/kg/h for 14 days on left ventricular systolic pressure (LVSP) in rats. Metoprolol (M) was administered in a dose of 1mg/kg/h, and the angiotensin II receptor blocker losartan (LOS) was applied in a dose of 12mg/kg/day. $^{*}p < 0.05$ vs. time-corresponding control. $^{\dagger}p < 0.05$ vs. time-corresponding NA infusion, $^{\ddagger}p < 0.05$ vs. NA + M, $^{\S}p < 0.05$ vs. LOS.

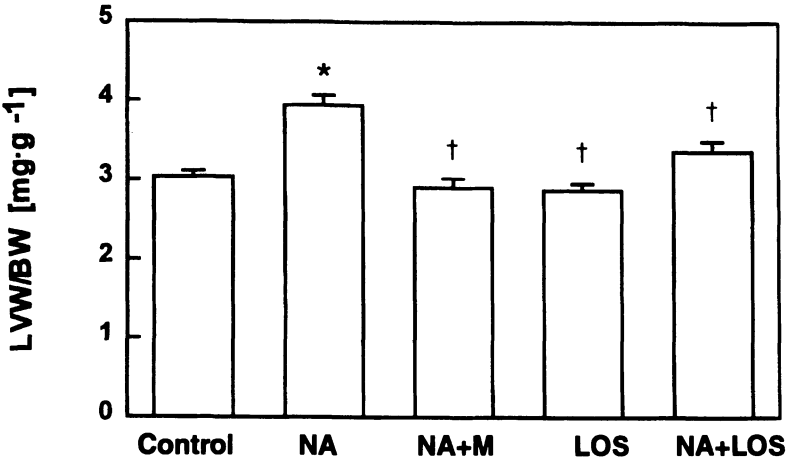


**Figure 7.** Effect of noradrenaline (NA) at 100μg/kg/h for 14 days on the left ventricular weight/body weight ratio (LVW/BW) in rats. Metoprolol (M) was administered in a dose of 1mg/kg/h and the angiotensin II receptor blocker losartan (LOS) was applied in a dose of 12mg/kg/day. $^{*}p < 0.05$ vs. time-corresponding control, $^{\dagger}p < 0.05$ vs. time-corresponding NA infusion.

LVSP. NA caused an increased in the left ventricular weight/body weight ratio (LVW/BW) of about 30% after two weeks. As illustrated in figure 7, the gain in LVW/BW was prevented by metoprolol and attenuated by losartan [31].

Thus, Ang II receptor blockade in this experimental model of continuous NA application attenuated LV hypertrophy. What is surprising is that metoprolol did not affect LVSP but did prevent cardiac hypertrophy. This is another example which demonstrates that pressure increase or elevation of total peripheral resistance does not necessarily induce cardiac hypertrophy [10]. When the increase in total peripheral resistance that was induced by NA was prevented by the calcium antagonist verapamil, the development of cardiac hypertrophy did still occur. On the other hand, although losartan did entirely prevent the NA pressure increase, it did only attenuate and not prevent cardiac hypertrophy.

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# EFFECTS OF INHIBITION OF ANGIOTENSIN-CONVERTING ENZYME ON MYOCARDIAL AND MYOCYTE REMODELING IN CHRONIC VOLUME OVERLOAD-INDUCED CARDIAC HYPERTROPHY IN THE DOG

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***Summary.*** The data from our studies with chronic mitral regurgitation (MR) in the dog demonstrate that the ventricular and myocyte remodeling in this model, characterized by ventricular dilation and myocyte elongation, are not returned toward normal by treatment with the converting enzyme inhibitor, ramipril. However, in spite of this failure to reduce cardiac hypertrophy or to prevent the remodeling associated with this model of chronic volume overload, there was functional improvement in the animals, as determined by the lower left ventricular filling pressures and pulmonary artery pressures in the treated animals compared with the controls. Ramipril also returned myocardial Ang II levels to control level. This suggests that there may have been a regulatory mechanism on contractile function acting through this chemical mediator.

Our results suggest that diastolic wall stress activates the cardiac renin-angiotensin system (RAS) and that angiotensin (Ang) II and angiotensin-converting enzyme (ACE) may play a functional role in the eccentric pattern of hypertrophy observed in the volume overload model of MR in the dog heart. Additional future studies are needed to further characterize the relative roles of ACE and chymase in Ang II formation in this model that is relevantly important to the human heart. MR produces an increase in intracardiac Ang II without the interstitial fibrosis that has been found in pressure overload. This finding is of particular interest because the heart is a target organ for Ang II, a growth factor for myocytes and fibroblasts [1–5], which has also been associated with myocyte necrosis [6]. Thus, the mechanisms by which the RAS may mediate eccentric hypertrophy in the low pressure volume overload of MR is open to question. The beneficial effect of converting enzyme inhibitor on myocardial function in this model of volume overload, but in the absence of morphological improvement, remains speculative but may be related to alterations in the RAS in the cardiac muscle produced by the treatment.

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## INTRODUCTION

The process of cardiac hypertrophy results in a variety of morphological and functional alterations of the myocardium, which depend on the type of overload. In pressure overload, a number of studies have demonstrated that chamber diameter tends to remain small, while there is marked thickening of the ventricular wall. On the other hand, volume overload tends to result in more marked chamber dilation with less pronounced wall thickening, in spite of increase in total ventricular mass. The hemodynamic consequences of these separate models with different ventricular remodeling result in different rates of development of congestive heart failure, different amounts of change in interstitial connective tissue, and different effects on myocyte remodeling.

There have been several clinical studies that have demonstrated a beneficial role for converting enzyme inhibitors in patients with developing congestive heart failure [7–9], but the mechanism for this clinical benefit remains unclear. The beneficial effects of converting enzyme inhibitors in asymptomatic patients could not be explained by the extent of blood pressure reduction [10]. Animal studies have clearly shown that in addition to the systemic renin angiotensin system (RAS), there are locally produced elements of the RAS in several organs, including the heart [11–16]. The role played by either the systemic or intracardiac RAS in remodeling of both the myocardium and the ventricular myocytes has received very little study and may also be different in these diverse models of cardiac hypertrophy.

Most of the animal studies that investigated the role of the cardiac RAS in the pathogenesis of myocardial hypertrophy and heart failure and its sensitivity to ACE inhibition has been performed in pressure overload models in the rat. Recent work in the rat heart has demonstrated that the increase in cardiac RAS gene expression seen in concentric left ventricular (LV) hypertrophy associated with pressure overload also occurs in eccentric hypertrophy related to the volume overload of aortocaval fistula [17]. In spite of the different effects on remodeling of the left ventricle (LV) and cardiac myocytes in pressure and volume overload, the cardiac RAS is upregulated in both processes. Further, the presumption that the beneficial effects of ACE inhibitors are mediated solely by decreasing intracardiac angiotensin (Ang) II generation has now been challenged by the identification of alternative Ang II-forming mechanisms that may vary across species, in particular heart chymase [18,19].

## LEFT VENTRICULAR HYPERTROPHY FROM PRESSURE VERSUS VOLUME OVERLOAD

Pressure overload results in the development of concentric LV hypertrophy, characterized by a marked increase in wall thickness, often with relative reduction in the ventricular lumen volume. It is hypothesized that increased systolic wall stress (stress = pressure $\times$ radius/2 thickness) causes this pattern of myocyte and chamber hypertrophy whereby increased wall thickness offsets increased pressure and normalizes stress [20,21]. Microscopic evaluation of cardiac tissue or measurements in isolated myocytes has shown that sarcomeres are added in parallel, resulting in an

increase in myocyte cross-sectional area, with little or no increase in cell length, depending on the model [22–26]. In contrast, volume overload with relatively less thickening of the ventricular wall because of dilation of the ventricular chamber increases diastolic wall stress and triggers an in series replication of sarcomeres. Studies of isolated cardiac myocytes from volume-overloaded and dilated models of cardiac hypertrophy have shown that the cell size predominantly increases in length, rather than width [27–33]. In compensated states of cardiac function, wall thickness (LV mass) increases in proportion to the volume overload so that adequate mass is present to pump the extra volume while wall stress remains normal. There is a distinction, however, between the low pressure volume overload of mitral regurgitation (MR) and the high pressure volume overload of aortic regurgitation and aortocaval fistula. MR presents a unique hemodynamic stress to the LV in that a large part of the excess volume is ejected into the low pressure left atrium. This form of chronic hemodynamic stress is very different from pressure overload, in which shortening load is increased throughout systole, and differs from the high pressure volume overload of aortocaval fistula and aortic regurgitation, in which the excess volume is ejected into the high pressure aorta. Consequently, MR places a much smaller systolic load on the LV and results in far less hypertrophy than pressure overload and high pressure volume overload [34].

Cardiac myocyte growth is the common denominator in myocardial hypertrophy. The pattern of myocyte remodeling and cardiac chamber geometry that develops in LV hypertrophy is determined by the hypertrophic stimulus. Furthermore, the hypertrophic remodeling of the myocardium may or may not include the growth of nonmyocyte cells (endothelial cells, macrophages, fibroblasts, smooth muscle cells), which include two-thirds of the cell population of the heart. Myocardial remodeling is mediated by locally and systemically generated trophic factors that are produced in response to hemodynamic stress. These trophic factors and the model-specific hemodynamic parameters combine to cause specific alterations in myocyte geometry and in the extent or lack of production of interstitial and perivascular fibrillar collagen in the different forms of cardiac hypertrophy.

In models of pressure overload cardiac hypertrophy with sudden onset, such as aortic banding in adult animals and in those which have developed congestive heart failure, there is an increase in the amount of interstitial and replacement-type myocardial fibrosis [35–39]. In animal models with a gradual onset of pressure overload, prior to the onset of congestive heart failure, there is much less accumulation of collagen [36,40]. In long standing or severe pressure overload, collagen accumulates in perivascular areas surrounding intramyocardial arteries and arterioles and extends from this perivascular location into the interstitial space. In addition, interstitial fibrosis develops unrelated to blood vessels, and microscopic foci of replacement-type fibrosis are found, especially in subendocardial locations [26,39,41–44].

Volume overload, on the other hand, while often accompanied by similar degrees of cardiac hypertrophy as in pressure overload, has most often been characterized by little or no increase in myocardial fibrosis. In dogs with aortocaval fistula, total

increases in LV mass ranged between 43 and 100%, depending on both shunt size and duration of the volume overload [45]. Weber and coworkers reported no increase in myocardial interstitial collagen content by the picrosirius polarization technique at 10 weeks following placement of aortocaval fistulae in the dog [46]. Volume overload induced by production of atrial septal defects in the cat also failed to result in an increase in myocardial collagen, in spite of an approximate 50% increase in right ventricular (RV) free wall weight [47]. However, in spite of the lack of increase in collagen, Covell and coworkers demonstrated a qualitative change in collagen content manifested by a greater degree of cross-linking between Types I and III collagen in dogs with aortocaval fistula, who had increased LV end-diastolic pressure and chamber stiffness at the time of sacrifice [48]. Thus, it appears that qualitative as well as quantitative examinations of structural collagen in the heart may be necessary to define changes in this extracellular supporting latticework that may affect chamber distensibility and stiffness. Whether the increase in collagen production progresses over the time course of a particular hemodynamic stress and whether it is a cause or a consequence of heart failure is unresolved.

The canine model of MR presents a lesser systolic stress to the LV than either high pressure volume overload or pressure overload because a large part of the excess volume is ejected into the low pressure left atrium. We and Carabello and coworkers have found that percutaneous rupture of the mitral valve in the dog produces a 20–30% increase in LV mass with an increase in LV chamber dimensions [34,49,50]. There is a significant decrease in the LV mass/volume ratio resulting from a 50–100% increase in LV end-diastolic volume (EDV) and a maximum 30% increase in LV mass at three to five months after chordal rupture.

## PRODUCTION OF CHRONIC MITRAL REGURGITATION IN THE DOG

In spite of the beneficial effects of converting enzyme inhibitors on myocardial function, little is known of the mechanisms involved or the effect on myocardial or myocyte remodeling. In order to study the effect of converting enzyme inhibitor therapy on myocardial chamber size, ventricular function, and myocyte remodeling in a volume overload model, we produced MR in the dog and treated animals with the converting enzyme inhibitor, ramipril.

All dogs used in this study had in vivo functional evaluation with magnetic resonance imaging (MRI) prior to creation of MR and again 5 months after creation of MR at the time of final study to compare functional performance and chamber morphology with changes in myocyte morphometry. Mitral regurgitation was induced by severing the chordae tendineae of the mitral valve with a catheter technique as previously described [49]. Briefly, each dog (18–25 kg) was anesthetized with intravenous Innovar-vet (droperidol and fentanyl 1–1.5 ml), intubated, and maintained on a surgical plane of anesthesia with isoflurane (0.75–1.5%) and oxygen (2 l/min) for the MRI studies and for percutaneous creation of MR. A flexible, rat-tooth grasping forceps (7 Fr.) (Cook Urological, Spencer, Indiana) was used to cut the chordae, and MR was judged to be sufficient when there was an increase in the

pulmonary arterial wedge pressure that was greater than 20 mmHg with V wave dominance, a decrease in arterial pressure, and a decrease in cardiac output of 50%.

Dogs were auscultated daily, and serial chest X-rays were performed to identify the onset of pulmonary venous congestion; furosemide was given at 2.2 mg/kg PO bid if needed. Seven MR dogs were treated with ramipril starting at 2.5 mg q day and increasing to 10 mg bid at one week (R-MR). Three dogs started ramipril therapy at 24 hours, and 4 dogs started ramipril therapy at three weeks after induction of MR. An additional 8 dogs with MR were not treated with the converting enzyme inhibitor (N-MR). Control dogs had a sham operation without cutting any chordae tendineae and were not given any drug treatment (CON).

### CINE-MAGNETIC RESONANCE IMAGING

MRI was recorded at baseline and 4–5 months after induction of MR with a 1.5 Tesla whole-body imaging system (Philips Gyroscan) utilizing a gradient echo pulse sequence, as previously described in our laboratory [49,51]. LV volumes and mass were calculated from summated serial short axis slices using a Simpson's rule algorithm, as previously described and validated in our laboratory [49,51].

### TISSUE PREPARATION FOR BIOCHEMICAL ASSAYS AND MOLECULAR BIOLOGY

At the time of sacrifice, a deep surgical plane of anesthesia was induced with isoflurane inhalation, and a thoracotomy was performed. The heart was arrested with a lethal dose of KCl, was removed from the chest, and rapidly cooled in ice cold Krebs-Henseleit buffer. The coronary arteries were retrogradely perfused from the aorta with cold Krebs-Henseleit solution, the atria and right ventricular free wall were dissected from the interventricular septum and LV, and the portions were weighed. Left and right ventricular tissue samples were immediately frozen in liquid nitrogen and subsequently analyzed for Ang II content, ACE and chymase-like activity, and $AT_1$ receptor mRNA levels.

### CARDIAC ANG II CONCENTRATIONS

Cardiac Ang II concentrations were determined by a method from our laboratory that was previously described which combines solid-phase extraction (SPE), high-performance liquid chromatography (HPLC), and radioimmunoassay (RIA) [49,52].

### HEART CHYMASE-LIKE ACTIVITY USING ANG I AS SUBSTRATE

Five hundred mg samples of LV midwall were assayed for chymase-like activity, as previously described in our laboratory [49,53]. Generated Ang II was quantitated using a reverse phase Alltima 5 micron phenyl-HPLC column. The peak area corresponding to a synthetic Ang II standard was integrated to calculate absolute Ang II formation. Chymase-like activity was defined as chymostatin inhibitable Ang II formation, expressed as nmoles of Ang II formed/gm/min of tissue (wet weight).

### CARDIAC ACE ACTIVITY USING HIPPURYL HISTIDYL LEUCINE AS SUBSTRATE

Cardiace ACE activity was measured using an assay developed in our laboratory. According to this method, ACE is extracted from homogenized cardiac tissue with detergent, and the reaction product, hippuric acid (HA), is isolated from the reaction mixture by reverse-phase HPLC, thus eliminating interference from the detergent, the substrate hippuryl histidyl leucine (HHL), and unreacted reaction byproducts [49,54].

### TISSUE FIXATION FOR MYOCARDIAL STRUCTURAL EVALUATION

After the heart was dissected and weighed, a polyethylene catheter was tied into a branch of the circumflex coronary artery, and this LV segment was perfused with saline followed by 3% phosphate buffered paraformaldehyde under gravity flow at 90 mmHg pressure. The right coronary artery was similarly prepared, and a portion of the RV free wall was also perfusion-fixed. Transmural sections of left and right ventricles were embedded in paraffin, and 5 μm sections were stained with hematoxylin and eosin, Gomori's aldehyde fuchsin trichrome, and picric acid sirius red. Additional perfusion fixed samples from the inner, mid, and outer thirds of the posterior wall of the LV and a sample of RV free wall were embedded in glycol methacrylate, sectioned at 1 μm thickness, and stained with methylene blue and with a silver methenamine-gold microwave technique for basement membranes (Accustain silver stain, Sigma diagnostics, St. Louis, MO).

For transmission electron microscopy, paraformaldehyde perfusion fixed tissues were dehydrated through alcohols, embedded in Spurr epoxy resin, thick sectioned at 1 μm thickness, and stained with toluidine blue for light microscopic examination. Suitable sections were thin sectioned at silver-gray interference color, stained with lead citrate and uranium oxide, and examined in a Philips 400 electron microscope.

### MYOCARDIAL CONNECTIVE TISSUE EVALUATION

Images of tissues stained with picric acid sirius red were obtained from a video monitor and CCD72 video camera attached to an Olympus AHT microscope and analyzed using Image-1 image analysis software (Universal Imaging Corporation, West Chester, PA). This system uses an 8-bit, 256 grey level discrimination with a CCD72 camera attached to an Olympus AT3 research microscope and interfaced to a 486DX computer with software supplied by the company, which is programmed to calculate the percentage of pixels exceeding a selected density in each field. A 540 nm (green) filter was used to provide contrast of the red-stained collagen with the background. Two or more transmural sections of LV and RV were first examined at low power (2× objective; 60× on the video screen; 3000 × 3000 μm tissue area) to measure replacement type fibrosis and perivascular collagen. The tissue was then examined at higher power (600× on the video screen; 300 × 300 μm tissue area) to measure interstitial connective tissue. Slides were marked with

a felt tip pen to demarcate endo, mid, and epicardial thirds of the LV, and 30–50 fields were measured from each tissue region. Mean volume percent connective tissue for each region of each animal was calculated from the total area measured, and these values were combined for determination of group means.

### MYOCYTE CROSS-SECTIONAL AREA

Myocyte cross-sectional area (CSA) was measured from video prints of silver-stained methacrylate sections of subendomyocardial, midmyocardial and, subepimyocardial of the LV and of RV myocardium. Suitable cross-sections were defined as having nearly circular capillary profiles and circular to oval myocyte cross-sections. Only myocytes with nuclei were measured. No correction of oblique sectioning was made. Video prints (1100 × final magnification) were used to trace the outline of at least 100 myocytes in each region, including all suitably sectioned myocytes in the print, using a sonic digitizer (Graf/Bar, Science Accessories, Southport, CT). Myocyte cross-sectional area was determined by using computer programs developed in our laboratory. The mean area was calculated for each region in each animal, and the group mean was calculated for each region and group.

### QUANTITATIVE EVALUATION OF MYOCARDIAL MAST CELLS

The density of mast cells was quantitatively determined for LV transmural thirds and for the RV using the methylene blue stained methacrylate sections. For each region, 30 fields, each 137,600 $\mu m^2$, were examined using the 10× objective of the microscope, and the number of mast cells per field were tabulated.

### SCANNING ELECTRON MICROSCOPIC EVALUATION OF COLLAGEN

Perfusion fixed tissue from the inner and outer thirds of the LV wall and from the RV were processed for scanning electron microscopy. Tissues were dehydrated with alcohols and acetone and critical point dried. Tissues were mounted on aluminum stubs with longitudinal-cut surfaces exposed, sputter-coated with gold, and examined in a Philips 515 scanning electron microscope.

Semiquantitative evaluation of each specimen was performed by assigning a grade from 1 to 5 for each of 40 consecutive adjacent areas, at both 1000× and 4000× magnification. Each field was graded at each magnification as follows: 1 = absent or very little collagen weave; 2 = moderately reduced collagen weave; 3 = normal collagen weave; 4 = moderately increased collagen weave; 5 = large increase in amount of collagen. Grades were determined from evaluation of normal and abnormal tissues, and the range for each grade was arbitrarily determined. Data were presented as a mean grade for each tissue and as a histogram of the percent of the total field examined for each subjective grade.

### MYOCYTE ISOLATION

Isolated myocytes were prepared by collagenase perfusion of a segment of the LV myocardium with collagenase media on a nonrecirculating Langendorff system from

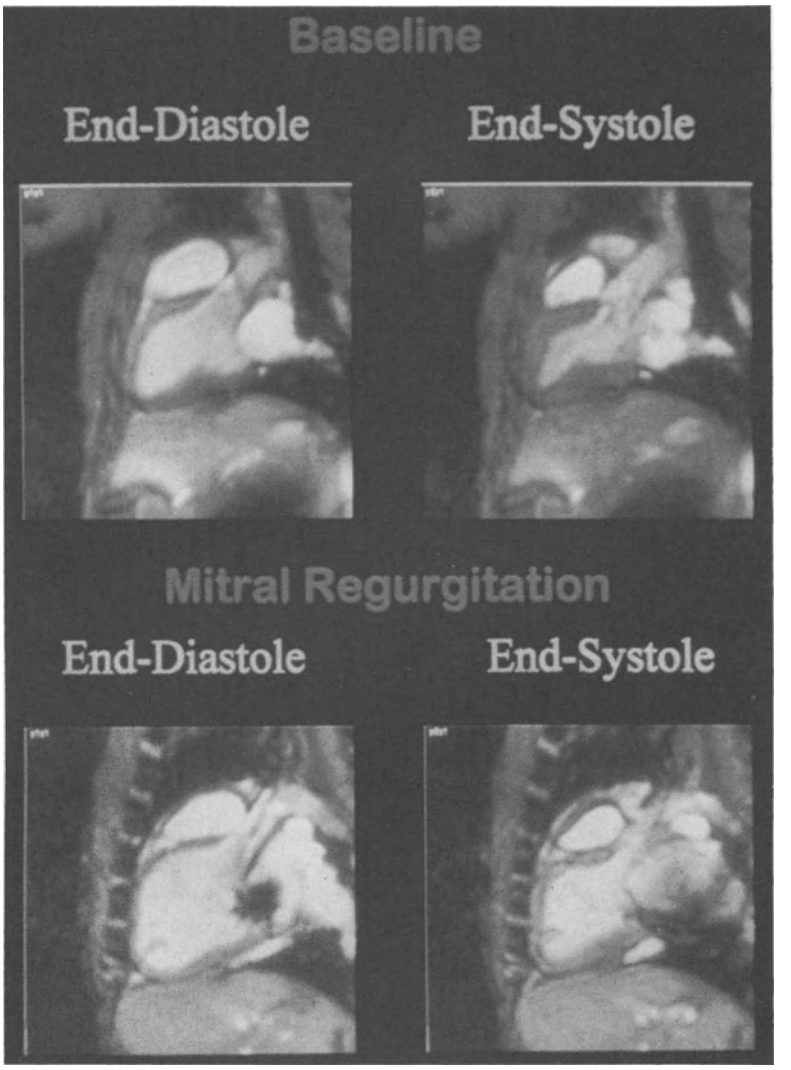


**Figure 1.** Long axis and short axis end-diastolic and end-systolic magnetic resonance images of the heart at baseline and 5 months after induction of mitral regurgitation, demonstrating the increase in end-diastolic volume and the decrease in wall thickness that occurs as a result of a decrease in the LV mass volume ratio. (Reproduced with permission from Le Jacq Communications, Inc.).

all MR dogs and 10 nonoperated normal dogs. A marginal branch of the left circumflex coronary artery in the KCl-arrested excised heart was cannulated with PE tubing and flushed with ice-cold tissue culture media. The heart tissue was mounted on a Langendorff perfusion apparatus, and the tissues were first perfused with a nominally $Ca^{++}$-free Joklik minimum essential medium containing EGTA,

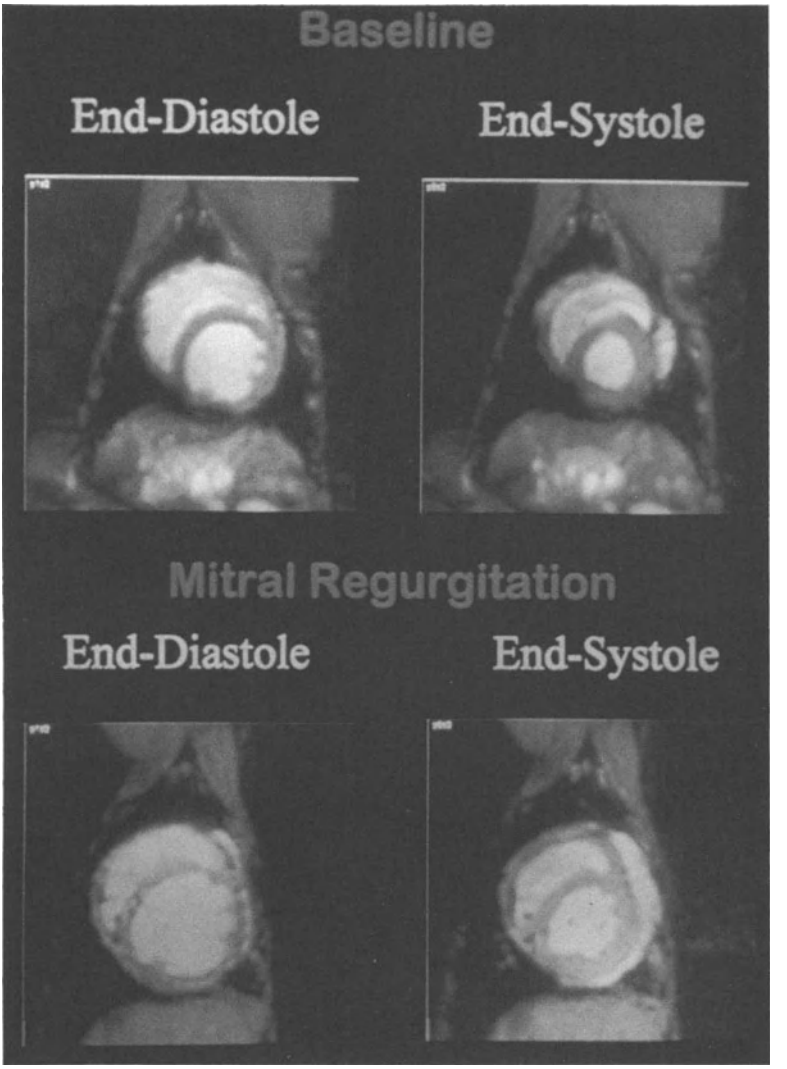


Figure 1 (continued)

followed by 25 minutes perfusion with the Joklik medium with 0.1% collagenase. The tissue was subdivided into inner and outer halves. Tissues were thoroughly minced with scissors, filtered through 250μm nylon mesh, and the myocytes fixed in a 1% final concentration phosphate buffered glutaraldehyde. Cell length was measured by light microscopy of fixed cells settled on a glass slide and examined with the video image system. Length of 100 cells was measured. Mean cell volume

was determined for each region by using a Coulter Counter system as previously described [55,56], and mean myocyte cross-sectional area was calculated as mean myocyte volume/mean myocyte length.

### EFFECT OF RAMIPRIL TREATMENT ON MYOCARDIAL FUNCTION AND VENTRICULAR SHAPE IN DOGS WITH CHRONIC MITRAL REGURGITATION

In the dogs with chronic MR, there was ventricular dilation and moderate increase in left ventricular mass (figure 1), which were not significantly attenuated by treatment with ramipirl. As shown in table 1, calculated values for LV end-diastolic volume, end-systolic volume, and stroke volume were increased to a similar extent in both treated and untreated MR dogs, and the increase in LV mass was not different between groups. The increase in LV mass to body weight measured at necropsy in the N-MR dogs was 38% and for the R-MR dogs, 27%, when compared to a group of 27 normal dogs from our laboratory with similar body weight (4.54 g/kg). LV mass by cine-MR correlated with the LV mass determined by heart weight at the time of sacrifice ($r = 0.92$, $p < 0.01$). The LV mass/EDV ratio decreased significantly in both N-MR (1.60 ± 0.07 to 1.13 ± 0.08 gm/ml, $p < 0.001$) and R-MR dogs (1.44 ± 0.06 to 1.20 ± 0.08 gm/ml, $p < 0.01$). These results demonstrated similar increases in LV volume and mass and a similar decrease in LV mass/EDV ratio in the R-MR and N-MR dogs.

In spite of the failure of ramipril to prevent cardiac hypertrophy or to significantly decrease LV chamber size, the hemodynamic status of the treated dogs was improved compared to the untreated group. Mean pulmonary artery pressure (13 ± 1 vs. 21 ± 3 mmHg, $p < 0.05$) and pulmonary arterial wedge pressure (9 ± 1 vs. 16

**Table 1.** MRI and postmortem cardiac measurements in dogs with chronic mitral regurgitation treated with Ramipril

| | N-MR | | R-MR | |
|---|---|---|---|---|
| | Baseline | MR | Baseline | MR |
| LVEDV | 58 ± 4 | 104 ± 10* | 55 ± 3 | 91 ± 6* |
| LVESV | 30 ± 3 | 46 ± 5* | 28 ± 3 | 46 ± 5* |
| LVSV | 28 ± 3 | 57 ± 5* | 27 ± 1 | 46 ± 5* |
| LVEF | 48 ± 3 | 56 ± 2* | 50 ± 4 | 50 ± 4 |
| LV Mass | 92 ± 7 | 112 ± 8* | 80 ± 4 | 108 ± 7* |
| LV Mass/EDV | 1.60 ± 0.07 | 1.13 ± 0.08* | 1.44 ± 0.06 | 1.20 ± 0.08* |
| Body weight (Kg) | | 20.7 ± 1.8 | | 20.1 ± 2.2 |
| LV + S/BW (necropsy) | | 6.29 ± 0.51 | | 5.80 ± 0.53 |
| RV/BW (necropsy) | | 2.01 ± 0.15 | | 1.85 ± 0.15 |

Note: Data are presented as mean ± SE. N-MR = mitral regurgitation untreated, R-MR = MR ramipril treated, LVEDV = left ventricular end-diastolic volume, ESV = end-systolic volume, SV = stroke volume, EF = ejection fraction, LV + S/BW = left ventricular septum weight (g)/body weight (Kg), RV/BW = right ventricular free wall weight (g)/body weight (Kg). * = $p < 0.01$, baseline vs. MR.

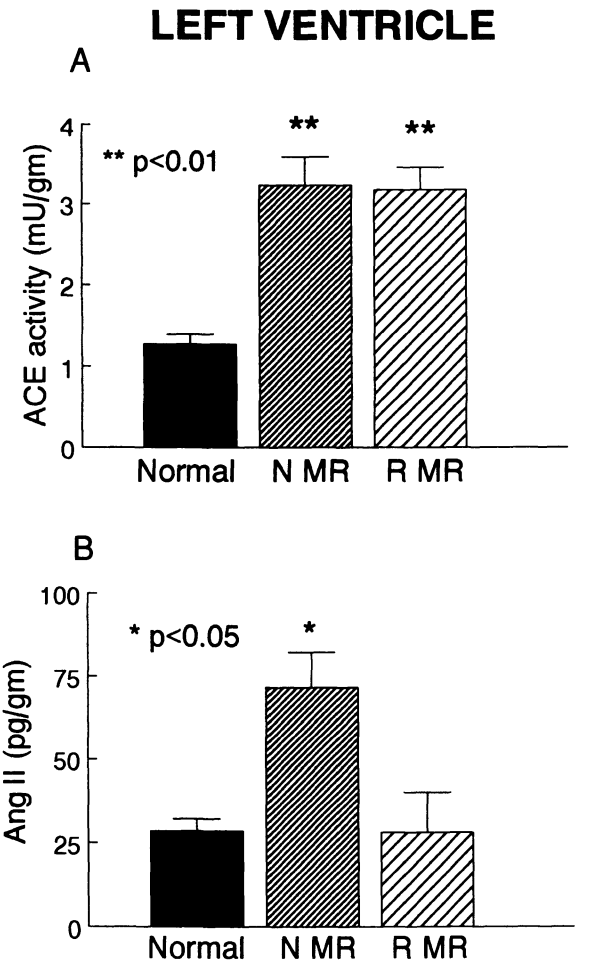


**Figure 2.** (*A*) illustrates left ventricular angiotensin-converting enzyme (ACE) activity, and (*B*) illustrates angiotensin (Ang) II levels in the Normal, nontreated mitral regurgitation (N-MR) and Ramipril-treated mitral regurgitation (R-MR) dogs. For ACE activity, ** = $p < 0.01$ compared to Normal. For Ang II levels, * = $p < 0.05$ compared to Normal. (Reproduced from [33] with permission).

± 2mmHg, $p < 0.05$) were lower in the R-MR compared to N-MR dogs at the time of sacrifice. As further subjective evidence of functional improvement of the dogs treated with ramipril, none of the treated dogs required furosamide treatment to control pulmonary congestion, while such treatment was often required in the nontreated MR dogs.

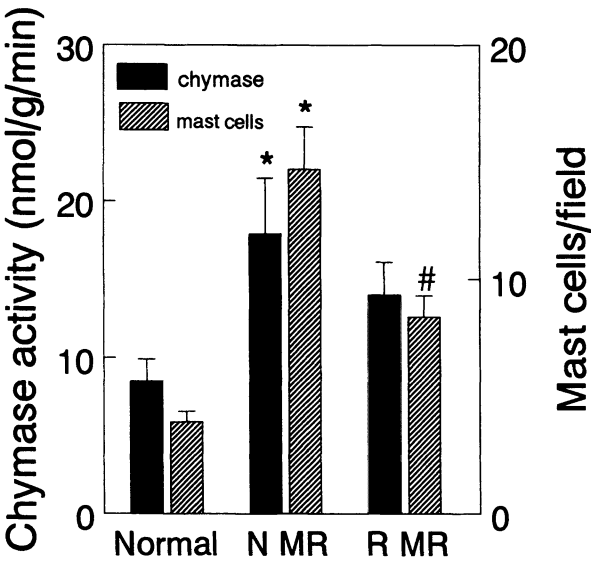


**Figure 3.** Graph demonstrating number of mast cells/field from LV subendocardial myocardium (right axis) and chymase activity (left axis) in normal, non-treated MR (N-MR) and Rampipril treated MR (R-MR) dogs. * = N-MR > Normal, $P < 0.05$ and # = Normal < R-MR < N-Mr, $p < 0.05$. (Reproduced from [33] with permission).

### CARDIAC ACE AND CHYMASE-LIKE ACTIVITY AND ANG II LEVELS

Ang II levels were significantly elevated in the LV of N-MR dogs compared to CON (72 ± 11 vs. 28 ± 4pg/gm, $p < 0.05$), but LV Ang II levels in the R-MR were not different from CON (28 ± 12pg/gm) (figure 2). LV membrane-bound ACE activity (figure 2) and chymase-like activity (figure 3) were elevated in both R-MR and N-MR compared to CON dogs.

Mast cells are known to be a source of cardiac chymase. Myocardial mast cell density was significantly increased in all regions of both ventricles in both MR groups compared to CON (figure 4). Mast cell density was moderately reduced in the R-MR dogs compared to N-MR dogs, but was still significantly greater than in CON. The increased mast cell number paralleled very closely the respective increases of chymase-like activity in R-MR and N-MR (figure 3).

### $AT_1$ RECEPTOR MRNA LEVELS IN THE LV

$AT_1$ mRNA content in LV was determined by Northern blot analysis using methods previously described [33]. Total RNA was isolated from each heart sample and analyzed separately. Blots for CON, N-MR, R-MR were simultaneously hybridized with the $AT_1$ receptor cDNA probe that was kindly supplied to us by Burns and coworkers [57]. Densitometric analysis demonstrated that the ratios of

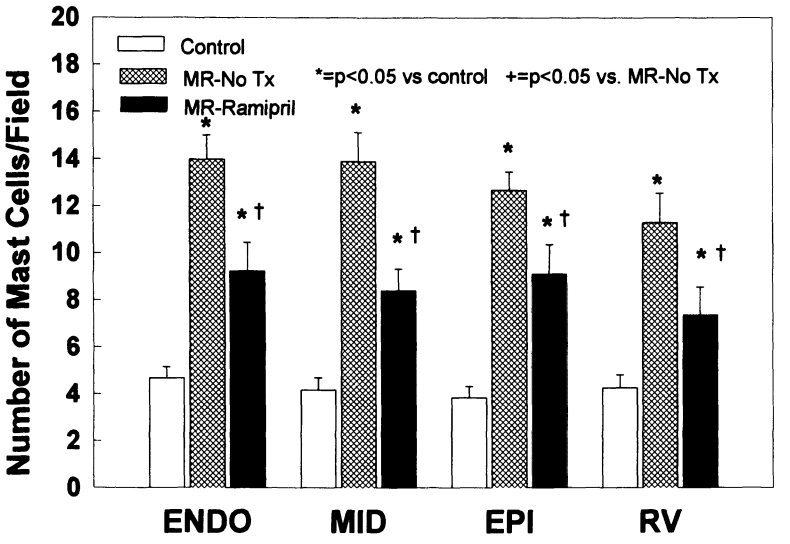


**Figure 4.** Number of mast cells per microscopic field (137,000 $\mu m^2$) for endomyocardial (ENDO), midmyocardial (MID), epimyocardial (EPI), and right ventricular (RV) myocardium for Control, non-treated mitral regurgitation (MR) and Rampiril treated MR dogs. * = $p < 0.01$ vs. normal; † = $p < 0.05$ compared to MR non-treated.

**Table 2.** Effect of ramipril treatmemt on myocyte dimensions

| | Cell Length (isolated myocytes) ($\mu$) | | Cross sectional area (CC volume/cell length) ($\mu^2$) | | Cross sectional area (digitizer) ($\mu^2$) | |
|---|---|---|---|---|---|---|
| | endo | epi | endo | epi | endo | epi |
| N-MR (n = 11) | 177 ± 10* | 180 ± 11* | 302 ± 11 | 283 ± 13 | 295 ± 19 | 244 ± 10 |
| R-MR (n = 7) | 203 ± 6* | 198 ± 4* | 318 ± 17 | 273 ± 11 | 306 ± 11 | 272 ± 13 |
| Normals (n = 7) | 144 ± 4 | 145 ± 4 | 344 ± 23 | 308 ± 22 | 271 ± 16 | 252 ± 15 |

Note: Data are presented as mean ± SE. N-MR = mitral regurgitation untreated, R-MR = MR ramipril treated, CC = Coulter Counter. * = $p < 0.05$ vs. Normals.

$AT_1$ receptor mRNA to GAPDH mRNA were significantly decreased in MR dogs compared to CON (0.28 ± 0.05 vs. 0.97 ± 0.09, $p < 0.001$). After treatment with ramipril, $AT_1$ receptor gene expression increased significantly in R-MR compared to CON (1.66 ± 0.07 vs. 0.97 ± 0.09, $p < 0.01$).

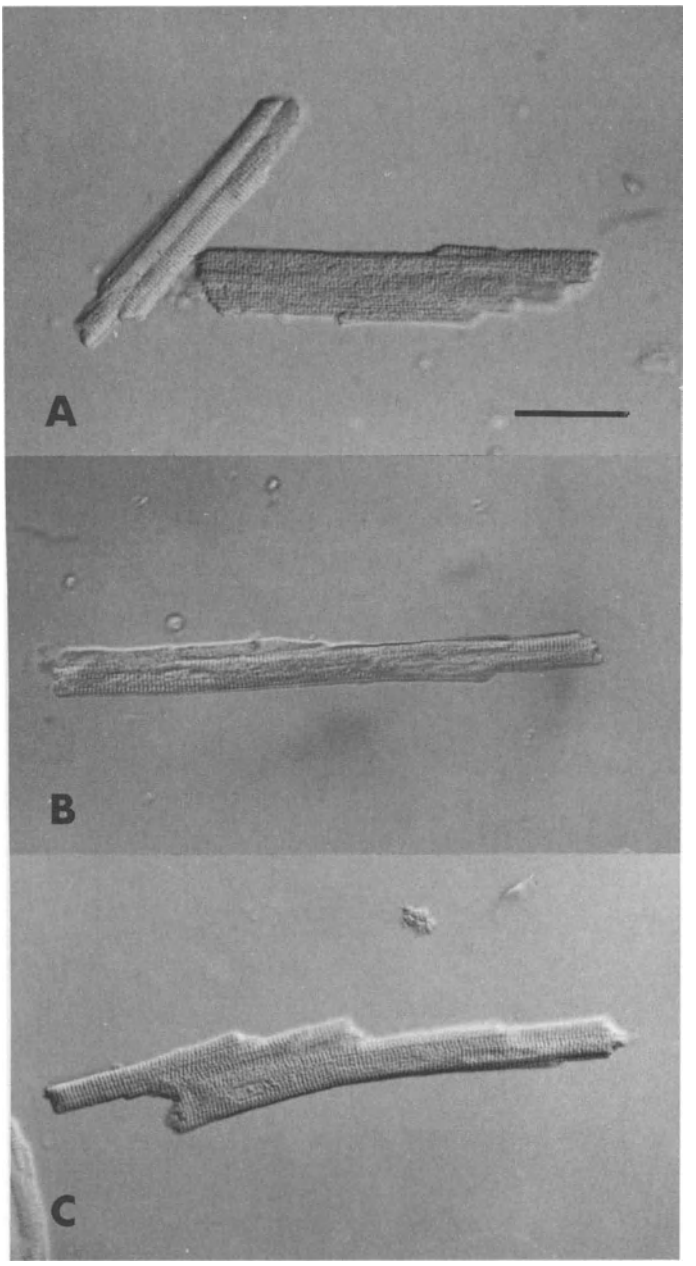


**Figure 5.** Isolated myocytes from a Normal dog (A), a nontreated dog with MR (B), and a Ramipril treated dog with MR (C). There is increased cell length in both groups of MR dogs compared to control. Bar in A = 50 μm. Magnification is the same in A, B, and C. (Reproduced from [33] with permission).

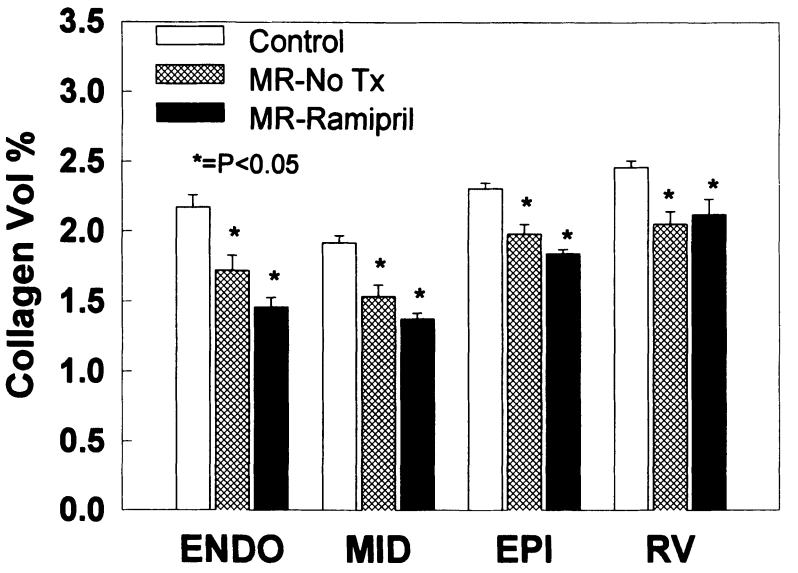


**Figure 6.** Quantitative evaluation of myocardial interstitial collagen measured by image analysis microscopy at high power using pricrosirius red-stained tissue sections for endomyocardial (ENDO), midmyocardial (MID), epimyocardial (EPI), and right ventricular (RV) myocardium for Control, non-treated mitral regurgitation, (MR) and Ramipril treated MR dogs. * = $p < 0.05$ compared to Normal.

### ISOLATED MYOCYTE MORPHOLOGY

The length of isolated myocytes was significantly greater in MR dogs than in CON and tended to be further increased in length in the R-MR dogs compared to N-MR dogs, although the difference was not statistically significant (table 2, figure 5). Myocyte cross-sectional area was similar, whether calculated from the Coulter Counter cell volume and mean cell length measured by microscopy or by planimetry by direct digitizer measurement of myocyte cross-sectional area in silver-stained methacrylate sections (table 2). Cross-sectional area in the endocardial and epicardial portions of the LV did not differ among the three groups.

### MYOCARDIAL COLLAGEN

Qualitative light microscopic examination of the myocardium revealed no discernible differences in the extent of fibrosis among the three groups. Low power quantitative evaluation of the LV and RV myocardium also revealed no differences in focal fibrosis or perivascular collagen among the three groups (CON LV = 1.51 ± 0.07 vol %, N-MR LV = 1.48 ± 0.06, R-MR LV = 1.40 ± 0.07; CON RV = 1.87 ± 0.09, N-MR RV = 1.71 ± 0.03, R-MR RV = 1.68 ± 0.07). However, interstitial connective tissue, measured at high magnification, was significantly reduced in both the R-MR and N-MR groups in all LV regions compared

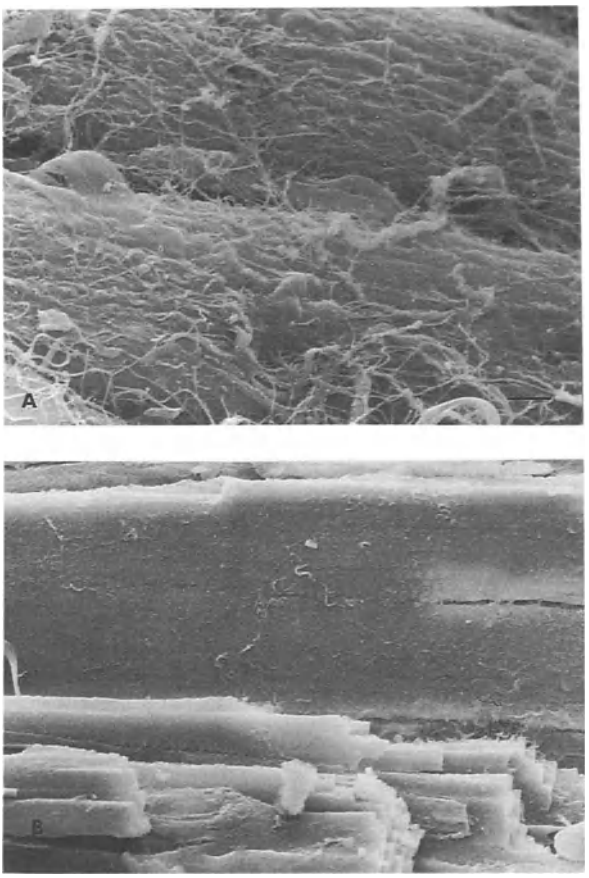


**Figure 7.** Scanning electron micrographs of a normal dog (A) and a dog with chronic mitral regurgitation (B). The normal collagen weave present in the normal dog is focally absent in both nontreated and treated dogs with MR. Bar in A = 5μm; magnification is the same in A and B.

to CON dog hearts (figure 6). In the RV, interstitial collagen also was mildly reduced in both MR groups compared to CON.

Scanning electron microscopy demonstrated a significant loss of the endomysial collagen weave in both the endocardial and epicardial regions of the LV of both R-MR and N-MR dogs compared to CON (figure 7). As analyzed by the semiquantitative grading system, the collagen weave was reduced or absent (grades 2 or 1) in many regions of the N-MR dogs, as demonstrated by the shift to the left of the peak values in the histogram (figure 8). LV subepicardial tissue had a similar shift to the left as in the subendocardium, but there was no significant difference among groups in the RV. Compared to CON, mean collagen grade was signifi-

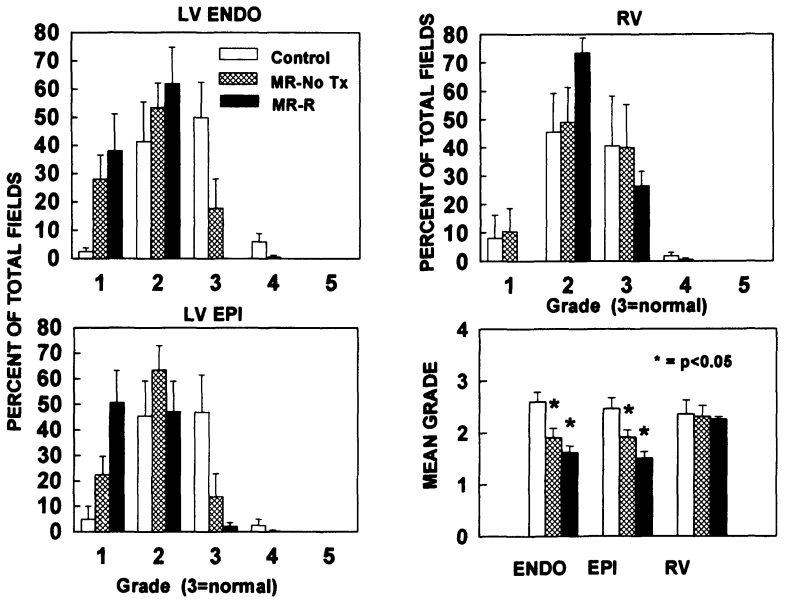


**Figure 8.** Histograms of percent of total fields examined having subjectively graded amounts of collagen as described in the text. Grade 3 = normal amount of collagen. In the left ventricular subendocardium (LV ENDO) or subepicardium (LV EPI), there is a shift to the left of grades, due to a higher percentage of fields with little or no collagen, while in the right ventricle, (RV) there is no shift in distribution of collagen weave grades. The mean grade for LV regions is reduced in both groups of MR dogs, but is not changed in the RV.

cantly reduced in LV subendocardium and subepicardium in both MR groups, but not different in the RV (figure 8).

### TRANSMISSION ELECTRON MICROSCOPY OF CARDIAC MYOCYTES

Compared to control myocardium, the myocytes of MR dogs were characterized by frequent disruption of the myofibrillar band pattern with distorted and thickened Z-bands, resulting in misalignment of sarcomeres (figure 9). This malalignment of sarcomeres was most prominent in those animals with the most severe signs of congestive heart failure and was much less common in the R-MR group of dogs, though not completely absent. The Z-band abnormalities and sarcomere loss of alignment illustrated in figure 9 were not found in the control animals.

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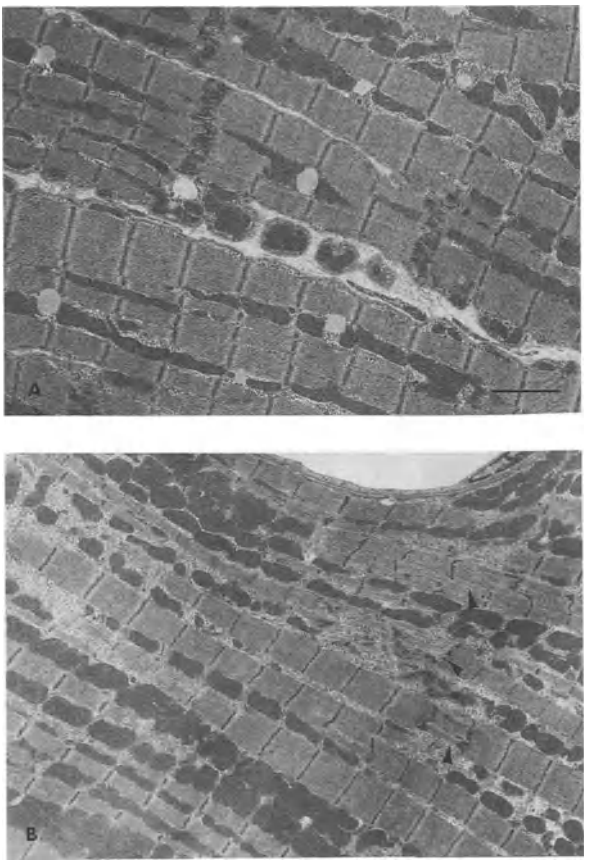


**Figure 9.** Transmission electron micrographs of Normal dog myocardium (A) and myocardium of a dog 5 months after surgical production of MR (B). In the MR dog, there is loss of sarcomere alignment and distortion of the Z-bands (arrowheads) compared to control. Bar in A = 2μm; magnification the same in A and B.

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# THE SITE OF ANGIOTENSIN GENERATION: FOCUS ON THE HEART

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***Summary.*** All renin-angiotensin system (RAS) components are present in cardiac tissue, and both angiotensin (Ang) I and II appear to be produced in the heart, either in cardiac interstitial fluid, on the surface of cardiac cells, or within cardiac cells. The renin responsible for this local angiotensin production, at least under normal circumstances, originates from the circulation and is therefore kidney-derived. Thus, a local RAS in the sense that the RAS components necessary for Ang II production are synthesized in situ, does not exist in the normal heart. Although cardiac angiotensin production depends on renin from the kidney, there are still many ways by which the heart may regulate its own Ang II production. Membrane binding could be a mechanism by which kidney-derived renin is sequestered in the heart. A renin receptor might be involved in this process. The density of such receptors may vary, and this could modify the tissue production of Ang I and II. Local concentrations of angiotensin-converting enzyme (ACE) may also vary. The ACE levels in human heart are, in part, determined by the insertion/deletion ACE gene polymorphism. Also, enzymatic degradation of Ang II and $AT_1$ receptor-mediated endocytosis could influence the Ang II concentrations at the cellular and subcellular level. Finally, under pathological conditions, renin may be produced in the heart itself, and this would create the possibility to synthesize Ang II at cardiac tissue sites independent of the kidney.

The RAS has traditionally been viewed as a circulating system. Kidney-derived renin cleaves liver-derived angiotensinogen in the circulation to form Ang I. Subsequently, Ang II is generated from Ang I by angiotensin-converting enzyme (ACE) located at the luminal side of the endothelium or by ACE in blood plasma. According to this classical concept, the RAS is a hormonal system, designed to

deliver the effector peptide Ang II to different tissue sites, where it exerts its effects via stimulation of Ang II receptors. Two types of Ang II receptors have been described so far, $AT_1$ and $AT_2$ [1].

Ang II plays an important role in the cardiovascular homeostasis. Not only is it a potent vasoconstrictor, it also stimulates renal sodium reabsorption and adrenal aldosterone production and release. Furthermore, it is believed to act as a growth factor [2]. All these effects are mediated via the $AT_1$ receptor. The role of the $AT_2$ receptor is less clear; it may oppose some of the $AT_1$ receptor-mediated effects [3–5]. The proportion of $AT_1$ and $AT_2$ receptors changes during development. In fetal tissues, $AT_2$ receptor expression is dominant, but this abundance is followed by a rapid decline immediately after birth [6].

In light of the vasoconstrictor and growth-stimulating effects of Ang II, it is not surprising that RAS blockade is beneficial in cardiovascular diseases. Renin inhibitors, although at present not further developed because of their poor oral bioavailability, reduce blood pressure effectively [7–9]. ACE inhibitors, the class of RAS inhibitors that was developed first, are effective blood pressure lowering agents and cause regression of cardiac hypertrophy and postinfarction remodeling in subjects with heart failure [10,11]. This also appears to apply to $AT_1$ receptor antagonists, the most recently developed class of RAS inhibitors [12–16].

The effects of ACE inhibitors on cardiac hypertrophy were shown to be independent, at least partly, from the blood pressure lowering effect of these drugs [10,17]. This may indicate that ACE inhibitors have local effects in the heart, in addition to their blood pressure lowering effects. Indeed, the existence of a so-called local RAS, as opposed to the circulating RAS, has been proposed in the heart [18]. In fact, such local renin-angiotensin systems may exist in many organs. RAS components and their messenger RNAs have now been identified in kidney, adrenal, brain, ovary, testis, eye, and heart [18–26]. Many investigators have speculated on the role and regulation of these local renin-angiotensin systems. So far it has been difficult to clearly separate the circulating and the local RAS. It is even possible that the circulating RAS serves to deliver renin and angiotensinogen to tissue sites, where local angiotensin production may then occur.

The cardiac RAS is believed to play an important role in cardiac growth and remodeling [18]. A better understanding of how and where angiotensins are produced in the heart may shed further light on the mechanism by which RAS inhibitors exert their effects in the heart. This review addresses angiotensin generation in the circulation and at cardiac tissue sites.

## CIRCULATING RENIN-ANGIOTENSIN SYSTEM

### Renin

The kidney releases both renin and its inactive precursor, prorenin, into the circulation. Renal renin release is influenced by blood pressure, the sodium load of the organism, the activity of the sympathetic nervous system, and a number of humoral or locally generated factors. Ang II inhibits renin release directly via $AT_1$

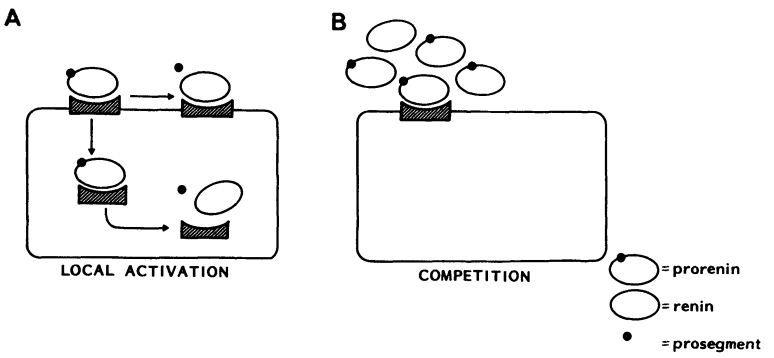


**Figure 1.** Prorenin binding to (pro)renin binding receptors may lead to prorenin activation (A), either extra- or intracellularly, thereby stimulating local angiotensin generation, or, when activation does not occur (B), it may competitively prevent renin from binding, thereby inhibiting local angiotensin generation.

receptors and indirectly via a rise in blood pressure and an increase of the sodium chloride load.

At present, the kidney is the only organ that is known to convert prorenin into renin. Normally, approximately 90% of plasma total renin (renin + prorenin) is present as prorenin. Following a bilateral nephrectomy, plasma renin, but not plasma prorenin, decreases to very low levels [27,28]. This suggests that the kidney is the main or only source of plasma renin, whereas prorenin may be synthesized extrarenally as well. Indeed, prorenin release into the circulation has been demonstrated for several extrarenal organs, e.g., the ovary, testis, and adrenal [29,30].

The function of prorenin remains unclear. Renin receptors, which have been recently described [31,32], bind renin and prorenin equally well [31]. Renin binding at tissue sites may be involved in the cascade leading to local angiotensin generation. Possibly prorenin competitively prevents renin from binding to these receptors, thereby blocking the local formation of Ang II [31] (figure 1). Alternatively, prorenin might be converted to renin at tissue sites following receptor binding. This activation may occur either on the cell surface or intracellularly (figure 1).

Renin appears to be metabolized mainly by the liver [33]. The half-life of circulating renin is approximately 30 min [28]. Renal clearance of renin is very low [34]. Renin has been detected immunohistochemically in the proximal tubule, and low levels can be measured in urine.

### Angiotensinogen

The liver is the main source of circulating angiotensinogen. Although angiotensinogen mRNA has been demonstrated in other organs (e.g., kidney, brain, and heart), [35] it remains uncertain whether extrahepatically produced angiotensinogen contributes to the plasma levels of angiotensinogen.

Angiotensinogen production in the liver is stimulated by estrogens, glucocorticoids, and Ang II [36,37]. Plasma angiotensinogen is cleared mainly by the kidneys via receptor-mediated endocytosis, its elimination half-life being approximately 10 hours [38].

### ACE

ACE has been detected on many cell types, but the conversion of circulating Ang I is probably mainly mediated by endothelial ACE. Plasma ACE appears to be of minor importance [39]. Its levels are partly determined by the so-called insertion/deletion ACE gene polymorphism [40]. This may also apply to tissue (i.e., endothelial) ACE [41].

ACE expression is subject to negative feedback by Ang II [42].

### Angiotensin I and II

Both Ang I and II are rapidly degraded by angiotensinases located in the vessel wall, their half-lives in the circulation being less than one minute. In early studies in sheep [43], Ang I and II clearance across the pulmonary and combined systemic vascular beds was calculated from the arteriovenous differences measured across these beds during infusion of high doses of Ang I or II into the pulmonary artery. These studies already indicated that the reaction of circulating renin with circulating angiotensinogen ("plasma renin activity", PRA) was insufficient to make up for the rapid degradation of both angiotensins.

More recently, the regional clearance and metabolism of Ang I and Ang II have been quantified, both in humans [44,45] and in pigs [39,46], by giving constant infusions of $^{125}$I-Ang I or $^{125}$I-Ang II. In humans, the infusions were given through an antecubital vein, and in pigs, into the left cardiac ventricle. Blood was obtained from various arterial and venous sampling sites for measurements of radiolabeled Ang I and II. Additional measurements of the levels of endogenous Ang I and II and measurements of PRA at physiological pH made it possible to estimate how much of the venous Ang I could be attributed to arterial delivery, how much could be attributed to de novo synthesis, and what proportion of de novo-synthesized Ang I depends on the action of circulating renin on circulating angiotensinogen. It was also possible to calculate how much of the venous Ang II originated from arterial delivery and how much originated from conversion of arterially delivered Ang I.

It was found that, in all vascular beds studied [44,46] (heart, kidney, limbs, liver, head, skin), a major proportion of venous Ang I originated from de novo production and that PRA contributed little to this production. One can therefore conclude that Ang I is produced at tissue sites and that part of it is released into the circulation. While most of venous Ang I appears not to be generated by the action of circulating renin on circulating angiotensinogen, the level of venous Ang I produced at tissue sites correlated strongly with the level of PRA [46]. This suggests that kidney-derived renin is responsible for the production of Ang I at tissue sites.

For Ang II, the situation is different. Most, if not all, venous Ang II appears to originate from Ang II delivered by the artery and from Ang II generated by conversion of arterially delivered Ang I [45]. Thus, Ang I produced at tissue sites and released into the circulation may have escaped conversion to Ang II. It is possible that Ang I produced in the tissue enters the blood at a level distal to the site where arterially delivered Ang I is converted to Ang II by the vascular endothelium, so that this conversion site is bypassed. Ang I formed at tissue sites may enter the circulation at the level of the capillaries or venules, whereas conversion of Ang I to Ang II occurs at the level of the arterioles. Alternatively, Ang II is produced in the tissue from locally generated Ang I without subsequent release into the circulation.

### Effect of RAS inhibitors on circulating RAS components

Inhibition of the RAS always results in the onset of feedback processes (table 1). Remikiren inhibits plasma renin activity, thereby leading to reduced plasma Ang I and Ang II concentrations [7]. Consequently, renin release from the kidney will increase, resulting in elevated immunoreactive renin levels [7,9]. However, because of the presence of the renin inhibitor, this renin is enzymatically inactive. ACE inhibitors also cause a rise in plasma renin and, as a result, a rise in plasma Ang I. Plasma Ang II is reduced initially, but it may rise to levels above normal during chronic treatment as a result of the increased renin and Ang I concentrations [47]. $AT_1$ receptor antagonists will not only increase plasma renin and Ang I but plasma Ang II as well [13,14].

## CARDIAC RENIN-ANGIOTENSIN SYSTEM

### Renin

Renin mRNA concentrations in normal hearts are very low or undetectable [48,49], which suggests that under normal conditions cardiac renin synthesis is unlikely to occur. It is possible, however, that during foetal development [50] or under pathological conditions [51], the renin gene is expressed in the heart. Cultured cardiac cells (cardiomyocytes, fibroblasts, vascular smooth muscle cells, and endothelial cells) have all been reported to synthesize renin [50,52,53]. However, the relevance of such findings for the in vivo situation may be questioned. Moreover, since appropriate control measurements with specific renin inhibitors were usually not performed, it is possible that renin-like enzymes such as cathepsin D [54], and not renin itself, were detected. Renin has been measured in the heart of normal and nephrectomized pigs [55]. Ang I-generating activity of cardiac tissue was identified as renin by its inhibition by a specific active site-directed renin inhibitor. The levels of renin in cardiac tissue (expressed per g wet weight) were found to be similar to those in plasma. The tissue levels of renin, therefore, could not be accounted for by trapped plasma, which suggests that renin may have been sequestered actively by the heart. Both in cardiac tissue and in plasma, renin fell to undetectable levels after nephrectomy, which suggests that under normal circumstances, most, if not all, renin present in the heart originates from the kidney.

Not much is known about cardiac renin sequestration. Renin, like ACE, was found to be enriched in a purified cardiac membrane fraction prepared from left ventricular tissue, which suggests that uptake of kidney-derived renin in the heart may occur through binding to cell membranes [55]. This would fit with the recently described renin receptors [31,32]. Binding to cell membranes is also suggested by chemical cross-linking studies using membrane fractions prepared from various tissues, including heart and blood vessels [56]. The possibility that prorenin, following its binding to these receptors, is locally activated cannot be excluded. However, such local activation has never been demonstrated.

**Angiotensinogen**

Angiotensinogen mRNA can be detected in the heart [22,57]. Its levels are approximately 1% of the angiotensinogen mRNA levels in the liver [22]. An increase in cardiac angiotensinogen mRNA has been reported following myocardial infarction [57], but this could not be confirmed by others [51]. Cultured neonatal rat cardiomyocytes and fibroblasts appeared to release angiotensinogen into the medium under serum-free conditions [50]. Other cells in the heart that have been reported to synthesize angiotensinogen are vascular smooth muscle cells [58] and endothelial cells [59].

The angiotensinogen concentrations in porcine cardiac tissue are 10–25% of the levels in plasma, which is compatible with its diffusion from plasma into the interstitium [55]. Angiotensinogen is generally believed to be distributed equally across the extracellular fluid [60]. It appears, therefore, that cardiac angiotensinogen is largely derived from the circulation. The contribution of locally synthesized angiotensinogen, if present at all, is probably small. In support of this assumption, the angiotensinogen concentrations in the coronary effluent of the isolated perfused rat Langendorff heart are $<0.1\%$ of the levels normally found in rat blood plasma [61].

**ACE**

ACE has been demonstrated in the rat heart by autoradiography, using a radiolabeled ACE inhibitor [62], as well as by measurements of its activity in cardiac homogenates [63]. ACE mRNA is readily detectable in cardiac tissue [63]. According to some investigators, cardiac ACE is normally limited to the coronary vascular endothelial cells and the endocardium [64]. Cardiac ACE protein and mRNA are increased following myocardial infarction [64–66] as well as during pressure overload induced left ventricular hypertrophy [63]. Under these conditions the localization of ACE may no longer be limited to the endothelium. In humans, following myocardial infarction, ACE can be detected in the remaining viable cardiomyocytes near the infarct scar of the aneurysmal left ventricle, as well as in fibroblasts, vascular smooth muscle cells, and macrophages in the scar area itself [65]. In rats, following coronary occlusion, ACE was demonstrated in fibroblasts in the healthy hypertrophying part of the heart [66].

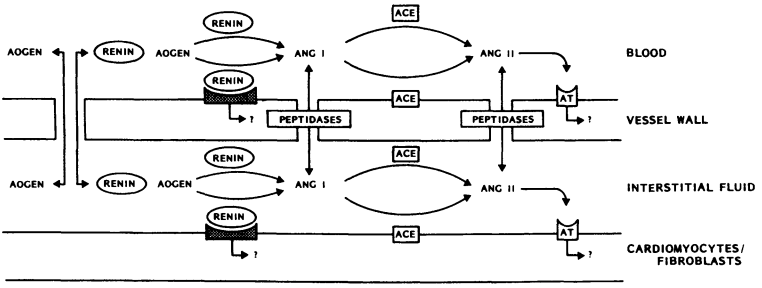


**Figure 2.** Origin of cardiac angiotensin I and II. See text (under Cardiac renin-angiotensin system, Angiotensin I and II) for a detailed description. Not addressed in the figure are (1) intracellular angiotensin generation by locally synthesized renin or renin taken up from the circulation (depicted by a question mark) and (2) how AT receptor-mediated endocytosis (also depicted by a question mark) might affect the intracellular Ang II levels.

## Angiotensin I and II

The concentration of Ang I in cardiac tissue (expressed per g wet weight) is similar to the level in plasma, whereas the cardiac tissue concentration of Ang II is two to three times the level in plasma [55,67]. The cardiac angiotensin levels, therefore, are far too high to be explained by trapped blood or by simple diffusion of angiotensins from plasma into the interstitial fluid. However, such relatively high concentrations cannot be taken as unequivocal evidence that Ang I and II are generated in cardiac tissue independently of the circulating RAS. For example, Ang II may have been sequestered from the circulation by a receptor-dependent process.

As mentioned above, measurements of circulating angiotensins during $^{125}$I-AngI infusion into the left cardiac ventricle provided evidence for the release of Ang I from cardiac tissue sites. These measurements of circulating angiotensins provided no evidence for the release of tissue Ang II into the circulation. Thus, taken together, the evidence available so far suggests that Ang I and possibly Ang II are generated in cardiac tissue, and that of these locally generated angiotensins, only Ang I is released into the circulation, Ang I and II may be formed in different tissue compartments that are not all capable of exchanging freely with the circulation. Levels of Ang I and II were undetectably low in both plasma and cardiac tissue following a bilateral nephrectomy [55]. This is a strong indication that cardiac angiotensin generation in vivo depends on renin from the kidney.

A scheme depicting the possible angiotensin production sites is shown in figure 2. Angiotensin production may either occur in extracellular fluid (blood plasma and/or interstitial fluid) or at the tissue-fluid interphase (i.e., on the cell membrane). A third possibility, not shown in figure 2, is intracellular production of angiotensin by renin that is locally synthesized or taken up from the circulation. Future investigation will have to address (1) what proportion of angiotensinogen cleavage occurs by intracellular renin, membrane-associated renin, or renin in the fluid phase and (2) the exact localization of Ang I and II in cardiac tissue (intracellular, extracellular, or

**Table 1.** Effect of renin inhibition, ACE inhibition, and $AT_1$ receptor blockade on renin–angiotensin system component levels in blood plasma and heart

| | Immunoreactive renin | Enzymatically active renin | Ang I | Ang II | Ang II/I ratio |
|---|---|---|---|---|---|
| *Plasma* | | | | | |
| Renin inhibition | ↑ | ↓ | ↓ | ↓ | N.D. |
| ACE inhibition | ↑ | ↑ | ↑ | ↓ | ↓ |
| $AT_1$ receptor blockade | ↑ | ↑ | ↑ | ↑ | = |
| *Heart* | | | | | |
| Renin inhibition | N.D. | N.D. | N.D. | N.D. | N.D. |
| ACE inhibition | ↑ | ↑ | ↑ | =↓ | ↓ |
| $AT_1$ receptor blockade | N.D. | N.D. | ↑ | ↑ | ↓ |

Source: Data are taken from references 7, 9, 13, 14, 68, 69, 70 and 71.
Note: N.D., not done.

membrane-bound). Extracellularly formed Ang II may, via receptor-mediated endocytosis, reach the intracellular compartment.

### Effect of RAS inhibitors on cardiac RAS levels

At present, not much is known about changes in cardiac RAS component levels during treatment with RAS inhibitors (table 1). Renin is elevated in cardiac tissue of both humans [68] and pigs [69] treated with ACE inhibitors, whereas cardiac angiotensinogen is decreased under these conditions [68,69]. ACE inhibition with perindopril led to a two- to fourfold increase in cardiac Ang I [70]. Cardiac Ang II did not change unless very high doses of quinalapril were applied. The $AT_1$ receptor antagonist losartan increased cardiac Ang I and II approximately sevenfold and twofold, respectively [71]. As a consequence of these nonparallel changes in cardiac Ang I and II, the cardiac AngII/I ratio decreased both with quinalapril and losartan. The decrease in cardiac AngII/I ratio during quinalapril treatment most likely illustrates the degree of ACE inhibition obtained in cardiac tissue. The decrease in cardiac AngII/I ratio during losartan treatment is more difficult to explain, especially since cardiac ACE activity was unchanged following losartan treatment [71]. The authors speculated that a proportion of the measured tissue level of Ang II may have been receptor-bound and protected from metabolism and that the displacement of receptor-bound Ang II by losartan may have accelerated local tissue metabolism of Ang II, with a consequent decrease in the AngII/I ratio.

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# ANGIOTENSIN II-MEDIATED STAT SIGNAL TRANSDUCTION: STUDIES IN NEONATAL RAT CARDIAC FIBROBLASTS AND CHO-K1 CELLS EXPRESSING $AT_{1A}$ RECEPTORS

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***Summary.*** The Signal Transducers and Activators of Transcription (STAT) proteins are a novel class of transcription factors activated by cytokines (Interleukin-6, IL-6) and growth factors (platelet-derived growth factor, PDGF). In cultured neonatal rat cardiac fibroblasts and in CHO-K1 cells expressing angiotensin (Ang) II type 1 receptors ($AT_{1A}$), called T3CHO/$AT_{1A}$ calls, Ang II stimulated predominantly the tyrosine phosphorylation of STAT3. In these cells, Ang II also tyrosine-phosphorylated STAT1, but tyrosine phosphorylation of STAT1 was significantly lower compared with that of STAT3. Angiotensin II-mediated activation of STAT3 compared with the rapid activation by the cytokine IL-6 was delayed (maximal 2 h). However, like cytokines, Ang II rapidly induced serine phosphorylation of STAT3. Using T3CHO/$AT_{1A}$ cells, we examined the potential reasons for the delayed tyrosine phosphorylation of STAT3 by Ang II. We tested the possibility that the delayed tyrosine phosphorylation of STAT3 was due to the induction of an inhibitory pathway, prior to stimulation. A short pretreatment of cells with Ang II transiently inhibited the rapid STAT3 tyrosine phosphorylation by IL-6, and this inhibition could be blocked by pre-exposing the cells with the $AT_1$ antagonist EXP3174. PD98059, a specific inhibitor of MAPK kinase 1, attenuated the inhibitory effects of Ang II. These results suggest that the inhibition by Ang II is a MAPK kinase 1-dependent process. The ability of Ang II to cross-talk with IL-6 signaling suggests a modulatory role for Ang II in cytokine-induced cellular responses.

## INTRODUCTION

Angiotensin (Ang) II is the biologically active component of the renin-angiotensin system. It mediates a variety of physiological responses including volume and fluid homeostasis, aldosterone production, and vascular smooth muscle contraction [1,2].

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Studies from our laboratory and others have shown that Ang II also acts as a growth-promoting factor in cardiac fibroblasts, cardiac myocytes, and vascular smooth muscle cells [3–7]. These effects of Ang II are mediated by specific receptors on the surface of its target cells. Two pharmacologically distinct subtypes of Ang II receptors have been defined by radiolabeled receptor binding studies using the nonpeptide antagonists Dup753 and PD123177 [8]. Dup753 has a greater affinity for type 1 receptor ($AT_1$), whereas PD123177 has a greater affinity for the type 2 receptor ($AT_2$). Additional subtypes exist in the $AT_1$ family; cDNA, representing $AT_1$ and its subtypes, and $AT_2$ have been cloned [9–12].

The $AT_1$, through which Ang II mediates most of its biological actions, is a G protein-coupled, seven transmembrane receptor [9,10]. Binding of Ang II to $AT_1$ stimulates several signal transduction pathways. These include stimulated increases in intracellular $Ca^{2+}$ and stimulation of phospholipase C, phospholipase $A_2$, phospholipase D, Raf-1 kinase, protein kinase C (PKC) and mitogen-activated protein kinases (MAPKs) [13–18]. Angiotensin II was also shown to activate the Janus family of tyrosine kinases (JAK kinases) and the Signal Transducers and Activators of transcription (STAT) family of transcription factors [19–22]. The STAT pathway is initiated by the binding of growth factors and cytokines to their cognate cell surface receptors [23]. This event results in the activation of cytoplasmic JAK kinases, which then tyrosine-phosphorylate STAT transcription factors in the cytoplasm. Upon tyrosine phosphorylation, STATs are activated and dimerize and translocate to the nucleus to induce gene transcription. Six STAT family transcription factors have been cloned and are designated STAT1 to STAT6, in the order of their discovery. Four JAK kinases have been identified, and they are as follows: JAK1, JAK2, TYK2, and JAK3 [24]. Recent evidence suggests that growth factors such as epidermal growth factor (EGF) can tyrosine-phosphorylate STAT proteins through a JAK kinase independent route [25,26]. STAT proteins regulate the expression of many genes, one of which is the proto-oncogene c-fos. The promoter of c-fos contains the regulatory element SIE, which is responsive to activated STAT proteins. The binding of STAT1 and STAT3 to the SIE results in the formation of DNA protein complexes, referred to as SIF. SIF appears in three different forms, namely, SIF-A, SIF-B, and SIF-C [23], and induction occurs in a ligand specific fashion. PDGF induces all three forms, IL-6-induces mainly SIF-A, and interferon γ (IFNγ) induces mainly SIF-C [27]. SIF-A contains homo-dimerized STAT3, SIF-B contains hetero-dimerized STAT1 and STAT3, and SIF-C contains homo-dimerized STAT1 [23]. Thus, by measuring the SIF complex formation, it is possible to determine whether a given ligand activates the STAT signal transduction pathway. This chapter discusses Ang II-induced activation of STAT signal transduction in neonatal rat cardiac fibroblasts and CHO-K1 cells expressing $AT_1$ receptors.

## RESULTS

### SIF induction by Ang II

Incubation of nuclear extracts from neonatal rat cardiac fibroblasts and T3CHO/$AT_{1A}$ cells resulted in the formation of SIF complexes (A, B, and C) [19]. Among

these, SIF-A was the predominant type in response to Ang II. Interestingly, the induction of SIF-A by Ang II was delayed, with initial stimulation occurring at 30 min and maximal stimulation occurring at two to three hours. The delayed SIF induction by Ang II was in contrast to the published reports of rapid induction by cytokines and growth factors [23,27]. We considered the possibility that Ang II stimulation caused the release of SIF-inducing growth factors/cytokines, which in turn was responsible for the delayed SIF-A induction by Ang II. To determine this, the conditioned medium from T3CHO/$AT_{1A}$ cells (treated with Ang II for 2 h) was tested for its ability to induce SIF activity at early (30 min) and delayed time points (2 h). We observed that conditioned media induced significant levels of SIF-A activity only at 2 h, but not at 30 min. This induction was completely blocked by pre-exposing the cells to the $AT_1$ receptor antagonist EXP 3174, which suggests that Ang II acts directly through the $AT_1$ receptor to induce SIF activity. These data demonstrate that the delayed induction is not due to a requirement for the release of other SIF-inducing factors.

**Detection of STAT3 in Ang II and IL-6-induced SIF-A**

To determine if T3CHO/$AT_{1A}$ cells respond normally to IL-6, we treated the cells with IL-6 for zero to two hours; nuclear extracts were prepared and tested for their ability to form SIF complexes. We observed that IL-6 predominantly induced SIF-A, consistent with previously published reports [28]. More importantly, unlike the delayed activation of SIF-A by Ang II, induction by IL-6 was rapid with maximal stimulation detected as early as 10 min [20]. In supershift assays, anti-Stat3 antibodies recognized both Ang II and IL-6-induced SIF-A complexes, which suggests that Stat3 was a common component [20]. Thus, although Ang II and IL-6 induced distinct kinetics of induction of SIF-A, both contained Stat3 as a constituent protein.

**Mechanism of delayed SIF-A/STAT3 induction by Ang II**

We hypothesized that the delayed stimulation of SIF-A by Ang II was due to the induction of an inhibitory pathway (0–30 min) prior to stimulation (1–2 h). To test this possibility, we determined whether a short pretreatment of the cells with Ang II would inhibit the rapid activation of SIF-A by IL-6. We observed that a 15 min pretreatment of cells with Ang II inhibited the rapid induction of SIF-A and Stat3 tyrosine phosphorylation by IL-6 [20]. The inhibition by Ang II was dose-dependent, and did not involve the degradation of Stat3 protein [21]. The inhibitory effect was completely blocked by pre-exposing the cells with EXP3174. Importantly, the inhibition was transient since at latter time points (2 h), the IL-6-induced SIF-A response reappeared [20]. These results raise the possibility that the delayed induction of STAT3 tyrosine phosphorylation/SIF-A response by Ang II may involve an inhibitory phase, prior to stimulation.

To determine if Ang II interfered with IL-6-induced Stat3 (92 kDa) tyrosine phosphorylation, we made nuclear extracts from untreated T3CHO/$AT_{1A}$ cells, T3CHO/$AT_{1A}$ cells treated with Ang II (25 min and 2 h) or IL-6 (10 min), or T3CHO/$AT_{1A}$ cells treated first with Ang II for 15 min and then with IL-6 for

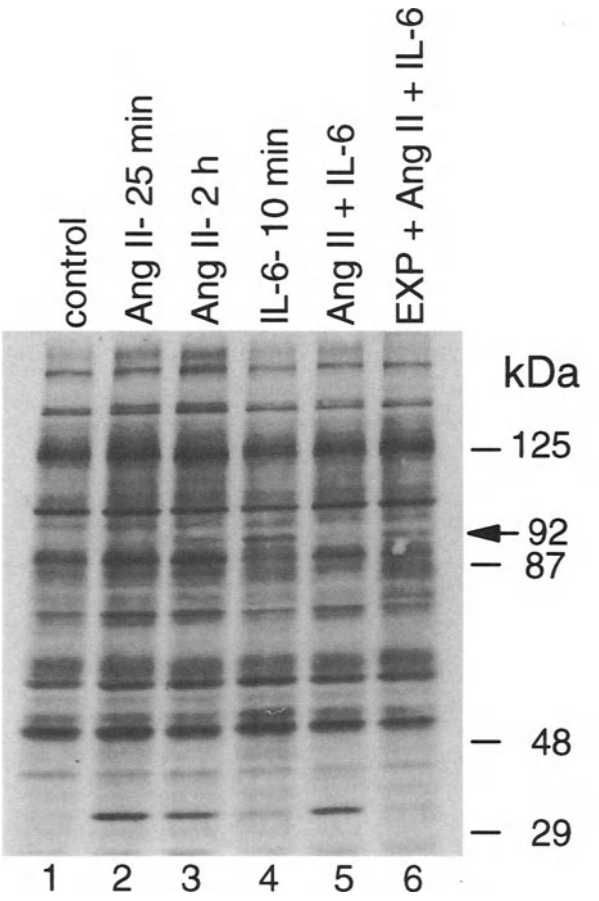


**Figure 1.** Effect of Ang II pretreatment on IL-6-induced nuclear protein tyrosine phosphorylation: T3CHO/$AT_{1A}$ cells were treated with Ang II alone (100 nM) for 25 min (lane 2) and 2 h (lane 3) and with IL-6 alone (10 ng/ml) for 10 min (lane 4). Alternatively, cells were treated with Ang II (100 nM) for 15 min followed by IL-6 (10 ng/ml) for 10 min (lane 5) or pretreated with EXP3174 ($1 \times 10^5$ M) for 30 min and then sequentially with Ang II (100 nM) for 10 min and IL-6 (10 ng/ml) for 10 min (lane 6). Nuclear extracts were prepared as previously described [20]; 10 μg of the extract was loaded onto a 8% SDS-polyacrylamide gel, transferred to nitrocellulose, and hybridized with antiphosphotyrosine antibodies. Tyrosine-phosphorylated bands were detected as previously described [20] using enhanced chemiluminescence (ECL, Amersham). The position of the 92 kDa protein is indicated by an arrow.

10 min. Nuclear extracts from these cells were analyzed in a Western blot by probing with antiphosphotyrosine antibodies. Figure 1 demonstrates that Ang II induces the tyrosine phosphorylation of a 92 kDa protein at two hours (lane 3), but not a 25 min (lane 2). IL-6 rapidly induced the tyrosine phosphorylation of this 92 kDa protein at 10 min (lane 4). In nuclear extracts from Ang II +IL-6 treated

cells, there was a marked decrease in the tyrosine phosphorylation of this protein (lane 5). If the cells were exposed to $AT_1$ receptor antagonist EXP3174, treatment with Ang II did not effect the IL-6-induced tyrosine phosphorylation (lane 6). Immunoprecipitation of these samples with anti-STAT3 antibody and subsequent phosphotyrosine blots indicated that this 92kDa protein was STAT3 [20]. Thus, figure 1 demonstrates that Ang II specifically affects the tyrosine phosphorylation status of a 92kDa protein, without significantly affecting others.

Since Ang II rapidly activates PKC [16,29], we determined whether this protein kinase was involved in Ang II-mediated inhibition of IL-6-induced responses. If this hypothesis was correct, pretreatment with the phorbol ester PMA (phorbol 12-myristate, 13-acetate) should inhibit IL-6-induced responses. We observed that pretreatment of cells with PMA for 15–25 min, inhibited the IL-6-induced SIF-A response and Stat3 tyrosine phosphorylation [20,21]. To establish a role for PKC, we treated the cells with PMA to downregulate PKC activity and then tested the ability of PMA and Ang II to inhibit IL-6-induced SIF-A/ Stat3 responses. We observed that in PKC downregulated cells, Ang II inhibited, but PMA failed to inhibit, IL-6-induced STAT3 tyrosine phosphorylation and SIF-A responses [20,21]. These data indicated that Ang II-mediated inhibition occurred independently of PMA-sensitive isoforms of PKC.

**Role of MAPK kinase 1**

Since PMA mimicked the actions of Ang II, we hypothesized that a protein kinase was involved in the Ang II-mediated inhibition of the IL-6-induced STAT3/SIF-A response. We hypothesized that signals elicited by Ang II and PMA converged at the level of MAPK kinase (see figure 2); therefore, inhibiting this step would prevent/attenuate the Ang II-mediated inhibition. To test this hypothesis, we used PD98059, a specific inhibitor of MAPK kinase 1. The use of PD98059 to block MAPK kinase 1 and, therefore, the MAPK pathway activated by various agents (PMA, PDGF, etc.,) has been previously described [30,31]. In T3CHO/$AT_{1A}$ cells, 20 μM PD98059 completely inhibited Ang II (1 nM)-mediated activation of MAPK. In cells pre-exposed to 20 μM PD98059, there was a significant attenuation of Ang II (1 nM)-mediated interference of IL-6-induced STAT3 tyrosine phosphorylation and SIF-A responses [21]. PD98059 also attenuated the PMA-mediated inhibition of the IL-6-induced STAT3/SIF-A response [21]. These results suggested that MAPK kinase 1 or downstream effector was involved in the inhibitory actions of Ang II and PMA.

**Serine phosphorylation of STAT3 by Ang II**

In addition to tyrosine phosphorylation, some STAT proteins also undergo serine phosphorylation. For example, STAT3 undergoes serine phosphorylation in response to cytokines and growth factors [32,33]. Although not proven, MAPKs have been implicated in this process [32,33]. It should be noted that mouse STAT3 contains a MAPK consensus site (PMSP, amino acid 725–728) [32]. Since Ang II is

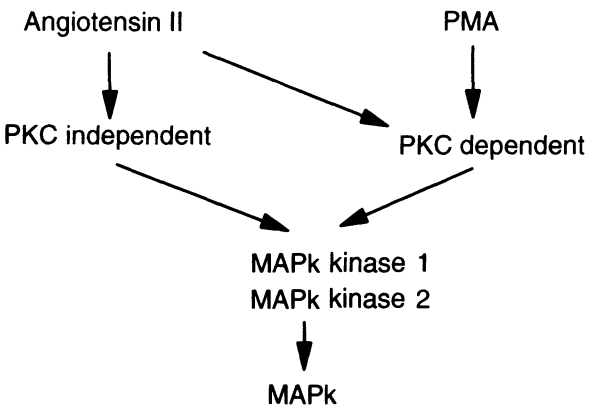


**Figure 2.** Convergence of Ang II- and PMA-induced signaling at the level of MAP kinase kinase: Angiotensin II activates MAPK kinase through both protein kinase C (PKC)-dependent and independent routes, whereas the phorbol ester PMA activates MAPK kinase exclusively through a PKC-dependent route.

a potent activator of the MAPK pathway, we determined whether stimulation of T3CHO/$AT_1$ cells would cause STAT3 serine phosphorylation. Immunoprecipitation of $^{32}$P-labeled STAT3 and subsequent phosphoamino acid analysis indicated that Ang II rapidly caused serine phosphorylation of STAT3 [34]. This was detected as early as 2 min following exposure to Ang II and was sensitive to treatment with PD98059 [34]. These data suggest that the MAPK pathway is involved in Ang II-mediated serine phosphorylation of Stat3.

## DISCUSSION

Using neonatal rat cardiac fibroblasts and T3CHO/$AT_{1A}$ cells, we demonstrated that Ang II induced a delayed (1–2 h) tyrosine phosphorylation of STAT3 and SIF-A formation. Since Ang II also transiently inhibited IL-6-induced STAT3 tyrosine phosphorylation and SIF-A formation, it is likely that the delayed tyrosine phosphorylation of STAT3 by Ang II results from the induction of an inhibitory pathway prior to stimulation. Despite the delayed tyrosine phosphorylation, Ang II rapidly (2 min) induced serine phosphorylation of STAT3. These distinct time phases of phosphorylation by Ang II are notably different from cytokine (IL-6, IFNγ)-induced tyrosine and serine phosphorylation, both of which occur within 10–15 min following agonist exposure. Such differential protein modification by Ang II may modulate the activity of STAT3 to regulate gene expression.

The ability of the MAPK kinase 1 inhibitor, PD98059, to attenuate the Ang II-mediated interference suggests a role for the Ang II-induced MAPK pathway in the negative regulation of STAT3 tyrosine phosphorylation by IL-6. The signaling intermediate responsible for this inhibitory action could be MAPK kinase 1, MAPK, or a downstream effector. This intermediate, in turn, may activate a kinase or

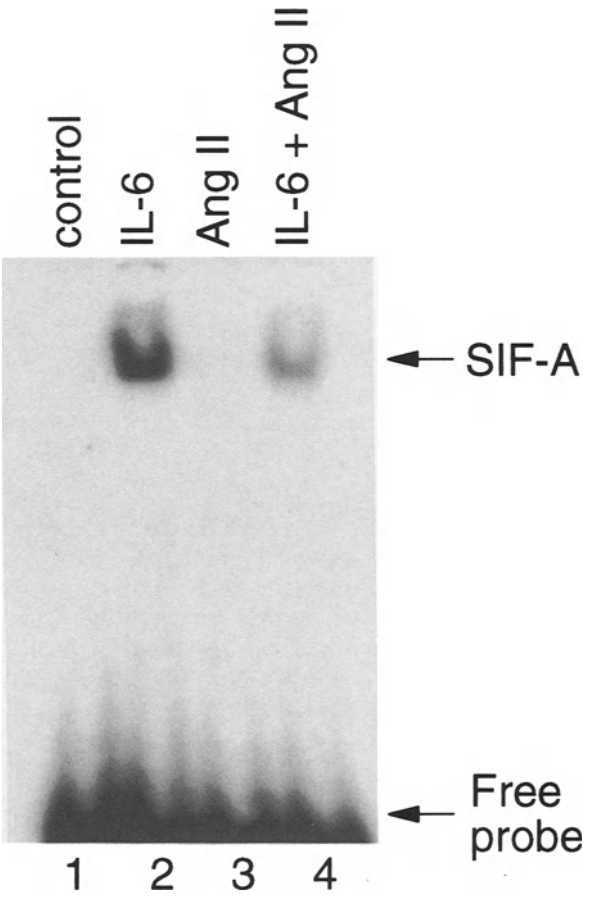


**Figure 3.** Effect of Ang II on the IL-6-induced SIF-A response: T3CHO/$AT_{1A}$ cells were left untreated (lane 1) or were treated with IL-6 (10 ng/ml) for 25 min (lane 2) or Ang II (100 nM) for 15 min (lane 3) or first with IL-6 (10 ng/ml) for 10 min and then with Ang II (100 nM) for 15 min (lane 4). Cells representing lane 4 were exposed to IL-6 for a total of 25 min and Ang II for 15 min. Nuclear extracts were prepared and analyzed by electrophoretic mobility shift assay using $^{32}$P-labeled SIE, as previously described [19, 20].

phosphatase to bring about the inhibitory effects of Ang II. If a kinase is involved, upon its activation by Ang II, it may directly alter the signal-transducing components of IL-6 (e.g., IL-6 receptor, signal transducing protein gp130, STAT3 etc.,) by protein modification. If a phosphatase is involved, it may dephosphorylate tyrosine-phosphorylated STAT3. In support of the role for a tyrosine phosphatase, we observed that the inhibition of SIF-A occurs even if Ang II is added 25 min after stimulation with IL-6 for 10 min (figure 3). We are currently investigating the role of a tyrosine phosphatase in this process.

How Ang II, through the activation of the MAPK pathway, can inhibit the IL-6-induced STAT3/SIF-A response is unclear. Interestingly, MAPK activation by IL-6 is not associated with the inhibition of STAT3 tyrosine phosphorylation. Examination of the MAPK activation profile in gel kinase assays indicated that the profiles are significantly different between Ang II and IL-6. For Ang II, it was rapid, maximal being 2 min (eightfold), and sustained [21]. For IL-6, it was slower, maximal being 10 min (1.7-fold), and transient. Several reports indicate that differences in the magnitude and duration of MAPK activation cause differential compartmentalization of MAPK activity, and this brings about distinct end responses [35]. For example, it was shown that in PC 12 cells, sustained activation (1 h) of MAPK by nerve growth factor caused the cells to undergo differentiation and transient activation (less than 20 min) by EGF stimulated proliferation [36]. Whether the rapid activation, higher magnitude, and sustained duration of MAPK activation by Ang II resulted in the compartmentalization of its kinase activity and whether this is responsible for the inhibition of IL-6-induced STAT3 tyrosine phosphorylation, requires further investigation.

The significance of the delayed STAT3 tyrosine phosphorylation by Ang II, the ability of Ang II to cross-talk with IL-6, and precisely what genes are controlled by the Ang II-induced STAT pathway requires further study. Some of the STAT-regulated genes include the proto-oncogene c-fos, those for acute phase response proteins such as α-2 macroglobulin, the tissue inhibitor of metallo proteinases-1 (TIMP-1), the serine protease inhibitor 3 (SP-3), and the gene for cell adhesion, intercellular adhesion molecule-1 (ICAM-1). All of these genes contain the promoter element to which STAT proteins bind [23,37–40]. Angiotensin II is known to induce c-fos mRNA in cardiovascular cells [14,41]. However, the timing of Ang II-mediated maximal c-fos induction (30 min) [41] is different from the ability of Ang II to induce maximal nuclear STAT3/SIF-A activity (2–3 h) [19]. These data suggest that c-fos-induction by Ang II is probably regulated to a large extent by other pathways, such as $Ca^{2+}$ and MAPK. A recent report on the ability of Ang II to induce TIMP-1 mRNA (maximal at 2 h) in rat aortic endothelial cells [42] suggests that STAT3 protein may be involved in regulation of the expression of this gene. Expression of TIMP-1 has been linked to pathological conditions such as the acute phase response, tissue remodeling, wound repair, and inflammation [38,43]. Studies are underway to identify the role of Ang II-induced STAT protein activation and the ability of Ang II to cross-talk with IL-6 and to investigate the regulation of gene expression.

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# CARDIAC ANGIOTENSIN II SUBTYPE 2 RECEPTOR SIGNAL TRANSDUCTION PATHWAYS:EMBRYONIC CARDIOMYOCYTES AND HUMAN HEART

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***Summary.*** The distribution of the Ang II-receptor subtype 2 ($AT_2$-R) in the heart of various species from chick embryo to neonatal rat, adult rat, rabbit and man is reviewed. Human heart has a predominance of $AT_2$-R while rat and rabbit have considerably less receptors suggesting caution in the extrapolation of data from species with few $AT_2$-R to human. $AT_2$-R signaling mechanisms in the heart utilize phosphoinositol hydrolysis, PKC activation, tyrosine kinase activation and likely PI 3 kinase activation as well. In contrast to $AT_1$-R, the $AT_2$-R is not linked with cAMP signaling. The development of cardiac hypertrophy and subsequent heart failure is associated with alterations of $AT_2$-R density and undoubtedly a disturbance of the usual mechanisms whereby the effect of angiotensin on the heart is manifested.

"It has been concluded that most of the well-known actions of Ang II are mediated by the $AT_1$-receptor subtype" Timmermans et al. [1].

Preliminary conclusions from scientific data may accelerate or retard the exploration of alternate explanations for biological phenomenon. Acceptance of the overwhelming importance of angiotensin (Ang) II subtype 1 receptor ($AT_1$-R) caused investigators to wonder whether there was any value in exploring the Ang II subtype 2 receptor ($AT_2$-R). However, the $AT_2$-R has recently been receiving increased attention. The role of the $AT_2$-R in the heart remains unclear, in part, because it has not been extensively studied. When it has been investigated, models with lower abundance of $AT_2$-R have been chosen. This chapter will highlight cardiomyocyte Ang II signal transduction pathways that are likely mediated through $AT_2$-R.

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The existance of multiple types of Ang II receptors has long been considered because of the highly diverse cellular actions of Ang II. Pharmacological studies with relatively selective antagonists uncovered the presence of the two major Ang II receptor subtypes, called $AT_1$-R and $AT_2$-R [2–5]. cDNA for the rat, mouse, and human $AT_1$-R and $AT_2$-R have been cloned [6–9]. The biochemical characterization of the $AT_2$-R was followed by in-depth examinations to determine its role(s). A considerable number of functions have been attributed to the $AT_2$-R. These include decreases in cyclic GMP and increases of $K^+$ currents in neuronal cells from neonatal rat brain [10], inhibition of basal and stimulated cGMP in rat adrenal glomerulosa cells and PC12W cells [11], reduction in polyinositol hydrolysis in the skin [12], release of $PGI_2$ in astrocytes [13], inhibition of proximal tubule sodium reabsorption in rat kidney [14], and other effects such as modulation of calcium transients in bovine medullary cells and secretion of luteinizing hormone and prolactin [1]. Despite the potential importance of the $AT_2$-R, the identification of its physiological and pathophysiological roles in the heart remain incompletely understood. This may be due to the difficulty in elucidating the role of the $AT_2$-R in the heart as a result of the model systems used in routine experimentation. The objective of this chapter is to bring together data on the $AT_2$-R in the heart, focusing on the cardiomyocyte element of the heart with the following specific objectives: (1) to discuss the distribution of $AT_2$-R receptors in the heart (2) to compare the differences in the distribution of $AT_2$-R in the heart in various species (3) to examine potential signal transduction pathways linked to the $AT_2$-R in the heart (4) to discuss potential role(s) of $AT_2$-R in cardiac disease.

## DISTRIBUTION OF $AT_2$-R IN THE HEART

That the relative amounts of the $AT_2$-R and $AT_1$-R in the heart vary markedly between species cannot be over emphasised. The ratio of $AT_2$-R to $AT_1$-R in the myocardium varies from 2:1 in human heart [15,16] to less than 1:1 in rat myocardium [17,18] and about 1:2 ratio in rabbit myocardium [19,20].

Let us specifically examine this issue, reviewing some of the studies that examine Ang II receptor subtypes in the heart. In the rabbit ventricle, the proportion of $AT_2$-R has been reported to vary from 20% [21] to 50% [22]. $AT_2$-R were evenly spread over the myocardium with a low number present per cell [21]. Interestingly, total Ang II receptor density in the atria and cardiac nervous tissue were respectively 4 and 9 times greater than in the ventricle [21].

In rat myocardium, the ratio of $AT_2$-R to $AT_1$-R is no greater than 1:1 [17,18], with some investigators reporting that the ratio is definitely less than 1:1 [17,18] and as low as 1:4 [23]. Hunt et al. [24] found that in the rat, Ang II-R were first detected in the myocardium on embryonic day 14 and reached a maximum density within the first postnatal week and declined thereafter. During the perinatal period, the density of Ang II-R was twofold higher in the atria than in the ventricle, but by adulthood the ratios were similar in the atrium and ventricle. In neonatal rat heart, Ang II binding sites were concentrated in the vasculature and conduction system and were mainly $AT_2$-R, while Ang II-R were barely detectable in the

**Table 1.** Angiotensin II receptor subtype distribution in human heart

| Tissue | Source | $AT_2$-R/$AT_1$-R | Comments | Reference |
|---|---|---|---|---|
| RA | 35pt heart surgery | 2/1(67 ± 10/33 ± 10%) | correlated Lapres | Rogg (1996) |
| RA | 5pt AC bypass | 82/18(84 ± 2/21 ± 1%) | related fibrosis | Brink (1996) |
| RV AND LV | Autopsy | $AT_2$-R predominates | decreased $AT_2$-R in LVH | Nozawa (1996) |

Notes: RA = right atrium, RV = right ventricle, LV = left ventricle.

myocardium [24]. Everett et al. [25] found in the Sprague-Dawley that after the neonatal period, there was no $AT_2$-R mRNA in the heart.

In dog and ferret ventricle, the $AT_1$-R subtype is also the predominant Ang II-R [22].

The distribution of Ang II-R subtypes is completely different in the adult human heart compared to the adult heart of other species. In human atria, $AT_2$-R are at least twice as common as $AT_1$-R, with the ratio reported as being 67%/33% [16] or 82%/18% [26] or about 2:1 [15] (table 1). About 70 percent of Ang II receptors in the human heart are $AT_2$-R. One may question whether experiments studying Ang II receptor signal transduction pathways from animal species with low prevalence of $AT_2$-R are relevant to man.

## ANG II SIGNAL TRANSDUCTION PATHWAYS IN THE HEART

The intracellular signal transduction mechanisms whereby Ang II produces its cellular effects are an area of intense interest. Ang II stimulates multiple distinct signaling pathways. These include

- hydrolysis of phosphotidylinositol 4,5-bisphosphate, yielding the two second messengers, inositol 1,4,5-triphosphate (1,4,5-$IP_3$) and diacylglycerol (DAG) [22,27–30]
- mobilization of sequestered intracellular calcium likely from 1,4,5-$IP_3$ and activation of protein kinase C (PKC) likely from DAG [31–34]
- modulation of cAMP and adenylate cyclase pathway in the heart [35–37]
- stimulation of platelet-derived growth factor (PDGF) A-chain expression and induction of proto-oncogenes such as c-fos and c-jun [38,39]
- stimulation of various protein kinases such as MAP kinases, JAK, and PKC [40–42]
- stimulation of tyrosine kinases to produce tyrosine phosphorylation [43–46]
- stimulation of various phospholipases such as $PLA_2$, PLD, and arachidonic acid metabolism [31–34]
- phosphorylation of Raf-1 likely through PKC [45]

Several of the above outlined signaling pathways will be examined from the perspective of those potentially linked to $AT_2$-R.

## $AT_2$-R SIGNAL TRANSDUCTION PATHWAYS

The $AT_2$-R is similar to only a few other seven membrane-spanning domain receptors that are not coupled to G proteins [7,8]. A similar receptor in *Drosophila* acts as a ligand for sevenless tyrosine kinase receptor during eye development [46].

### $IP_3$ signaling

Ang II produces phosphoinositide hydrolysis in the ventricle of a number of different species, in which $AT_1$-R is the predominant Ang II-R subtype. In neonatal rat cardiomyocytes, the results are somewhat controversial. Some evidence suggest that Ang II does not produce inositol 1,4,5-triphosphate ($IP_3$) but rather produces $IP_1$ and $IP_2$ [30], while other studies suggest that Ang II may induce formation of $IP_3$ [36,47]. As one might have anticipated, $AT_2$-R blockade did not have a major impact on blocking the action of Ang II on phosphoinositide hydrolysis in neonatal rat myocardium [32,48], a cell type in which the effects of Ang II on inositol pathway have not been consistent [28,34,47] and in which there are fewer $AT_2$-R than $AT_1$-R. Sadoshima and Izumo [39] reported that in neonatal rat ventricle, $AT_1$-R antagonism with losartan, but not $AT_2$-R antagonism, blocked Ang II-induced activation of $IP_3$. Lokuta et al. [40] reported that Ang II-induced $IP_2$ formation was blocked by $AT_1$-R, but not $AT_2$-R antagonists, in neonatal rat ventricular cells, but they did not study $IP_3$ formation. For comparison, in neonatal rat fibroblasts, Ang II increased total inositol production, which was blocked by losartan, but not by an $AT_2$-R antagonist [49]. Ang II-induced increases in intracellular calcium, which are mediated in part through calcium release from sarcoplasmic reticulum calcium stores that are modulated by $IP_3$, are blocked by $AT_1$-R but not $AT_2$-R antagonism, in neonatal rat myocytes in culture [50]. Neonatal cardiomyocytes, however, not only have lower concentrations of $AT_2$-R but also show considerable loss of $AT_2$-R binding in culture perhaps because of internalization of $AT_2$-R [51].

In the rabbit myocardium, Ishihata and Endoh [30] did not show a link between $AT_2$-R; however, they examined inositol monophosphate. The density of $AT_2$-R is very low in rabbit myocardium [19,20].

Embryonic chick ventricular myocytes have well-defined and abundant Ang II-R [52,53]. In addition, chick cardiomyocytes have well-defined G proteins [54], linked to Ang II receptors [55]. In chick embryonic cardiomyocytes, Ang II significantly increased intracellular levels of $IP_1$, $IP_2$, and $IP_3$ [38,56,57]. The effects were evident within 1 minute, supporting a role for this pathway in the signal transduction mechanisms whereby Ang II induces its cellular effects. $AT_1$-R and $AT_2$-R blockers produced dose-dependent anatgonism of Ang II-induced $IP_3$ production (figure 1). The $AT_1$-R may be more closely linked to $IP_3$ production than the $AT_2$-R, based on the greater effect of losartan at equimolar concentrations. Ang II-induced $IP_3$ formation was not inhibited completely by $AT_1$-R blockade with losartan [56]. Ang II-induced $IP_3$ formation was reduced by $AT_2$-R antagonism. Since a combination of losartan and $AT_2$-R blockade was

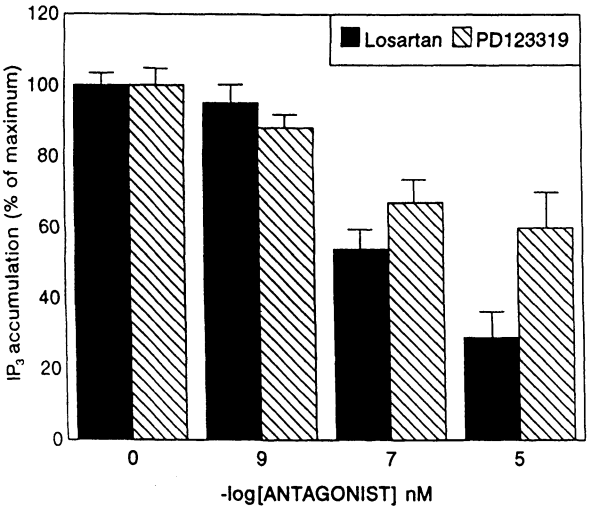


**Figure 1.** Effect of $AT_1$ and $AT_2$ receptor blockers on Ang II-induced $IP_3$ accumulation: [$^3$H] inositol-prelabeled myocytes were stimulated with Ang II (1000 nM, 1 min), and the accumulation of $IP_3$ was quantitated. A 30-min preincubation with increasing concentrations of antagonist ($10^{-9}$, $10^{-7}$, $10^{-5}$ mM) in medium prior to the 1 min 1000 nM Ang II stimulation is shown. Stimulation values were compared directly to parallel experiments in which antagonist was not added. Shown are the dose-response curves for losartan and the $AT_2$-R antagonist PD123319. Results represent mean ± SEM of five experiments done in triplicate (from Gen Pharm, 1998).

necessary to almost completely blocked $IP_3$ formation, it can be suggested that phosphotidylinositol hydrolysis in these cardiomyocytes is mediated through both $AT_1$-R and $AT_2$-R (figure 2). While this cell model has the problems of its avian origin and the precise characterization of its Ang II receptors, its embryonic origin ensures a higher prevalence of $AT_2$-R, so that it may be closer to the prevalence of $AT_2$-R in human heart compared to rabbit or neonatal rat cardiomyocytes in culture.

### cAMP signaling

Ang II inhibits β-adrenergic-R-mediated stimulation of cyclic adenosine monophosphate (cAMP) production in the heart. Ang II inhibits isoproterenol and glucagon-induced increases in cAMP in isloated cardiomyocytes and cardiac sarcolemmal membrane from adult rat and rabbit heart [28,35,37]. This effect of Ang II is part of a generalized response, as it has been found in various other tissues [59,60], and is operative through G inhibitory protein [35]. This action of Ang II on cAMP in cardiomyocytes was not mediated through the $AT_2$-R, but rather the $AT_1$-R (figure 3) [61]. This is consistent with the findings in renal artery smooth muscle [10], human myometrium [10], rat glomeruli [60], and rat adrenal glomerulosa [3]. In contrast, $AT_1$-R blockade completely prevented the effects of

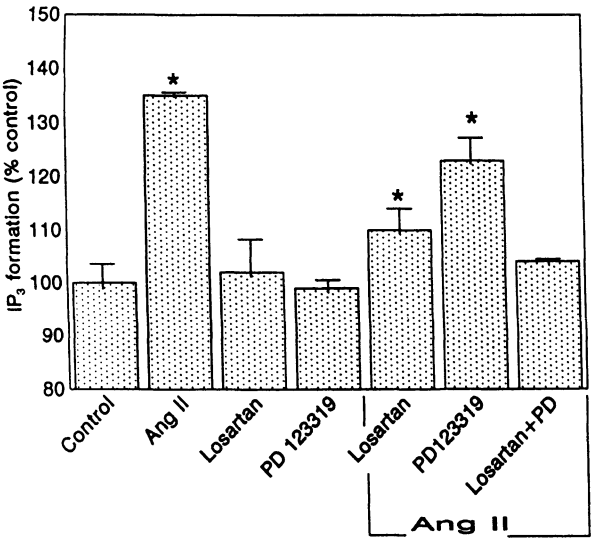


**Figure 2.** Effect of $AT_1$ and $AT_2$ receptor blockade on Ang II-induced formation of $IP_3$ Cardiac myocytes were prelabeled with [$^3$H] inositolwere stimulated with Ang II (1000 nM) for 1 min in the presence of LiCl (10 mM). The accumulation of $IP_3$ was determined by Dowex column chromatography. For antagonist experiments, cells were pretreated with losartan ($10^{-5}$M) or PD123319 ($10^{-5}$, M) or both ($10^{-5}$M) prior to stimulation with Ang II. Some cells were incubated with blockers and harvested without being stimulated by Ang II. The amount of $IP_3$ in the control was defined as a 100% value. All values represent the mean ± SEM of 3–5 experiments performed in tripicate (*$p < 0.05$; **$p < 0.01$). (from Gen Pharm, 1998).

Ang II to inhibit isoproterenol-mediated increase in cAMP production in the heart [61].

The lack of coupling between the $AT_2$-R and adenylyl cyclase to generate cAMP remains somewhat perplexing as the Ang II-R has a seven-transmembrane domain topology, the type which is generally linked to G proteins. The $AT_2$-R appears to belong to a unique class of seven-transmembrane receptors, which include the somatostatin, SSTR1, and dopamine $D_3$, for which G protein coupling has not been demonstrated [7,8].

### PKC signaling

Ang II activation of PKC occurs over a 30 min time frame in embryonic chick hearts [61] and in neonatal rat heart [34]. The magnitude of Ang II-induced translocation of PKC from cytosol to membrane in cardiomyocytes is consistent with that reported in other tissues [62]. Ang II-induced increases in membrane PKC activity and translocation of PKC from cytosol to membrane were antagonized by competitive blockade of the $AT_2$-R [61] (figure 4). Blockade of Ang II-induced PKC activation was evident at a low concentration of an $AT_2$-R antagonist, supporting the contention that it is due to an effect on $AT_2$-R and not through an

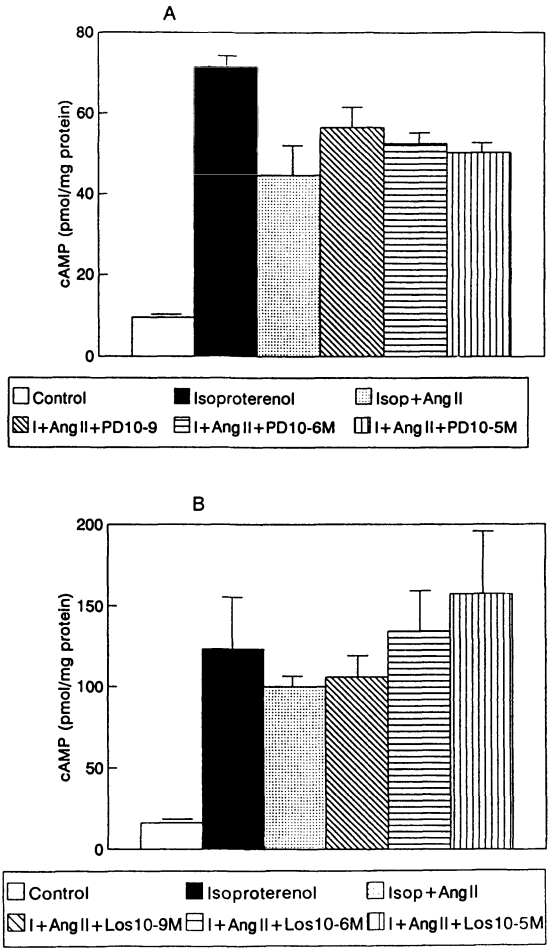


**Figure 3.** Effect of Ang II-R antagonists on isoprpterenol-induced increases in cAMP: (*A*) Intracellular cAMP in ventricular cardiomyocytes that were stimulated with isoproterenol (I) in the presence of angiotensin (Ang) II for 5 min and different concentrations of PD 123319 (PD) at $10^{-9}$M, $10^{-6}$M, and $10^{-5}$M. Myocytes were pretreated with PD123319 for 30 min as well as the phosphodiesterase inhibitor 3-isobutyl-1-methyl xanthine prior to exposure to isoproterenol. Myocytes were treated with isoproterenol, 1 mM, for 1 min and Ang II for 5 min.
(*B*) Intracellular cAMP in ventricular cardiomyocytes that were stimulated with isoprtoterenol in the presence of Ang for 5 min and different concentrations of losartan. Myocytes were pretreated with losartan for 30 min as well as the phosphodiesterase inhibitor 3-isobutyl-1-methyl xanthine, 1 mM, prior to exposure to isoproterenol. Myocytes were treated with isoproterenol, 1 mM, for 1 for 5 min. The data, in panels A and B, are the mean ±1 SEM. (From Canadian Journal of Physiology and Pharmacology 1996.)

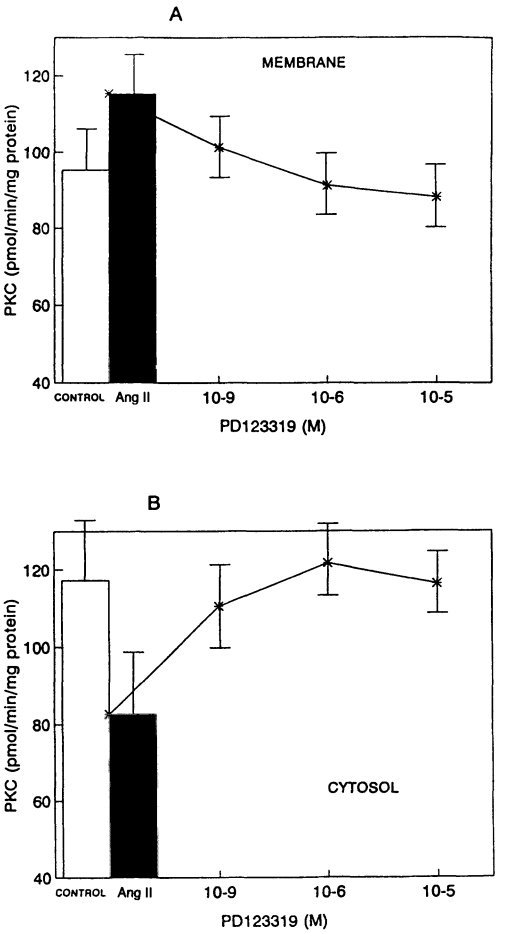


**Figure 4.** The effect of $AT_2$ receptor antagonism on Ang II-induced translocation of PKC from cytosol to membrane. PKC activity in cytosolic and membrane fractions of ventricular cardiomyocytes that were treated with different concentrations of the $AT_2$ receptor antagonist PD 123319, $10^{-9}$M to $10^{-5}$M, (panel A) beginning 30 min prior to measurement of PKC. The bars represent control, no angiotensin or PD123319 (open bar), and angiotensin II (Ang II), 1 μM without PD123319 (solid bar). The data are presented as mean ± 1 SEM for each concentration. Control represents no Ang II or antagonist, and Ang II represents Ang II in the absencee of the antagonist. (From Canadian J Physiol and Pharm 1996.)

effect on another Ang II-R that might possibly occur at high concentrations of the $AT_2$-R antagonist. $AT_2$-R antagonism did not affect PKC activity in the basal state.

### Tyrosine kinase signaling pathways

There have been few studies of Ang II-induced tyrosine phosphorylation in the heart. Sadoshima et al. [63] reported that Ang II-induced tyr phos of cellular

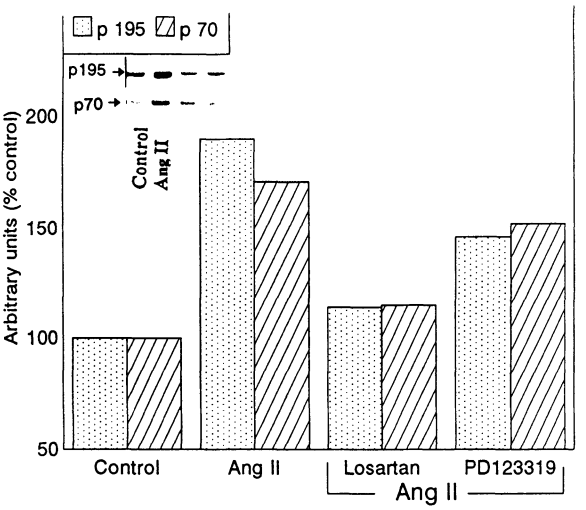


**Figure 5.** Effects of $AT_1$ and $AT_2$ receptor blockers on Ang II-stimulated protein tyrosine phosphorylation. Embryonic chick cardiomyocytes, incubated in 818A medium containing 2% FBS, were treated with 1000nM Ang II for 1 minute. For inhibition experiments, the cells were preincubated for 1 hour in the presence of losartan or PD123319 (both $10^{-5}$M). Proteins were immunoprecipitated with anti-P-Tyr antibody, separated by SDS-PAGE. The results of tyrosine phosphorylation of 195 and 70kDa proteins are shown. They were compared with control (untreated cells) which was defined as 100%. Data are representative of independent experiments that were performed at least three times. The insert shows the data from the autoradiogram. (From Cellular Signalling, 1997.)

proteins with molecular weights of 42,44,75–80, and 120–130 in cultured neonatal rat cardiomyocytes and fibroblasts. The 42 and 44kDa proteins were mitogen-activated protein kinases (MAPKs), and the 90kDa was S6 kinase (RSK), but they were not able to identify the other proteins. They subsequently described Ang II activation of $p21^{ras}$ via the tyrosine kinase-Shc-Grb2-Sos pathway in these cardiomyocytes. Schorb et al. [64] found tyrosine phosphorylation of 44, 46–60, 95, and 125kDa proteins in response to Ang II in neonatal rat fibroblasts and demonstrated that these were MAPKs, $p56^{SHC}$, $p46^{SHC}$ and $p125^{FAK}$. They did not examine cardiomyocytes. We have shown tyr phosphorylation of various proteins in cytosolic extracts of Dahl salt-sensitive rat [65]. Saad et al. [66] reported increased tyrosine phosphorylation of a 85kDa protein in rat heart extracts. Ang II-induced tyrosine phosphorylation of cardiac proteins, at least in embryonic cardiomyocytes, is mediated through both $AT_1$-R and $AT_2$-R [56] (figure 5).

We have recently shown that in embryonic chick cadiomyocytes, Ang II-induced phosphoinositol generation is mediated, at least in part, through a pathway involving tyrosine phosphorylation [57]. This was based on several lines of evidence. First, the time course of Ang II-stimulated protein tyrosine phosphorylation was within the time course of Ang II-induced inositol phosphate formation in these

cardiomyocytes, supporting the contention of a linkage of the two pathways. A causal link between tyrosine phosphorylation pathway and Ang II-induced inositol phosphate generation was most conclusively demonstrated by the ability of the tyrosine kinase inhibitor genistein to markedly and significantly reduce Ang II-induced inositol phosphate formation. In contrast, its structural analog, daidzein, which has limited tyrosine kinase inhibitory ability, did not block Ang II-induced phosphoinositol formation. Furthermore, genistein reduced Ang II-induced tyrosine phosphorylation of cardiomyocyte proteins. In vascular smooth muscle cells, genistein has been reported to either reduce [67] or have no effect [68] on Ang II-induced $IP_3$ production. The rapid time course of Ang II induced-tyr phos suggests either a linkage between the Ang II-R and a tyrosine kinase receptor [69] or a tyrosine phosphoprotein activation linked to $AT_2$-R signaling.

Demonstration of Ang II tyrosine phosphorylation pathways in the heart is difficult because the heart contains comparatively small amounts of tyrosine kinases compared to other tissues. Tremblay and Beliveau [70] found that the heart has the least amount of tyrosine kinase activity, in both soluble and particulate fractions, compared to the brain, thymus, liver, spleen, kidney, pancreas, or testes [70]. Elberg et al. [71] also found that the heart had lowest cytosolic tyrosine kinases compared to other tissues. This may be due to the especially low levels of tyrosine kinase substrate in the heart.

Ang II-induced protein tyrosine phosphorylation was inhibited by both $AT_1$ and $AT_2$ receptor blockade. The $AT_1$-R lacks intrinsic tyrosine kinase activity [72], and only a small amount of tyrosine phosphorylation of this receptor occurs, with a relatively long time frame (20 min) after Ang II stimulation [73].

### Other signal transduction pathways

*Phosphatidylinositol 3-kinase (PI3K) activation*

A potentially important pathway that may be linked to $AT_2$-R signaling is PI3K. PI3K refers to a family of enzymes that phosphorylate the membrane lipids PtdIns, PtdIns 4-P, and PtdIns 4,5-$P_2$ on the third position of the inositol ring, yielding PtdIns 3-P, PtdIns 3,4-P, and PtdIns 3,4,5-P [74,75]. By phosphorylating the 3 position rather than the 4 position, this kinase generates new phosphoinositides that are not on the pathway for production of $IP_3$ and DAG, through the action of phospholipase C (PLC). PI3K acts as a biochemical link between a novel phosphatidylinositol pathway and a number of proteins containing intrinsic or associated tyrosine kinase activities, such as the PDGF receptor [76,77], insulin [78], colony-stimulating factor-1 [79], and the products of oncogenes v-src [80] and v-abl [79]. The ability of Ang II to induce tyrosine phosphorylation in cardiac and noncardiac tissues [56,66] and the involvement of tyrosine phosphorylation with PI3K led us to hypothesize that Ang II would activate PI3K in the heart [80].

We have recently found that Ang II activated PI3K in embryonic chick cardiomyocytes [81]. Furthermore, the link between Ang II and protein synthesis was related to Ang II-induced activation of PI3K as the inhibitor of this enzyme

significantly decreased the action of Ang II to increase protein synthesis, as demonstrated by [$^{35}$S] methionine incorporation into cardiomyocytes [81].

*Tyrosine phosphatase*

The $AT_2$-R was initially found to induce a phosphotyrosine phosphatase based on the ability of orthovanadate to protein block tyr phos in PC12W cells [82]. This has also been demonstrated in N1E-115 neuroblastoma cells [83]. Subsequently, the cloning of the $AT_2$-R demonstrated that the receptor was linked to phosphotyrosine phosphatase inhibition [7].

*Arachadonic acid release*

Ang II increases the production of arachadonic acid in the heart, utilizing pathways discrete from $IP_3$ formation [48]. In neonatal rat heart, $AT_2$-R antagonism influenced the expression of the $AT_2$-R, but not $AT_1$-R-mediated Ang II-induced arachadonic acid release [48].

*Na+/HCO3− symport*

Ang II produces alkalinization of neonatal cardiomyocytes through a Na/HCO3 symport and not through the Na+/H+ exchanger [84]. This action of Ang II was mediated through the $AT_2$-R and appeared to involve arachadonic acid [84].

## $AT_2$-R AND CARDIAC DISEASE

### $AT_2$-R and myocardial infarction

In the rat, Nio et al. [85] found that there was a 3.1-fold increase in $AT_2$-R mRNA in infarcted myocardium and a 1.9-fold increase in noninfarcted myocardium after coronary artery ligation compared to the myocardium from control animals. The transcriptional rates for $AT_2$-R and $AT_1$-R were increased significantly in the infarcted heart. The $AT_2$-R number was increased 2.3-fold, only slightly less that the increase in the $AT_1$-R, while the receptor affinity was unchanged. $AT_2$-R antagonism did not affect $AT_1$-R mRNA, blood pressure, or infarct size. Interestingly $AT_1$-R antagonism also did not affect infarct size. However $AT_1$-R antagonism influenced the expression of the $AT_2$-R in the infarcted myocardium [85]. The $AT_2$-R may mediate the adverse effect of Ang II on the recovery of left ventricular function after ischemia and during reperfusion, as $AT_2$-R antagonism improves left ventricular recovery in this circumstance [85].

The potential role of Ang II on apoptotic cell death is controversial with studies supporting and refuting this possibility [86–88]. Furthermore, whether this possible action of Ang II is mediated by $AT_1$-R or $AT_2$-R is unresolved as there is opposite data from different investigators [86,87].

### $AT_2$-R in cardiac hypertrophy

The potential role of the $AT_2$-R in cardiac hypertrophy is controversial. There is limited data available in humans; however, Nozawa et al. [89] examined Ang II-R

in hearts that were examined at autopsy within 3 h of death. There was a marked reduction in $AT_2$-R in cardiac hypertrophy. Whether this represents downregulation of Ang II-R to prevent Ang II-R-mediated cardiac hypertrophy or whether this is unrelated to the pathophysiology of cardiac hypertrophy remains to be determined.

The proposal for a causal role of $AT_2$-R in cardiac hypertrophy is supported by several lines of evidence. First, $AT_2$-R is the most abundant Ang II-R in embryo, which is a state of rapid growth. Second, $AT_2$-R is linked to several growth-promoting pathways, namely, PKC and tyrosine kinase pathways. The PKC pathway may be involved in mediating the effects of Ang II on cardiac hypertrophy, as PKC can mediate cell growth [90], and PKC participates in the induction of myocardial β-myosin heavy-chain and myosin light-chain [91,92]. $AT_2$-R does not mediate the effects of Ang II to increase cardiac contractility [20,30] as one would anticipate, given that PKC activation in the heart, by phorbol esters, can produce a transient negative inotropic response [93,94]. Interestingly, cardiac hypertrophy in response to hypertension, in the spontaneously hypertensive rat or in the two kidney one clip model of renovascular hypertension, is associated with an approximately twofold increase in both $AT_2$-R in the heart [18].

Experimental data that does not support a causal role for $AT_2$-R in cardiac hypertrophy originates from animals with a much lower density of the $AT_2$-R in the heart compared to man. Ang II receptor gene expression was not altered by the development of cardiac hypertrophy. The abundance of $AT_2$-R, as well as AT1a and AT1b mRNA levels, was not changed with the development of cardiac hypertrophy after aortic banding in the rat [95]. In the Tsukuba hypertensive mice that carry the human gene for renin and angiotensinogen, no changes in $AT_2$-R were found while there were definite upregulation of $AT_1$-R in the heart [96]. The development of cardiac hypertrophy produced by coarctation of the abdominal aorta in Sprague-Dawley rat was not affected by treatment with an $AT_2$-R antagonist [97].

### $AT_2$-R in cardiac failure

The number of Ang II binding sites in sarcolemmal fractions was significantly reduced in explanted end-stage failing human hearts [15] compared to mild heart failure or normal controls. The degree of heart failure influences the Ang II receptors, as cardiac Ang II receptor density is reduced in some patients with heart failure [98,99]. Indeed the reduction in Ang II-R binding was directly related to the degree of reduction in left ventricular function, as reflected by ejection fraction [98]. This was not due to internalization of receptors, as a similar change was found in vesicle fractions. Rather the reduction in Ang II receptors can be ascribed to a decrease in steady-state mRNA abundance [15]. In heart failure, the proportion of $AT_2$-R in human heart may increase. Rogg et al. [16] reported that in human right atrium, the proportion of $AT_2$-R was higher in patients with elevated right atrial

pressure. Indeed, there was a significant linear correlation between the $AT_2$-R density and left ventricular ejection fraction [16].

## CONCLUSION

The data present in this chapter has attempted to draw attention to the importance of the $AT_2$-R in the heart. Previous studies that dismissed or minimized the role of $AT_2$-R in the heart were done mainly in species that had a low density of $AT_2$-R in the heart. This problem is further compounded by the rapid downregulation or internalization of $AT_2$-R when cardiomyocytes are maintained in culture. In the embryonic heart, $AT_2$-R is more closely linked to PKC activation than the $AT_1$-R [61]. In these cardiomyocytes, the $AT_2$-R is *not* linked to cAMP dependent pathways [61]. $AT_2$-R are functionally coupled to protein tyrosine dephosphorylation [7,83]. Thus, $AT_2$-R is linked more closely to pathways similar to growth hormone receptor pathways that utilize PKC and tyrosine kinases. Inferences drawn about the role of the $AT_2$-R in Ang II single transduction in the heart of some species may not be relevant to human heart. In human atria and ventricle, the $AT_2$-R is the predominant Ang II receptor [15,16,21,99]. In human heart, heart failure and cardiac hypertrophy are associated with a decrease in Ang II-R binding. Furthermore, in cardiac hypertrophy, a major predictor of cardiovascular morbidity and mortality in hypertension, $AT_2$-R density may increase proportionately more than the $AT_1$-R [18]. In myocardial infarction, there is a two- to threefold increase in $AT_2$-R mRNA and receptor number in the infarcted myocardium. Given the role of Ang II in cardiac hypertrophy [100,101] and myocardial infarction, the data reviewed here suggest that $AT_2$-R merits considerable further investigation in cardiac disease in man.

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# ANGIOTENSIN II STIMULATES CONTRACTILITY AND C-FOS GENE EXPRESSION IN ISOLATED ATRIAL HUMAN MYOCARDIUM

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***Summary.*** The renin-angiotensin system plays an important role in the pathogenesis of many cardiovascular disorders. Althougth the acute effect of angiotensin II on myocardial contractility, as well as the chronic effect of angiotensin II on cardiac growth, has been studied in several species, little is known about these effects in human myocardium.

We therefore studied (1) the acute effects of angiotensin II on myocardial contractility in different types of human tissues: Angiotensin II had a positive inotropic effect only in atrial human myocardium but not in right or left ventricular myocardium of human hearts with different diseases. (2) In atrial myocardium, angiotensin II increases mRNA expression of c-fos which can be blocked by losartan, an $AT_1$-receptor antagonist.

In human hearts, Ang II exerts positive inotropic effects only in atrial but not in ventricular myocardium. The trophic effect of Ang II can be demonstrated in atrial human myocardium. Studies are necessary to clearly demonstrate trophic effects of Ang II in isolated human left ventricular myocardium in the absence of positive inotropic effects.

## INTRODUCTION

The renin-angiotensin system (RAS) is important in the pathogenesis of many cardiovascular disorders [1,2]. There is now clear evidence that angiotensin receptors exist in mammalian cardiac tissue [3,4], including that of humans [5–7], and that there is a localized cardiac RAS [8]. Stimulation of angiotensin receptors from increased levels of angiotensin (Ang) II may result in an acute inotropic effect and may exert the induction of cardiac hypertrophy.

In some species, Ang II has been shown to exert a positive inotropic effect [9–12], which could not be found in others [4,13,14]. Even negative inotropic effects

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were observed in cultured neonatal rat myocytes [15], and different results were obtained in dog myocardium [16]. Because the inotropic effect of Ang II may also depend on the experimental conditions, we studied the influence of Ang II on right atrial human myocardium at physiological conditions, i.e., at an experimental temperature of 37°C and a stimulation rate of 60 beats per min [17].

Ang II has been shown to stimulate the expression of proto-oncogenes in neonatal rat cardiomyocytes [18–21] but not in isolated adult rat heart [22]. Because c-fos was the most sensitive proto-oncogene for Ang II stimulation in these studies, we analyzed mRNA of c-fos in right atrial human myocardium, which was exposed to Ang II for 45 min, in addition to conducting the contractility studies.

The present study provides support that Ang II increases contractility and stimulates expression of the proto-oncogene c-fos in human atrial myocardium.

## METHODS

### Patients

Myocardial tissues used in the present study for contractility measurements and northern blot analysis of c-fos mRNA were obtained from patients undergoing routine aortocoronary bypass surgery because of two- or three-vessel disease. These patients had sinus rhythm and normal ejection fraction and did not show any symptomes of congestive heart failure. Most of the patients were treated with β-blockers (metroprolol, atenolol, bisoprolol), aspirin (100 mg per day), nitrates, and calcium antagonists (nifedipine, nisoldipine, diltiazem). One third of the patients received an angiotensin-converting enzyme (ACE) inhibitor (captopril, enalapril, lisinopril), but no patients were on diuretic medication. All patients had given written informed consent.

### Mechanical experiments

Immediately after excision of a small piece of the right atrium, the tissue was submerged into KREBS-RINGER solution containing 30 mM BDM. Transportation plus preparation time prior to the start of the experiment was about 30 minutes. The solution had the following composition: $Na^+$ 152 mmol/l, $K^+$ 3.6 mmol/l, $Cl^-$ 135 mmol/l, $HCO_3^-$ 25 mmol/l, $Mg^{2+}$ 0.6 mmol/l, $H_2PO_4^-$ 1.3 mmol/l, $SO_4^{2-}$ 0.6 mmol/l, $Ca^{2+}$ 2.5 mmol/l, Glucose 11.2 mmol/l, and Insulin 10 IU/l. This solution was constantly bubbled with a gas mixture of 5% $CO_2$ and 95% $O_2$. Solutions that were used for transportation and dissection purposes additionally contained 30 mmol/l BDM to protect the myocardium against ischemic and cutting injury [23,24].

Preparations were performed in a special dissection chamber and with the help of a stereo microscope (VMT Olympus). Both ends of atrial trabeculae were attached to fine steel hooks. Mean muscle length was 3.4 ± 0.2 mm, and mean cross-sectional area was measured to be 0.48 ± 0.05 $mm^2$.

The muscle preparations were placed in a muscle bath and prestetched by a passive load of maximally 2.5 mN. Stimulation was started at 60 beats per minute via

two parallel platinum electrodes that were located on both sides of the muscle and connected to a stimulation unit (Hugo Sachs Elektronik Type 215/1). Stimulation duration was 5 milliseconds, and voltage was set to 25% above therehold. Passive and active force were measured by a force transducer, F 30 type 372 (Hugo Sachs Elektronik), and recorded on a linearcorder, Mark VII Graphotec. After developed force had reached a steady state, the preparations were carefully stretched to $l_{max}$, the optimum length at which developed force is maximum, by 0.10 and 0.05 mm stretches. By using a special electronic feedback system, bath temperature was controlled and regulated to be 37°C.

BDM and Ang I and II were obtained from Sigma Chemical Co., whereas losartan was kindly provided by Merck Sharp & Dohme Research Laboratories.

All values are given as mean ±SEM in text and figures.

### RNA preparation and analysis

Total RNA was isolated from right atrial myocardium by the method of Chomczynski and Sacchi [25]. Aliquots of twenty micrograms of total RNA were size-fractionated by electrophoresis on 1.2% agarose/3% formaldehyde gels and transferred to nitrocellulose filters using a Posi Blot pressure blotter (Stratagene). After UV-crosslinking, the filters were prehybridized in a solution containing 25 mM $KPO_4$, pH 7.4; 5× SSC; 5X Denhardt's solution (1% Ficoll 400, 1% polyvinylpyrrolidon, 1% BSA); and 50% formamide at 42°C for at least 2 hours. The filters were then hybridized with p32-labeled DNA probes in the same solution at 42°C for at least 12 hours.

The blots were washed at a final stringency of 0.2X standard saline citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) at 65°C. The level of c-fos expression was quantified on a Phospho Imager, referring to the GAPDH hybridization signal as a loading control. In addition, filters were exposed to Kodak X-Omat-XAR-5 films for 2 hours to 1 week at −80°C.

DNA probes used in this study were as follows:

1. As a probe for c-fos, we used Hind II/Kpn I fragment of pBFH 480 [26].
2. Gyceraldehyde-3-phosphate dehydrogenase (GAPDH), derived as a BamH I/ EcoR I fragment from pTRI-GAPDH (Ambion), was included as a control for RNA quantity and integrity.

## RESULTS

### Mechanical experiments

As shown in figure 1A, both Ang I and II exert positive inotropic effects in preparations of human right atrial tissues. This effect is concentration-dependent and is maximum at $10^{-7}$M Ang II and $10^{-6}$M Ang I. The effect can be blocked by saralasin, but not by a combination of propranolol ($10^{-6}$M) and prazosin ($10^{-5}$M) (see figure 1B).

These data indicate that (1) the positive inotropic effect of Ang I and II is

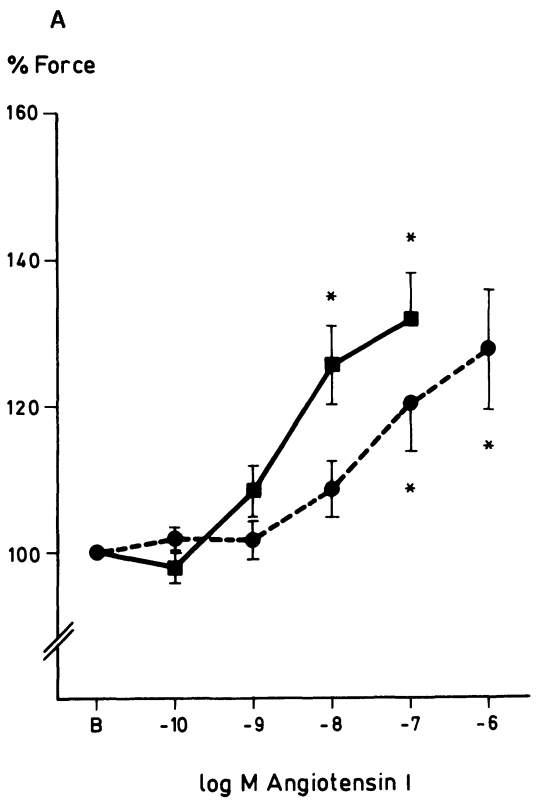


**Figure 1.** *A*, Dose-response curves for angiotensin I and II in nonfailing human right atrial myocardium (n = 8 experiments for each curve). There is a clear concentration-dependent effect on contractile force, which is maximum at $10^{-7}$M angiotensin II and at $10^{-6}$M angiotensin I.

mediated by specific angiotensin receptor and (2) this effect is independent of noradrenaline release. Furthermore, we were able to show that the positive inotropic effect of Ang I can be blocked by the ACE inhibitor enalaprilate (no figure) [17,27].

### C-fos gene expression

A typical example of a northern blot analysis of c-fos and GAPDH, as a control, is shown in figure 2. In this example, the incubation with angiotensin obviously increases c-fos mRNA. Figure 3 shows a similar experiment: in addition to Ang II, however, experiments were carried out after preincubation with $10^{-6}$M losartan, a specific subtype 1 angiotensin receptor blocker. From these experiments in figure 3, it is evident that the c-fos mRNA values are lowest after preincubation with losartan (with or without later Ang II application), are highest after Ang II incubation alone,

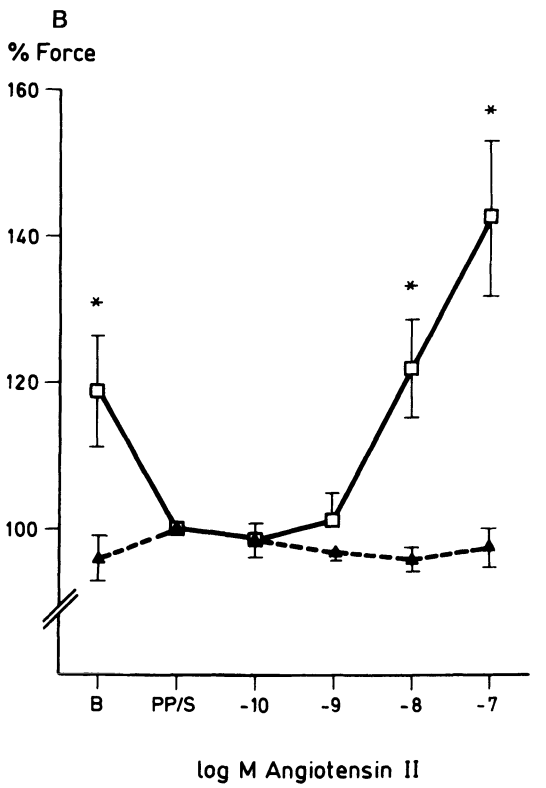


**Figure 1.** *B*, Dose-response curves for angiotensin II in nonfailing human right atrial myocardium after treatment with saralasin ($10^{-5}$M) and a combination of propranolol ($10^{-6}$M) and prazosin ($10^{-5}$M) (n = 8 experiments for each curve). Saralasin blocks the positive inotropic effect of angiotensin II, but prazosin and propranolol do not.

and may vary in the control situation (angiotensin-independent c-fos expression). Losartan pretreatment shows the lowest c-fos mRNA values, whereas Ang II treatment shows the highest (figure 4).

## DISCUSSION

### Contractility

The effect of Ang II on the contractile force of myocardium has been demonstrated to vary greatly between species and to depend critically on experimental temperature and stimulation rate. For example, positive inotropic effects of Ang II have been described for the myocardium of cat [9,10], rabbit [3], chicken [28] and especially hamster [12,17], while noninotropic effects were observed in myocardium of guinea pig [4] and dog [16], and even negative inotropic effects could be detected in isolated rat myocytes [15]. Therefore, we studied the influence of Ang II in

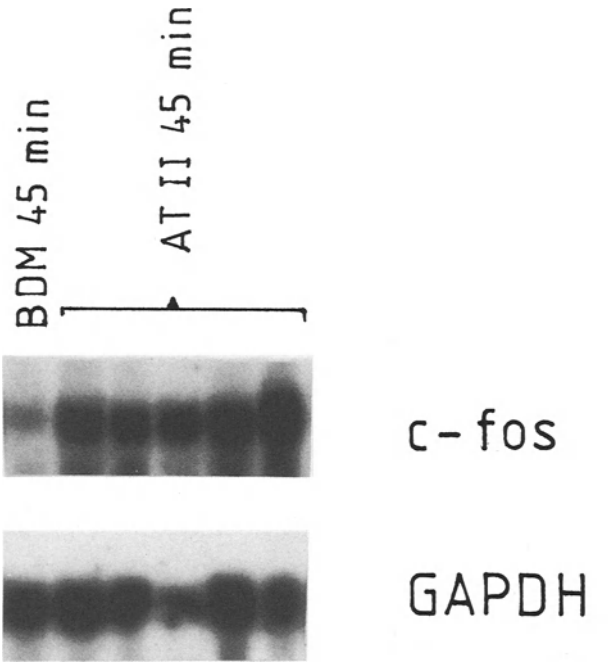


**Figure 2.** A typical example of a northern blot analysis of c-fos and GAPDH. Muscle strips were either incubated in BDM solution as a control (left) or in angiotensin II-containing solution (right).

human atrial myocardium by simulating physiological experimental conditions (37°C, 60 beats per min) [21–23]. Two important observations were made: 1 atrial human myocardium responds to Ang I and II by an increase in peak systolic force development; 2 this effect has been shown to be angiotensin receptor-mediated and independent of endogenous catecholamines.

Furthermore, we have shown previously that the positive inotropic effect of Ang I can be completely blocked by preincubation with enalaprilate, an ACE inhibitor [17,27]. This indicates the presence of the converting enzyme within the human myocardium.

### C-fos gene expression

We have demonstrated that Ang II-induced increase in c-fos mRNA can be inhibited by losartan. The problem in these types of experiments is the observation that c-fos may be expressed, to different degrees, without stimulation by exogenous Ang II. This nonangiotensin related effect may be explained by several mechanisms:

1. It is unlikely that it is stimulated already in vivo by Ang II because none of the patients were in heart failure.

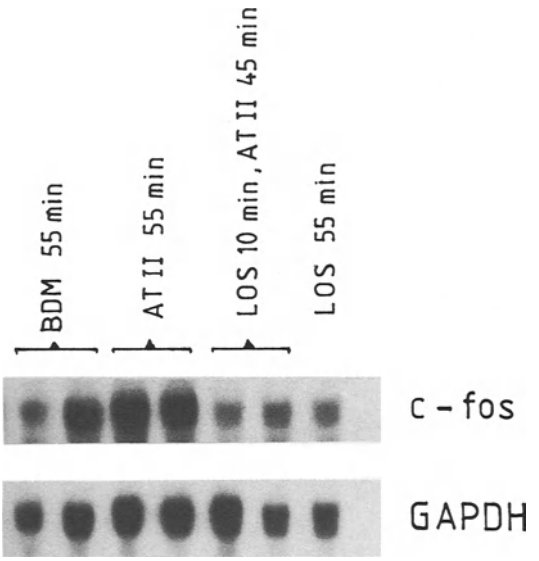


**Figure 3.** A typical experiment: from the right atrial appendix of one patient, specimens were prepared and subjected to four different conditions. Two specimens were incubated in BDM solution as a control (left), two specimens were incubated in angiotensin II (middle left), two specimens were incubated in angiotensin II after pretreatment with losartan (middle right), and one specimen was incubated in losartan alone (right). Northern blots of c-fos and GAPDH (as a control) are shown.

2. It might be possible that the preoperative application of diuretics might have stimulated the RAS, which might have induced the expression of c-fos. However, none of the patients from which the tissues were obtained had received a diuretic compound.
3. C-fos expression may be induced unspecifically to different degrees by anesthesia or cardioplegia [29].
4. The likely explanation is that parts of the atrial tissue were stretched during surgical and excision procedure. Whether or not such a stretch-induced c-fos expression may be Ang II-mediated is speculative but is supported by our data (figure 4) and the literature [21,30].
5. Protooncogene expression during ischemic conditions has clearly been described [31,32]. Ischemia may have occured during the operation procedure as well as during transportation. Therefore, the variation in c-fos expression under control conditions may also be attributed to ischemic conditions. However, this variation in the control experiments does not invalidate the data on angiotensin-induced expression of c-fos and its competetive inhibition by losartan.

**Signal transduction pathways**

In atrial human myocardium, Ang II stimulates both myocardial contractile force and c-fos expression. The most likely explanation for both observations is an

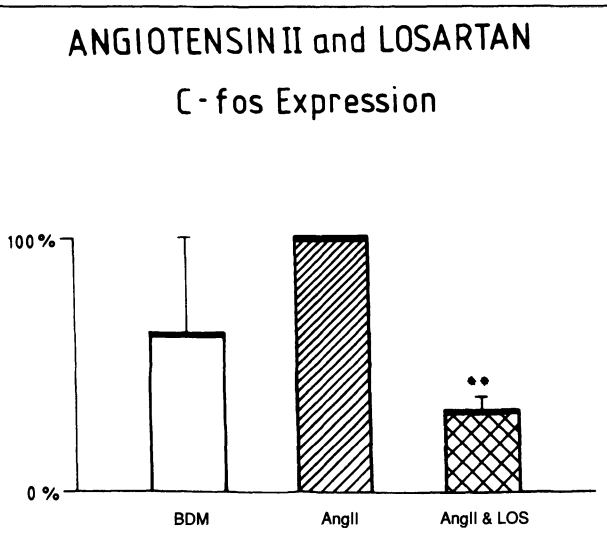


**Figure 4.** Statistical summary of the experiments of figure 3. N = 10 specimen for each condition from n = 5 atrial appendices. Differences between control (BDM solution) and angiotensin II incubation were not significant; however, preincubation in losartan significantly ($p < 0.01$) reduced c-fos expression.

activation of the PLC by angiotensin receptor-coupled G protein which results in increased levels of $IP_3$ and PKC (see figure 5).

**Ventricular human myocardium**

Regarding myocardial contractility measurements, studies have also been performed in human ventricular myocardium. But no data are presently available regarding c-fos expression in human ventricular myocardium. Despite the presence of angiotensin receptors in human myocardium [5–7], we could not find any positive inotropic effect of Ang II in human ventricular myocardium [17,27].

The number of receptors in failing left ventricular myocardium has been shown to be about one-fifth of that in right atrial human myocardium [5,6]. If these receptors would be active regarding contractility, our force measurements would be sensitive enough to detect an increase in force development induced by this small number of receptors. However, it may well be that the angiotensin receptors are exclusively localized on fibrocytes in the left ventricle. In this case, neither force development nor growth may be directly influenced by Ang II (Hypothesis 1). However, another hypothesis is possible: Provided the reduced number of angiotensin receptors is localized on myocytes, the signal transduction cascade for c-fos expression and induction of cardiac hypertrophy via $IP_3$ and PKC may be present. Such a signaling pathway may not be coupled to contractility in left ventricular human myocardium, for example, by the absence of $IP_3$ receptors in the sarcoplas-

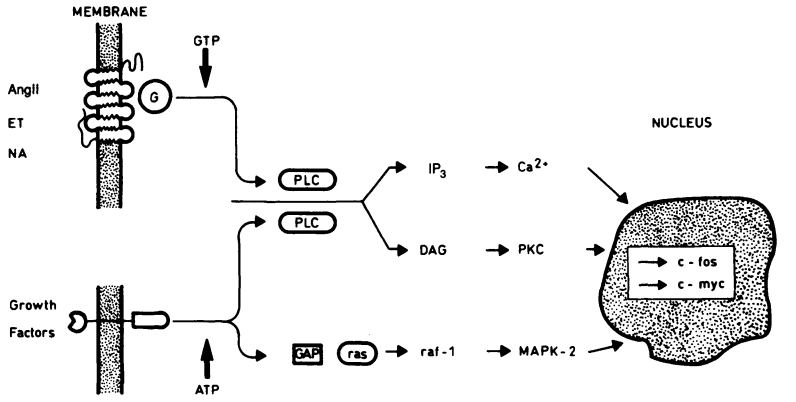


**Figure 5.** These receptor agonists bind to 7-membrane-spanning receptors, which are coupled to GTP-binding proteins (G) to activate PLC, phospholipase C. An alternative pathway may be activated via thyrosine kinase-linked receptors by growth factors. PLC generates $IP_3$ inositol (1,4,5)-triphosphate, and DAG, and diacylglycerol. Whereas $IP_3$ may release calcium via $IP_3$-receptors, DAG activates PKC, and protein kinase C, which may have direct effects on the nucleus: Ang II = angiotensin II; ET = endothelin; NA = noradrenalin (according to Ref. 32).

matic reticulum (Hypothesis 2). Further studies are necessary to find out how angiotensin receptors of human left ventricular myocardium are coupled to contractility and cell growth.

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# STIMULATION OF THE $NA^+/CA^{2+}$ EXCHANGER BY ANGIOTENSIN II

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***Summary.*** Angiotensin (Ang) II exerts an inotropic, a lusiotropic, and a hypertrophic response in the heart. All of these actions have been attributed, in part, to Ang II-mediated changes in the calcium movement within the myocyte. While the effect of Ang II on some calcium transporters has been widely studied, information regarding the effect of Ang II on $Na^+/Ca^{2+}$ exchanger activity is lacking. Therefore, the aim of the present study was to examine the potential regulation of the $Na^+/Ca^{2+}$ exchanger by Ang II. Isolated sarcolemmal membranes were incubated with varying concentrations of Ang II at temperatures ranging from 10°C to 45°C and then assayed for $Na^+/Ca^{2+}$ exchanger activity. At 37°C, Ang II exhibited a dose-dependent stimulation of the $Na^+/Ca^{2+}$ exchanger, reaching a maximal activity of 4.51 ± 0.10 nmol $Ca^{2+}$ mg/sec, which was 125% greater than activity in the unstimulated membranes. The underlying mechanism by which this stimulation occurred was through a decrease in the activation energy for the $Na^+/Ca^{2+}$ exchanger reaction. Interestingly, both losartan, an $AT_1$ receptor antagonist, and chelerythrine, a protein kinase C inhibitor, significantly inhibited stimulation of the exchanger by Ang II. The potential contribution of the $Na^+/Ca^{2+}$ exchanger stimulation to the actions of Ang II in the normal and failing heart are discussed.

## INTRODUCTION

The hypertrophic, inotropic, and lusiotropic actions of Ang II in cardiac tissue are well recognized and are thought to occur via $AT_1$ receptors coupled to phosphoinositide hydrolysis. The mechanism by which phosphoinositide hydrolysis mediates this cardiac function, however, is unclear. Several investigators have proposed that alterations in calcium movement by the cardiomyocyte may play a role

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in Ang II action, an idea supported by the observation that disruption of calcium movement by use of intracellular and extracellular calcium chelators inhibits Ang II-mediated mitogenesis [1–3]. Understandably, the contractile effects of Ang II have also been linked to calcium movement [4]. Yet, the effect of Ang II on $[Ca^{2+}]_i$ is complex and species-dependent. While Baker et al. [5] and Miyata and Haneda [6] have demonstrated an elevation in $[Ca^{2+}]_i$ upon Ang II application, Kinugawa et al. [2] and Kem et al. [7] have reported Ang II-mediated decreases in calcium transients. Still other groups have seen no effect of Ang II on intracellular calcium levels [8].

The confusion surrounding the effects of Ang II on calcium handling stem in part from the complexity of its signal transduction pathway. Most studies examining Ang II regulation of calcium transporters have focused on the L-type calcium channel. The earliest studies consistently reported an increase in the slow inward calcium current following exposure of either rat heart or chick heart myocytes to Ang II [9,10]. However, Ikenouchi et al. [8] recently found no effect of Ang II on inward calcium current of beating rabbit myocytes. Ang II has also been reported to promote calcium release from the sarcoplasmic reticulum [11], but this effect cannot account for many of the actions of Ang II.

Another limitation in evaluating the calcium-modulating actions of Ang II is the paucity of information regarding potential regulation of individual calcium transporters by Ang II. Particularly noteworthy is the lack of information regarding the acute effects of Ang II on $Na^+/Ca^{2+}$ exchanger and sarcoplasmic reticular $Ca^{2+}$ pump activity. Both transporters play intimate roles in cardiac function, which is significantly altered by Ang II [4]. Thus, the aim of this study has been to examine regulation of $Na^+/Ca^{2+}$ exchanger activity by Ang II.

## METHODS

### Sarcolemmal vesicle preparation

Enriched sarcolemmal vesicles were prepared from Wistar rat hearts using the method of Pitts [12]. To verify the purity of each preparation, assays for standard marker enzymes were routinely performed. All preparations exhibited relatively high ouabain-sensitive $Na^+/K^+$ ATPase and adenylate cyclase activity but low levels of cytochrome c oxidase, oxalate-facilitated calcium transport, and p-nitrophenylphosphatase activity. The purity factor relative to the homogenate for $Na^+/K^+$ ATPase and cytochrome c oxidase was 12.2 and 0.6, respectively. Thus, the amount of sarcoplasmic reticular and mitochondrial contamination in the sarcolemmal preparation was minimal. Protein concentration was determined by the Bradford method [13].

### $Na^+/Ca^{2+}$ exchanger assay

The isolated sarcolemmal vesicles were assayed for $Na^+/Ca^{2+}$ exchanger activity using a modification of the method of Reeves and Sutko [14]. Briefly, the vesicles were loaded with a sodium buffer containing 160 mM NaCl, 20 mM MOPS,

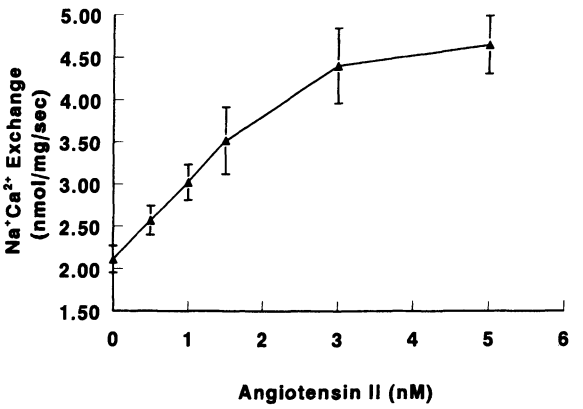


**Figure 1.** Dose-response relationship for stimulation of the $Na^+/Ca^{2+}$ exchanger by angiotensin II. Isolated rat heart sarcolemmal vesicles were loaded with a sodium buffer containing 100 μM Gpp(NH)p, and $Na^+/Ca^{2+}$ exchanger activity was assayed in the absence or presence of 0.5 to 5 nM angiotensin II. The values shown represent the means ± S.E.M of three to five preparations. All values were statistically significant relative to controls lacking angiotensin II ($P < 0.05$).

100 μM Gpp(NH)p, and 1 mM $MgCl_2$. The sarcolemmal vesicles were preincubated to reach the appropriate assay temperature (10–45°C) before Ang II (0.5 to 5 nM) or an equal volume of sodium buffer was added. After a 10 minute incubation, the $Na^+/Ca^{2+}$ exchanger reaction was initiated by the addition of the membrane (5–7 μg) to 500 μl of a potassium buffer containing 160 mM KCl, 20 mM MOPS, 40 μM $^{45}CaCl_2$, and 5 μM valinomycin. After 2 seconds, the reaction was terminated by the addition of 3 ml of ice-cold MOPS buffer containing 160 mM KCl and 1 mM $LaCl_3$ followed by rapid filtration. The filters were washed five times with the $LaCl_3$ containing buffer before the filters were dried and counted for radioactivity. All data were corrected for nonspecific binding, which is defined as $^{45}Ca^{2+}$ associated with the membrane in the absence of a sodium gradient. For the Arrhenius plot, the data were replotted according to the Arrhenius equation, and the activation energy for each condition was determined from the slope of the line.

## RESULTS

One of the first questions addressed in this study was the effect of Ang II on $Na^+/Ca^{2+}$ exchanger activity. As shown in figure 1, Ang II dose-dependently stimulated $Na^+/Ca^{2+}$ exchange with maximal stimulation occurring at an Ang II concentration of 5 nM. This value is comparable to the concentration of Ang II utilized for maximal effect in most cardiac studies [2,8]. Using the method of Hill, the $EC_{50}$ for the Ang II effect was found to be 1.0 nM.

Since the activity of the $Na^+/Ca^{2+}$ exchanger is quite sensitive to the phospholipid environment [15], we next examined whether Ang II could alter the transition temperature for $Na^+/Ca^{2+}$ exchange, which is indicative of a change in membrane

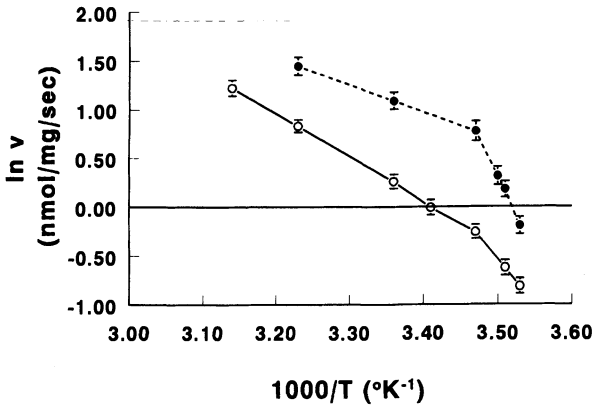


**Figure 2.** Arrhenius plot of the $Na^+/Ca^{2+}$ exchange reaction. $Na^+/Ca^{2+}$ exchanger activity was assayed in rat heart sarcolemmal vesicles over a temperature range of 10°C to 45°C in the absence (O) or presence (●) of 2nM angiotensin II. $Na^+/Ca^{2+}$ exchanger activity data was expressed as In of the initial velocity at the indicated inverse temperatures ($°K^{-1}$). Values shown represent the means ± S.E.M. of four preparations.

environment. In order to obtain information on the transition temperature, $Na^+/Ca^{2+}$ exchanger activity was evaluated from 10 to 45°C, and the data were plotted according to the Arrhenius equation. As expected from the temperature dependence of other membrane functions, a characteristic break occurred at 15°C, which is referred to as the transition temperature (figure 2). Interestingly, treatment of sarcolemmal vesicles with 2nM Ang II did not alter the $Na^+/Ca^{2+}$ exchange transition temperature. Based on the Arrhenius plot, the activation energy of the $Na^+/Ca^{2+}$ exchange reaction in the absence and presence of 2nM Ang II was 9.09kcal/mol and 5.18kcal/mol, respectively.

To further investigate the mechanism by which Ang II might enhance exchanger activity, the specific receptor subtype mediating the Ang II effect was determined using the $AT_1$ receptor antagonist losartan. Shown in figure 3, 10μM losartan significantly attenuated the stimulation of $Na^+/Ca^{2+}$ exchange by 5nM Ang II, implicating the $AT_1$ receptor in Ang II action. Since the $AT_1$ receptor is coupled to phosphoinositide hydrolysis with the subsequent activation of protein kinase C [16], the involvement of protein kinase C in the stimulatory effect of Ang II on $Na^+/Ca^{2+}$ exchange was determined. For this study, the protein kinase C inhibitor chelerythrine was used. Ang II-mediated stimulation of the $Na^+/Ca^{2+}$ exchanger was significantly depressed from 125% to only 15% by 25μM chelerythrine. Since the concentration of chelerythrine utilized in this study is reported to selectively inhibit protein kinase C [17], this result suggests that Ang II may activate the exchanger through a protein kinase C-dependent mechanism. This finding is supported by Iwamoto et al. [18], who found that protein kinase C phosphorylation enhances $Na^+/Ca^{2+}$ exchanger activity.

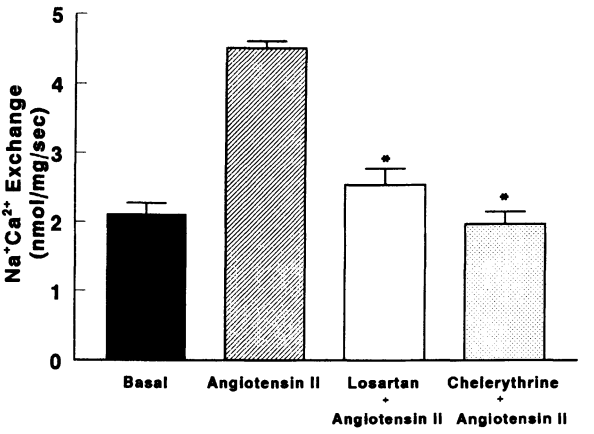


**Figure 3.** Effect of regulators of angiotensin II action on $Na^+/Ca^{2+}$ exchanger activity. Sarcolemmal vesicles were isolated from rat hearts and loaded with sodium buffer containing 100 μM Gpp(NH)p. $Na^+/Ca^{2+}$ exchange activity was assayed in the absence of angiotensin II (solid bar), in the presence of 5 nM angiotensin II alone (striped bar), or in the presence of 5 nM angiotensin II plus either 10 μM losartan, an AT1 receptor antagonist (open bar), or 25 μM chelerythrine, a protein kinase C antagonist (stippled bar). Values shown represent the means ± S.E.M. of four preparations. *significant difference from angiotensin II group.

## DISCUSSION

The most significant finding of this study is the acute stimulation of $Na^+/Ca^{2+}$ exchanger activity by Ang II. Since the $Na^+/Ca^{2+}$ exchanger is intimately involved in the regulation of cardiac function, this finding has particular relevance to the cardiac effects of Ang II, which include inotropic, lusiotropic, and hypertropic effects.

Although widely studied, the mechanism underlying the inotropic action of Ang II is still controversial, partly because of the complexity and species-dependent variations of Ang II action. Two hypotheses have been developed to explain the positive inotropic effect of Ang II. Baker et al. [5] proposed that elevations in intracellular calcium might mediate the positive inotropic action of Ang II. This hypothesis was initially based on the observation of several groups that Ang II augmented L-type calcium channel current [9,10]. However, in a more recent study, Ikenouchi et al. [8] reported no effect of Ang II on this channel. These conflicting data suggests that perhaps other calcium transporters may be involved in Ang II action. Since the results of the present study clearly demonstrate a stimulatory action of Ang II on $Na^+/Ca^{2+}$ exchange, it is possible that reverse mode $Na^+/Ca^{2+}$ exchange may contribute to the positive inotropic effect of Ang II by increasing calcium influx during the initial phase of the action potential. Although controversial, this idea is feasible because calcium entry via $Na^+/Ca^{2+}$ exchange is thermodynamically possible and has been shown by several groups to presumably trigger calcium release from the sarcoplasmic reticulum [19,20].

The second hypothesis is based on the observation of Ikenouchi et al. [8], who demonstrated that the positive inotropic effect of Ang II can occur in the absence of increased $[Ca^{2+}]_i$. Thus, it has been proposed that Ang II may increase contractility by enhancing calcium sensitivity of the myofilaments through intracellular alkalinization [8,21]. In agreement with this hypothesis, Ang II-mediated stimulation of $Na^+/H^+$ exchange and cytosolic alkalinization has been shown to coincide with enhanced contractile function [8]. In addition, Matsui et al. [21] found that inhibition of $Na^+/H^+$ exchange by amiloride attenuated the positive inotropic effect of Ang II. An interesting caveat is that activation of the $Na^+/H^+$ exchanger should also cause a net increase in $[Na^+]_i$, thereby promoting calcium entry through reverse mode $Na^+/Ca^{2+}$ exchange. Thus, the positive inotropic action of Ang II may somehow involve reverse $Na^+/Ca^{2+}$ exchange.

Unlike the inotropic effects of Ang II, the lusiotropic action of Ang II is not caused by $Na^+/Ca^{2+}$ exchange stimulation. Nonetheless, since the primary function of the exchanger is calcium efflux during relaxation, $Na^+/Ca^{2+}$ exchanger activation may limit the extent of the negative lusiotropic action of Ang II.

The growth promoting action of Ang II is well recognized, although the particular biochemical process responsible for this action of Ang II is unclear. A few investigators have proposed that the hypertropic action of Ang II may involve a calcium-dependent MAPK phosphorylation cascade [3,22]. While the calcium requirement may conceivably be met by activation of the $Na^+/Ca^{2+}$ exchanger, this idea has not been investigated. Also, Allo et al. [23] have demonstrated that contraction is a potent mediator of cardiomyocyte cell growth, which provides a more direct link to the $Na^+/Ca^{2+}$ exchanger.

Since hypertrophy is commonly observed in numerous pathological conditions of the heart, the widespread usage of ACE inhibitors in the treatment of myocardial disease states such as congestive heart failure is not surprising [24]. However, the link between Ang II action and congestive heart failure is unclear. Several investigators have proposed that altered calcium handling by the myocyte contributes to the etiology of this disease. Recently, it has been reported that a depression in sarcoplasmic reticular CaATPase mRNA levels and an elevation in $Na^+/Ca^{2+}$ exchanger mRNA levels are associated with the development of heart failure [25,26]. This compensatory increase in $Na^+/Ca^{2+}$ exchanger mRNA levels, coupled with Ang II-mediated activation of the exchanger (figure 1), has major implications for the failing heart. Normally, a competition develops between the $Na^+/Ca^{2+}$ exchanger and the sarcoplasmic reticular calcium pump for the removal of calcium from the cytoplasm during diastole. Since the $Na^+/Ca^{2+}$ exchanger is the major transporter extruding calcium from the cell, dominance of the exchanger relative to the sarcoplasmic reticular calcium pump can lead to a net removal of calcium from the myocyte. This depletion of the intracellular calcium pool would be expected to adversely affect the contractile function of the heart and to contribute to the development of heart failure.

Ang II appears to play a major role in the development of the state of heart failure by enhancing the activity of the $Na^+/Ca^{2+}$ exchanger relative to the sarcoplasmic

reticular calcium pump. Therefore, the most important consequence of the present finding is that Ang II-mediated stimulation of $Na^+/Ca^{2+}$ exchanger activity should exacerbate the congestive heart failure state.

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# DEVELOPMENTAL REGULATION OF THE CARDIAC RENIN-ANGIOTENSIN SYSTEM: EXPRESSION AND ASSOCIATION WITH GROWTH AND APOPTOSIS

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***Summary.*** Identification and elucidation of the roles of humoral factors involved in cardiogenesis and other aspects of cardiac growth will be important for the overall understanding of how the heart develops and functions. Angiotensin (Ang) II has been demonstrated to have important in vivo and in vitro growth-related effects in the neonate and adult myocardium. However, very little is known regarding the regulation of the cardiac renin-angiotensin system (RAS) and the role of Ang II receptors at any developmental stage. Enhanced expression of RAS during fetal and neonatal development suggests that locally produced Ang II exerts autocrine and paracrine influences on cardiogenesis and cardiac growth in these stages of development. Utilization of transgenic models and specific nonpeptide Ang II receptor antagonists will be useful in determining the roles of $AT_1$ and $AT_2$ receptors, respectively, in the regulation of the cardiac RAS, myocardial growth, and apoptosis in the developing rat heart. These studies may provide a better understanding of the effects of Ang II receptors on cellular and molecular aspects of cardiac development and thus lead to improved therapeutic interventions for cardiovascular disease.

Although the physiological properties of the myocardium have been the focus of intense research during the past two decades, biochemical and molecular correlates underlying cardiac development and performance remain poorly understood. The recent development of cellular, molecular, and pharmacological tools has made it possible to study the mechanisms involved in cardiac development and to better understand the basis for important clinical and experimental problems in cardiovascular physiology. It has become apparent that cardiac growth is regulated/mediated in part through the actions of various paracrine, autocrine, and/or endocrine factors [1–3]. In the adult rat, Ang II is a direct and potent in vivo stimulus for the

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production of cardiac hypertrophy [4]. Regulation of the RAS and the specific roles of $AT_1$ and $AT_2$ receptors in mediating cardiac growth in the fetus and neonate remain to be more clearly defined. We and others have reported growth-related effects associated with $AT_1$ and/or $AT_2$ receptors utilizing cultures of neonatal cardiac cells [5,6]. In neonatal rat cardiac cells, the $AT_1$ receptor has been shown to couple to myocyte hypertrophy [6] and fibroblast proliferation [5], whereas the $AT_2$ receptor inhibits myocyte hypertrophy [6]. Since a portion of the growth effects are likely to result from locally produced Ang II, the scope of this review will be to provide a discussion of the expression of the cardiac RAS and the potential roles of the local RAS with an emphasis on growth regulation in the developing heart.

## CARDIAC MORPHOGENESIS AND GROWTH

Embryogenesis, fetal development, and growth are controlled by the coordinated actions of several humoral regulators. In the last decade, morphological, biochemical, and physiological studies have improved our understanding of how structure and function of the heart changes in sequential stages of cardiac growth and development [7–10]. The heart is the first organ to form in vertebrates and arises through a complex series of morphogenic interactions involving cells from several embryonic origins [11,12]. Soon after gastrulation (embryonic days 20 and 8 in human and rat, respectively), cardiac development is initiated by a commitment of mesodermally-derived progenitor cells to the cardiac muscle cell lineage and is followed by the formation of a primordial heart tube. Organogenesis proceeds through a series of involutions of the heart tube resulting in distinct cardiac chambers having regional-specific atrial, ventricular, and conduction system cells. Shown in figure 1 are major developmental events in the fetal heart which include formation of the cardiogenic plate (8–9 days) and primitive cardiac tube (9–10 days), initiation of beating and regionalization of the tubular heart (10–11 days), cardiac septation (11-16 days), and subsequent cardiac enlargement as a result of hyperplasia and cellular hypertrophy (17–21 days) [11,12]. Neonatal growth of the rodent heart involves three phases [11,12]. During fetal and early neonatal periods (birth to four days postpartum), the heart enlarges as a result of cardiogenesis, hyperplasia, and hypertrophy. After five days postpartum, a transition from hyperplastic to hypertrophic growth occurs [13], which results from karyokinesis without cytokinesis. Thus, in early stages of development, enlargement of the embryonic heart occurs primarily by an increase in myocyte numbers, which continues until shortly after birth, after which cardiac myocytes lose their proliferative capacity and acquire the terminally differentiated phenotype of adult cardiac muscle cells [10].

Acquisition of the divergent morphological, biochemical, electrochemical, and contractile properties of these specialized cardiac cells is primarily due to the activation of specific programs of gene expression [14]. Humoral stimulation is the major regulatory determinant of cardiac growth in early stages of cardiac development, whereas in later stages of development, both humoral and mechanical stimulation serve to modulate growth [1–3,15]. Work performed by left and right

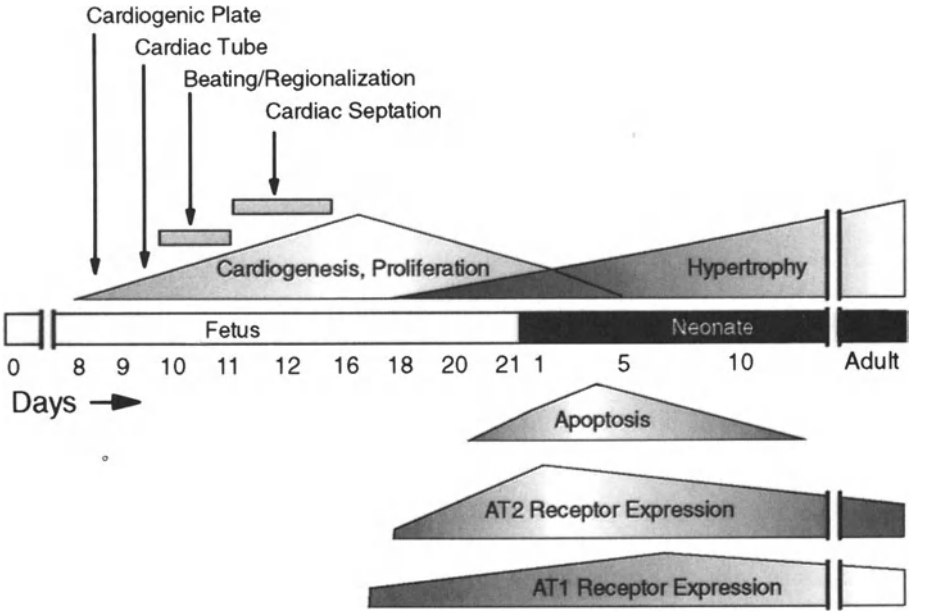


**Figure 1.** Temporal Relationship of Cardiac Development and Expression of Angiotensin II Receptors in the Rat. Following gastrulation (day 8), mesodermally-derived progenitor cells form a cardiogenic plate that is followed by the formation of a primordial heart tube at 9–10 days. Beating and regionalization of the tubular heart occurs (10–11 days), followed by cardiac septation (11-16 days) and subsequent cardiac enlargement as a result of hyperplasia and cellular hypertrophy (17–21 days). In late fetal and early neonatal periods, the heart enlarges as a result of cardiogenesis, hyperplasia, and hypertrophy. After 4 days, the neonate heart enlarges primarily as a result of hypertrophic growth. Apoptosis of cardiac myocytes and fibroblasts in the right ventricle during late fetal and early neonatal development parallels the expression of $AT_1$ and $AT_2$ receptors. At birth, the level of $AT_2$ receptor expression in cardiac tissue diminishes, whereas the level of $AT_1$ receptor increases. At 5–10 days after birth, expression of the $AT_1$ receptor also begins to decrease in the myocardium. In the adult myocardium, $AT_1$ and $AT_2$ receptors each account for 50% of the specific binding.

ventricles as they pump in parallel during fetal development is approximately equal [16] and is associated with comparable ventricular weights [17]. Following closure of the *foramen ovale* and *ductus arteriosis* after birth, blood flow is in series through the right and left ventricles, resulting in a greater volume workload on each ventricle. In the same period, pulmonary resistance is lowered by expansion of the collapsed lungs, peripheral resistance increases with loss of the placental circulation, and the pressure load on the left ventricle becomes significantly greater compared with that on the right ventricle [16,18]. Morphometric studies have documented that differences between left and right postnatal ventricular growth is primarily the result of a higher total number of myocytes in the left ventricle [19]. The number of myocytes in the left ventricular free-wall becomes greater principally one to five days after birth, and by eleven days, the left ventricle contains approximately twice the

number of myocytes as the right ventricle. This proportion between the number of myocytes in the two ventricles persists later in life, so that an approximate twofold difference is also found in the adult heart [20]. In contrast, the myocyte volume varies little in the left and right ventricles in early [17] and late postnatal maturation and in aged and senescent myocardium [20]. Similar observations have been made in humans [21]. These observations suggest that the magnitude and the distribution of programmed myocyte cell death are crucial factors in modulating the number of muscle cells in the left and the right ventricles and the interventricular septum of the myocardium. In vitro studies have demonstrated that cardiac workload affects the rate of gene transcription [14] and selectively regulates the expression of specific genes, including those of the RAS [22–24].

## IN VITRO LOCALIZATION AND REGULATION OF RENIN-ANGIOTENSIN SYSTEM COMPONENTS IN CARDIAC CELLS

Our laboratory and others have demonstrated the presence of Ang II receptors in cultured cardiac myocytes and fibroblasts [5,6,25–29]. Ang II receptor subtypes have been identified and characterized utilizing biochemical, molecular biological, pharmacological, functional, and radioligand binding studies [30]. Plasma membrane angiotensin receptors are characterized as $AT_1$ or $AT_2$, based on the binding affinity for nonpeptide antagonists, such as losartan and PD123177 (or PD123319), respectively [31–34]. Screening of the rat genomic library by utilizing molecular approaches has demonstrated that two subtypes of $AT_1$ receptor (A and B) exist [35–37]. These two $AT_1$ receptor isoforms exhibit no difference in binding to Ang II analogs, have a high degree of nucleotide sequence homology (91%) within the coding region, and lower sequence homology within the 5′- and 3′-untranslated regions (58% and 62%, respectively). High affinity $AT_1$ binding sites have been demonstrated on cultured neonatal rat cardiac myocytes [38] and fibroblasts [39]. The $AT_1$ receptor has biological actions in cardiac tissue and couples to G proteins, intracellular $Ca^{2+}$ mobilization, and activation of serine, threonine, and tyrosine kinase-mediated signaling pathways [40,41]. In contrast, less is known about the biological effects and coupling mechanisms associated with activation of the $AT_2$ receptor in cardiac tissue. Inhibition of DNA synthesis and proliferation in vascular smooth muscle and coupling of the third intracellular loop of the $AT_2$ receptor to Gi and protein-tyrosine phosphatase suggest that this receptor is a negative regulator/modulator of DNA synthesis and cell proliferation [42–44].

During development, components of the cardiac RAS undergo significant changes in levels of expression. However, determinants responsible for the regulation of RAS components in fetal and neonatal hearts are unknown. In vitro studies [23,27,28] and unpublished data from our laboratory have shown that passive mechanical stretch (simulates increased afterload) upregulates several components (renin, angiotensinogen, $AT_1$ and $AT_2$ receptors) of the cardiac RAS. It is likely that residual stress (i.e., forces remaining when external load is removed), which increase ventricular wall stress because of differential growth of the myocardium during morphogenesis [45], serves to regulate regional expression of cardiac RAS compo-

nents. Humoral stimuli, including α- and β-adrenergic, endothelin, glucocorticoids, and Ang II also appear to be important regulators of the cardiac RAS [46–48]. Expression of cardiac RAS precursors in neonatal or adult rat heart are much lower than other RAS-producing tissues, such kidney and liver [49]. This suggests that circulating or locally-produced Ang II may exert negative feedback effects on this humoral system. This postulate is supported by the observation that exogenous Ang II results in significant downregulation of both renin and Ao mRNA levels in cultured neonatal rat cardiac fibroblasts [48]. Ang II has also been reported to cause time- and dose-dependent decreases in $AT_1$ receptor mRNA levels in cultured neonatal rat cardiac myocytes and fibroblasts [50]. However, in vivo effects of Ang II on regulation of RAS precursor components and Ang II receptors remain to be determined in fetal and neonatal rat hearts.

## THE RENIN-ANGIOTENSIN SYSTEM IN FETAL HEART DEVELOPMENT

The presence of Ang II receptors in fetal [34,49,51], neonatal [5,6], and adult heart [52] suggests that Ang II has a functional role in this organ, Support for a role of Ang II in fetal growth is given by clinical studies, in which administration of ACE inhibitors during pregnancy results in a high frequency of serious developmental problems, including cardiac defects [53]. However, these effects are difficult to interpret since ACE inhibitors not only block Ang II production but also affect metabolism of other humoral systems such as enkephalins, substance P, and the kinins [54]. During embryogenesis and immediate postnatal development, rat myocardium almost exclusively expresses $AT_2$ receptors [51,55]. At birth, the level of $AT_2$ receptor in cardiac tissue greatly diminishes, whereas the level of $AT_1$ receptor increases [34,49,51,55]. This dramatic shift from $AT_2$ to $AT_1$ receptors suggests that the roles of Ang II are different in fetal heart than its roles in neonatal heart. Changes in receptor subtype expression are presumably due to changes in endocrine and mechanical environments that are present after birth. Although coupling mechanisms of the $AT_2$ receptor are still under investigation, increased abundance and transient expression of the $AT_2$ receptor in the fetus suggest that these receptors have important regulator/modulatory roles in late fetal and early neonatal development [53]. Changes in the mechanism of cardiac growth (i.e., hyperplasia vs. hypertrophy) during the perinatal period [56] may reflect, and/or contribute to, changes in the relative expression of $AT_1$ and $AT_2$ receptors, similar to what has been reported in vascular smooth muscle cells during development [57]. Abundant $AT_2$ receptors on less differentiated mesenchymal cells of the rat fetus [53] suggest that Ang II may act through these receptors to regulate differentiation of cardiac cells. The induction of collagen synthesis in human cardiac fibroblasts [26] also suggests that the $AT_2$ receptor could have a role in matrix formation of the fetal heart. Because Ang II affects the mechanical activity of the heart, it is also possible that the peptide is an important regulator of the mechanical activity of the heart until the sympathetic nervous system develops and becomes functional. Sympathetic innervation of the rat heart becomes functionally mature during the early postnatal

period [58,59]. Thus, changes in the density of Ang II receptors could reflect a transition in the balance between hormonal and neural control of cardiac function that takes place during ontogeny.

## THE RENIN-ANGIOTENSIN SYSTEM IN NEONATE DEVELOPMENT

In the neonate rat heart (0–3 days), we have shown that the right ventricle contains the highest levels of renin and Ao mRNA, followed by the left ventricle and atria [60]. Convincing evidence that Ang II mediates hypertrophic growth in neonates has been obtained from studies in which enalapril and losartan were used to inhibit ACE and $AT_1$ receptors, respectively [61–63]. In vivo studies are supported by results from in vitro studies that demonstrate Ang II causes proliferation of neonatal rat cardiac fibroblasts and hypertrophy of chick cardiac myocytes [25,62–64]. In a recent study in which losartan was given to pregnant rats (in drinking water five days prior to parturition), left ventricular thickening of the neonate heart was prevented within the first 24 hours after birth [61]. Mechanisms by which the $AT_1$ receptor may mediate growth in the neonate heart are unknown. Both direct and indirect mechanisms probably contribute to this stage of cardiac growth since Ang II not only stimulates growth-related kinases, such as MAPK, and proto-oncogenes, such as c-myc, c-fos, and c-jun but also increases expression of platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-β) [41,65]. In cardiac fibroblasts [66] and myocytes [67,68], Ang II has been shown to activate the Signal Transducers and Activators of Transcription (STAT) signaling pathway, the stimulation of which has been primarily ascribed to cytokine receptors [69]. The STAT signaling pathway constitutes a direct link between the membrane receptor and gene transcription. The JAK-STAT pathway has been shown to regulate embryonic development in *Drosophila* [2,70,71], and constitutive phosphorylation of STAT3 has been associated with hypertrophic events in the myocardium [72].

Both $AT_1$ and $AT_2$ receptors appear to regulate growth of neonatal rat cardiac myocytes in that the $AT_1$ receptors stimulate growth and $AT_2$ receptors inhibit growth in these cells [6]. In vitro effects of $AT_2$ receptors on cardiac fibroblast growth are unknown since these cells do not appear to express the $AT_2$ receptor under culture conditions [5]. One of the mechanisms by which the antigrowth effects of $AT_2$ receptor stimulation is exerted is by programmed cell death (i.e., apoptosis) [73]. The transient expression of $AT_2$ receptors during the perinatal period (see figure 1) is consistent with a hypothesized role of this receptor in apoptosis [73] and coincides with programmed cell death that occurs in the heart during normal development [74]. Neonatal cardiac myocytes possess components of the RAS and are capable of releasing Ang II [13,75]. Stretching of neonatal myocytes in vitro, which mimics an elevation in diastolic stress in vivo, leads to upregulation of the $AT_1$ and $AT_2$ receptors [28], renin (D.E. Dostal, unpublished), and Ao [27] mRNA expression and autocrine formation of Ang II [27,76]. Myocardial stretching is associated with activation of apoptosis of myocytes in vitro [77]. The role of Ang II under these conditions remains to be determined. The increases

in preload that occur shortly after birth in the mammalian heart are characterized by a significant increase of apoptotic myocyte death in the ventricular myocardium [74]. Similarly, diastolic overloading of the heart, following coronary artery constriction, is characterized by an upregulation of the myocyte RAS in vivo [78] and diffuse myocyte cell death [79]. These observations suggest that a link exists between stretching of sarcomeres, synthesis and release of Ang II, and activation of apoptosis of cardiac myocytes.

In the adult rat, downregulation of bcl-2 and upregulation of bax in myocytes is associated with apoptotic cell death in myocardium surviving an acute infarct [80]. In the neonate rat, expression of bcl-2 in myocytes is inversely related to the extent of apoptosis [74]. Thus, lower levels of bcl-2 mRNA correspond to a higher incidence of programmed cell death and, therefore, appear to be a key factor in regulating the number of myocytes in the ventricular wall of the neonate, The bcl-2 proto-oncogene forms heterodimers with other members of the bcl-2 family, including bax, which promotes apoptosis [81]. The lack of heterodimerization of bcl-2 with bax eliminates its protective effect against apoptosis [81]. Thus, if bax homodimers predominate, cell death occurs, whereas if bcl-2 protein in the cell exceeds the amount of *bax* protein, apoptosis is prevented. However, an excess of bax with respect to bcl-2 triggers the suicide program of cells. The $AT_2$ receptor has been demonstrated to stimulate apoptosis and increase in vitro expression of the *bax* gene [82]. Although Ang II appears to stimulate apoptosis in vitro, further studies are needed to assess direct and indirect roles of the cardiac RAS in producing apoptosis in vivo. Since Ang II is an initial mediator following cardiac myocyte stretch, it could trigger autocrine and/or paracrine production of secondary factors which may act in concert to enhance or produce apoptosis.

## EXPRESSION AND EFFECTS OF THE CARDIAC RENIN-ANGIOTENSIN SYSTEM IN ADULT HEART

Both $AT_1$ and $AT_2$ receptor subtypes are present in adult ventricular myocardium [52,83], albeit at much lower numbers than late fetal and early neonatal stages. In adult rat myocardium, $AT_1$ and $AT_2$ receptors each account for 50% of the specific binding [37,52]. In adult heart, the spatial distribution of Ang II receptors has been well characterized. A high density of Ang II receptors is present in the atrioventricular node, cells of the intracardiac ganglia, and on parasympathetic nerve bundles in rat heart [84]. Fewer receptors have been found associated with atrioventricular bundles, and fewer have been found in the atria, ventricles, and media of the aorta, pulmonary arteries, and superior vena cava [84]. These locations of receptors are consistent with the local actions of the peptide which include inotropic and chronotropic effects. The peptide potentiates sympathetic function and inhibits vagal efferent nerve activity [85,86]. In blood vessels, Ang II is a potent vasoconstrictor, the actions of which are mediated by direct effects on vascular smooth muscle and indirectly by facilitating release and inhibition of norepinephrine at sympathetic nerve terminals [85,86]. We have previously demonstrated, using subpressor doses, that Ang II, via the $AT_1$ receptor, is a potent stimulus for inducing cardiac

hypertrophy in the rat [4]. This hypertrophic effect of Ang II is separate from increases in vascular resistance (increased afterload) caused by the peptide. Little is known regarding the functional role of the $AT_2$ receptor in adult heart. Although expression of the $AT_2$ receptor in adult rat heart is very low, it is upregulated in spontaneously hypertensive rats with the development of cardiac hypertrophy [87]. There is also a several-fold increase in angiotensinogen and ACE mRNA levels in left ventricular myocardium of the senescent rat heart [88], suggesting that expression of these genes may be altered by changes in arterial compliance that occur normally during aging. Upregulation of components of the cardiac RAS could also have a functional role by increasing local Ang II production that may compensate, at least in part, for decreased circulating levels of Ang II observed during senescence [88].

## TRANSGENIC STUDIES

Several transgenic animal models have been developed to determine the role of RAS components on biological function in the mouse and rat. The transgenic approach has distinct advantages over analysis of gene regulation using in vitro cardiac cell culture systems, since it incorporates complex cell-specific regulatory factor-DNA interactions with overall physiological conditions that affect the animal. Recently, the murine Ao gene has been deleted by homologous recombination [89]. The Ao null mice experienced high mortality by the time of weaning. Survivors were hypotensive and had evidence of vascular proliferative lesions in the kidney and cortical atrophy, possibly as a consequence of vascular insufficiency. In contrast, $AT_{1A}$ and $AT_2$ receptor null mice had no obvious morphological changes in heart and several other tissues related to abnormal development [90–93]. The large disparity between the overall developmental effects observed in Ao null mice compared with that in $AT_{1A}$ or $AT_2$ null mice suggests that Ang II is important for normal development and that development can be mediated/modulated by Ang II receptors other than $AT_{1A}$ and $AT_2$.

## FUTURE DIRECTIONS

Much remains to be determined regarding regulation of developmental growth processes of embryonic cardiac cells and hypertrophic growth of the neonatal heart. Malformation of the heart and blood vessels account for the largest number of human birth defects, with an incidence of approximately 1% of live births and 10% among stillbirths [94,95].

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# ANGIOTENSIN II REGULATED APOPTOSIS IN CARDIOVASCULAR REMODELING

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***Summary.*** Angiotensin (Ang) II exerts major influences on the heart and blood vessels via its effects on systemic hemodynamics and blood volume as well as structural effects. The major cardiovascular actions of Ang II have been reported to be mediated by the type 1 Ang II receptor or $AT_1$ receptor. Recently, we have cloned a second receptor subtype known as $AT_2$ receptor. The existence and differential expression of two different subtypes of Ang II receptors in the human myocardium and the reciprocal expression of $AT_1$ and $AT_2$ receptor in myocardial infarction and cardiac failure suggest pathophysiological importance of these receptors in cardiovascular disease and remodeling. Moreover, documented evidences suggest that the cellular composition of the heart and blood vessels is determined by the balance between apoptotic cell death (programmed cell death) and cell survival, and the unbalanced cell death plays a critical role in the pathogenesis of cardiovascular diseases and remodeling. Our successful clonings of $AT_2$ receptor cDNAs have provided a unique opportunity to study the biology and function of this receptor. Indeed, in our previous experiments, we have demonstrated that $AT_2$ receptor activates tyrosine phosphatase(s) and inhibits mitogen-activated protein kinase (MAPK) activation, thereby exerting the proapoptotic and antigrowth effects in several cells including cardiomyocytes and vascular smooth muscle cells. These antagonistic actions may contribute to the pathogenesis of cardiovascular diseases and remodeling.

Angiotensin (Ang) II, a key regulator of cardiovascular homeostasis, exerts various actions in its diverse target tissues that control vascular tone, hormone secretion, tissue growth, and neuronal activity [1]. Multiple lines of evidence have suggested the existence of Ang II receptor subtypes. At least two distinct receptor subtypes

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were defined, based on their differential pharmacological and biochemical properties, and designated as type 1 ($AT_1$) and type 2 ($AT_2$) [2,3], To date, extensive pharmacological evidence indicates that most of the known effects of Ang II in adult tissues are attributable to the $AT_1$ receptor [4–7]. In contrast, less is known about the $AT_2$ receptor. $AT_2$ receptor is abundantly and widely expressed in fetal tissues, but present only in scant levels in adult tissues including adrenal gland, brain, uterine myometrium, and atretic ovarian follicles [2,3,8–12]. We and Inagami's group recently reported the successful cloning of the $AT_2$ receptor [13,14]. Interestingly, both receptors belong to the seven-transmembrane, G protein-coupled receptor family. However, recent evidences revealed that the function and signaling mechanism of these receptor subtypes are quite different, and these receptors exert opposite effects in terms of cell growth and blood pressure regulation [15–21].

## ANTIGROWTH EFFECT OF $AT_2$ RECEPTOR

We examined the effects of expression of the $AT_2$ receptor in cultured vascular smooth muscle cells (VSMC) [15]. Rat aortic VSMC, isolated from 3-month-old rats, were transfected with the $AT_2$ receptor expression vector (fused with β-actin promoter and cytomegalovirus enhancer)-hemaggulutinating virus of Japan (HVJ)-liposome complex, since the density of endogenous $AT_2$ receptor is very low in the cultured adult VSMC. We examined the effects of this $AT_2$ receptor transgene expression on cell growth. Subconfluent $AT_2$ receptor-transfected and control vector-transfected VSMC were grown in 5% fetal calf serum. Cell number was determined daily. The $AT_2$ receptor-transfected VSMC accumulated at a significantly slower rate than the control vector transfected VSMC. We next studied the effect of the $AT_2$ expression on the growth of confluent, quiescent VSMC. VSMC were plated, grown to confluence, and transfected with the control vector or the $AT_2$ receptor expression vector. The cells were then switched to a defined serum-free condition for 3 days to induce quiescence. The cells were then treated with Ang II ($10^{-7}$M) or vehicle. Consistent with our previous results with this cell line [22], Ang II significantly increased the cell number in the control vector-transfected VSMC. This increase was abolished with the $AT_1$ receptor antagonist, DuP753, demonstrating that $AT_1$ receptor activation enhances VSMC growth in this culture. On the other hand, in the cells expressing the $AT_2$ receptor, Ang II treatment had little or no effect on cell number. However, in $AT_2$ receptor-expressing cells treated with the $AT_2$ receptor antagonist, PD123319, Ang II increased the cell number to that observed in the control vector-transfected cells. DuP 753 did not affect the "antigrowth" effect of the $AT_2$ receptor.

We next examined the effects of the expression of the transfected $AT_2$ receptor expression vector on adult VSMC in vivo using the rat carotid injury model [15]. The $AT_2$ receptor vector-HVJ-liposome complex or control vector-HVJ liposome complex was transfected into the balloon-injured rat carotid artery at the time of surgery. In some animals, we implanted PD123319 (20mg/kg/day) in osmotic minipumps intraperitoneally at the time of injury and transfection. Animals were

killed 14 days later, and the vessels were examined. $AT_2$ receptor mRNA was clearly observed in the $AT_2$ receptor-transfected vessel but only slightly in the control vector-transfected and uninjured vessels. The level of expression was comparable to that observed in whole rat fetus. The neointimal area (expressed as a ratio of medial area) in the injured vessels transfected with the control vector was not significantly different from those in the nontransfected injured vessel. Interestingly, the neointimal area of the vessels transfected with the expressing the $AT_2$ receptor transgene was significantly smaller (70% decrease) than that of the untransfected or the control vector-transfected vessels. This inhibitory effect on the development of the neointimal lesion could be blocked with the $AT_2$ receptor antagonist PD123319.

Consistent with our results, Stoll et al. [20] demonstrated that the $AT_2$ receptor exerts an antigrowth effect on coronary endothelial cells. Moreover, Booz and Berk [21] recently demonstrated that the $AT_2$ receptor in cultured cardiomyocytes shows antagonistic effect against the $AT_1$ receptor, resulting in the antigrowth effect. Taken together, these results provided us the new idea that Ang II exerts the opposite effects on cell growth in cardiovascular system via two different subtypes of the receptors.

## ROLE OF $AT_2$ RECEPTOR IN VASCULAR DEVELOPMENT

We have shown that rat vascular $AT_2$ receptor mRNA is expressed at very low levels in the rat aorta during early rat embryonic development (up to embryonic day 15, E15) but at high levels during the latter stages of development (E16–21) and in the neonatal rat [21]. Recently, Shanmugaun et al. [23] confirmed this using in situ. During this time, the rates of DNA synthesis are undergoing dramatic reductions, decreasing from 75–80% labeling rate on E14 to <5% on postnatal day 7 [15]. To examine the role of $AT_2$ receptor, we studied the effect of $AT_2$ receptor blockade on the rate of DNA synthesis in the developing rat aorta during this time window of $AT_2$ receptor expression [15]. The $AT_2$ receptor antagonist PD123319 was administered in utero 3 days prior to tissue harvest, and BrdU incorporation was measured during a 24-hour period prior to tissue harvest. At early times, when the $AT_2$ receptor is not or is minimally expressed and aortic DNA synthesis rate is at near maximum (E 15), PD123319 has no effect on DNA synthesis. However, between E16 to E21, as DNA synthesis rate falls and $AT_2$ receptor expression increases, PD123319 treatment significantly attenuates the reduction in aortic DNA synthesis. Based on these data, one would conclude that the $AT_2$ receptor modulates the growth of the developing blood vessel and thus contributes to vascular remodeling in late gestation [15].

The structural consequences of vascular $AT_2$ receptor expression in vascular remodeling await detailed analysis. An examination of the mice harboring disruptions in the gene encoding the $AT_2$ receptor offers a unique opportunity to study the embryonic actions of the $AT_2$ receptor. We and Inagami's group have reported that the $AT_2$ knockout mouse exhibits an enhanced acute blood pressure response to low-dose Ang II infusion [18,19]. Inagami's group also reported that the knock-

out mouse was hypertensive compared to the wild type animal. These data suggest that the transient and developmentally regulated $AT_2$ receptor expression in the fetus exerts a long-term effect on blood pressure, possibly via its influence on vascular structure.

## ANGIOTENSIN II-REGULATED APOPTOSIS

Interestingly, greater than 99.9% of the ovarian follicles present at birth undergo atresia, an event dependent on apoptosis or "programmed cell death", and the $AT_2$ receptor expression is tightly associated with atresia [10,24]. $AT_2$ receptor is also abundantly expressed in immature brain and some specific regions of the adult brain [11,12]. Approximately half of the neurons produced during embryogenesis normally die by apoptosis before adulthood. Moreover, Langille and colleagues [25] demonstrated that postpartum remodeling of the aorta and umbilical artery were due, in part, to apoptosis. Interestingly, this apoptosis also occurs at a time when the $AT_2$ receptor in abundantly expressed. These results lead us to examine the effect of $AT_2$ receptor on the induction of the apoptosis.

We have examined the effect of Ang II on the apoptosis in cultured VSMC. After serum growth factor depletion, VSMC showed typical morphological changes of apoptosis and internucleosomal DNA fragmentation, and Ang II inhibited the apoptosis via $AT_1$ receptor [26]. We also observed that nitric oxide (NO)-donor molecules, *S*-nitroso-*N*-acetylpenicillamine or sodium nitroprusside, induced apoptosis in cultured rabbit VSMC, and $AT_1$ receptor stimulation blocked apoptosis, suggesting that countervailing balance between NO and Ang II may determine the overall cell population within the vessel wall by regulating genetic programs that determine cell death as well as cell growth [26]. In contrast to the effect of the $AT_1$ receptor, we demonstrated by using the $AT_2$ receptor transfected VSMC that selective $AT_2$ receptor stimulation enhanced apoptosis [16]. The function of the $AT_2$ receptor in the myocardium has not been well defined. A preliminary study has confirmed an antigrowth action for this receptor on the cardiac myocyte [21]. Consistent with this observation, our preliminary experiments demonstrated that $AT_1$ receptor blocked apoptosis and $AT_2$ receptor enhanced apoptosis in cultured neonatal cardiomyocytes [27].

In order to examine the molecular and cellular mechanism of $AT_2$ receptor-induced apoptosis, we applied PC12W cells, which express abundant $AT_2$ receptor but not $AT_1$ receptor. Ang II counteracted survival effect of nerve growth factor (NGF) and induced apoptosis in a time- and dose-dependent manner [17]. This Ang II-induced apoptosis was blocked by a selective $AT_2$ receptor antagonist, PD123319. We studied the effect of $AT_2$ receptor on mitogen-activated protein kinase (MAPK), which is a key regulator in several kinase cascades and a cell survival signal for cell growth and apoptosis [28], and observed that Ang II-inactivated MAPK ($p42^{MAPK}$ and $p44^{MAPK}$). Next we examined the effects of vanadate and PTX on the $AT_2$ receptor-mediated apoptosis. The addition of vanadate or PTX, in fact, attenuated the $AT_2$ receptor-mediated apoptotic changes. However, okadaic acid did not

show any effect on the $AT_2$ receptor-mediated apoptosis. These results demonstrate that the G protein-coupling mechanism and resultant PTPase activation participate in the induction of $AT_2$ receptor-mediated apoptosis in PC12W cells. We next examined the effects of $AT_2$ receptor on Bcl-2 activation by prelabeling the cells with $^{32}$P-Pi followed by immunoprecipitation with Bcl-2 antibody and observed that NGF activated Bcl-2 by phosphorylation, whereas $AT_2$ receptor stimulation blocked this NGF effect [29]. Pretreatment with antisense oligonucleotide of MKP-1 inhibited the effect of $AT_2$ receptor on the inactivation of MAPK as well as Bcl-2 dephosphorylation. MKP-1 antisense treatment also attenuated the $AT_2$ receptor-induced apoptosis. Taken together, these results suggest that $AT_2$ receptor activates MKP-1 via G protein coupling, resulting in the inactivation of Bcl-2 and the induction of apoptosis. In addition, we also observed that $AT_2$ receptor upregulates Bax expression.

## THE POSSIBLE ROLE OF ANGIOTENSIN II-REGULATED APOPTOSIS IN CARDIOVASCULAR DISEASES

Aoptosis plays an important role during development of many structures. This process occurs in the adult as well as during development. Apoptosis has been extensively studied for its involvement in the immune response, tumor cell growth and regulation, and many other physiological processes. Documented evidence of apoptosis in cardiovascular tissues has been limited until quite recently. Accumulating evidences suggest that the cellular composition of the heart and blood vessels are determined by the balance between apoptotic cell death and cell survival. Moreover, the contribution of unbalanced cell death to some diseased states in human and animal models have been demonstrated [30–34]. We have shown [16,17,27] that the $AT_2$ receptor modulates apoptosis in several different cell lines such as PC12W cells, VSMC, cardiomyocytes and ovarian follicular granulosa cells, and R3T3 cells (mouse fibroblast cell line).

The involvement of the $AT_2$ receptor in cardiac pathophysiology remains ill defined. Increasing evidences suggest the involvement of $AT_2$ receptor in cardiovascular disease. Recently, the density of the myocardial $AT_2$ receptor has been shown to be increased in experimental myocardial infarction [35] in the hypertrophied heart [36]. $AT_2$ receptor has been showed to be increased in cardiac fibroblasts as well as in cardiomyocytes in the failing heart [37]. We postulate that the $AT_2$ receptor may contribute to this phenomenon. The existence and differential expression of two different subtypes of Ang II receptors in the human myocardium may have pharmacological implications, and the reciprocal relationship between $AT_1$ and $AT_2$ receptor densities (i.e., increased $AT_2$ and reduced $AT_1$ receptor levels) in conditions of impaired ventricular function as reported [38] would suggest pathophysiological importance of these receptors in cardiac remodeling. If apoptosis is indeed an important action of the myocardial $AT_2$ receptor, then treatment with selective $AT_1$ receptor antagonist may have interesting cardiac remodeling effects that have not heretofore been appreciated. Thus, it is intriguing to question if the regression of cardiac hypertrophy in response to $AT_1$ receptor antagonist therapy is

in part due to the apoptotic action of $AT_2$ receptor activation. Furthermore, our observation of a functional antagonism between $AT_1$ and $AT_2$ receptors in vascular smooth muscle cells may have significant pathophysiological and pharmacological implications in the myocardium as well.

**ACKNOWLEDGMENT**

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# THE ROLE OF ANGIOTENSIN II IN STRETCH-ACTIVATED SIGNAL TRANSDUCTION OF THE NORMAL, HYPERTROPHIED, AND FAILING ADULT HEART

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***Summary.*** Pathological cardiac hypertrophy is in a critical initial adaptive response that is common to human heart failure syndromes of diverse etiologies. It is clear from work using neonatal myocytes that static stretch can produce an increase in protein synthesis and recapitulate the fetal gene program characteristic of rodent cardiac hypertrophy. Some data suggest that this may be entirely a consequence of an autocrine action of angiotensin II. In order to address this issue in the adult whole heart, we produced pathophysiological levels of stretch and isolated buffer-perfused isovolumically contracting guinea pig left ventricles. Our results demonstrate that stretch can activate the phospholipase C signaling pathway in the adult heart. Inositol phosphate accumulation, but not protein Kinase C (PKC) translocation, is abolished by angiotensin II receptor blockade. Based upon these results, we studied transgenic mice that overexpress Gαq and PKC-$\beta_2$ in a cardiac-specific postnatal manner. The results demonstrate a dose-dependent induction of cardiac hypertrophy and heart failure in genetically engineered mice. This suggests that phospholipase C activation plays a critical role in hypertrophy and failure. Treatment of the PKC-$\beta_2$ transgenic mouse with a highly specific inhibitor largely reverses the phenotype. Taken together, these results demonstrate that angiotensin II-independent mechanisms may play a crucial role in the activation of the hypertrophy program and suggest new directions for pharmacotherapy of congestive heart failure.

## PHYSIOLOGICAL AND PATHOLOGICAL HYPERTROPHY

Pathological cardiac hypertrophy is a critical initial adoptive response to abnormal global or regional increases in cardiac work. Initially, the increase in cardiac chamber mass serves to normalize wall stress and to permit normal cardiovascular function at

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rest and during exercise in compensated hypertrophy. Clinicians have long understood that if the stimulus for pathological hypertrophy is sufficiently intense or prolonged, decompensated hypertrophy and heart failure may ensue.

Decompensated pathological hypertrophy is characterized by an increase in chamber wall stress despite an increase in cardiac mass and is associated with symptoms and signs of pulmonary and circulatory congestion (figure 1). The molecular events that are unique to compensated and decompensated hypertrophy are being elucidated by studies that employ genetically engineered mice, conventional experimental animal models, and clinical investigation [1]. The increase in cardiac mass in pathological hypertrophy is largely a consequence of an increase in the size of terminally differentiated cardiomyocytes, which comprise only one-third of the total cell number in the heart but are responsible for over 70% of cardiac volume. Vascular smooth muscle cells, endothelial cells, and fibroblasts can undergo hyperplasia, but there is little data to suggest that cardiomyocytes are capable of reentering the cell cycle. There is, however, some evidence to suggest that human end-stage cardiac hypertrophy is associated with some degree of apoptosis (figure 2). The precise mechanism(s) wherein the physical stimulus of increased external work is converted to the subcellular biochemical processes that are responsible for change in the cardiac phenotype are poorly understood.

## MECHANOTRANSDUCTION IN NEONATAL CARDIOMYOCYTES

Dynamic or static stretch of the neonatal or adult cardiomyocyte, papillary muscle, isolated or intact heart appears to be necessary and sufficient to produce an increase in protein synthesis and resultant cardiac hypertrophy. This process whereby stimuli in the physical domain activate intracellular growth-signaling pathways is known as mechanotransduction [2]. Data from a number of laboratories have indicated that this process may be mediated in part in the cardiomyocyte by stretch-activated sarcolemmal ion channels, $Na^+/H^+$ antiporters, tyrosine kinase containing receptors, an extracellular matrix integrin linked pathway, or G protein-coupled receptors. These mechanotransducers can then activate cytosolic signaling pathways that initiate gene transcription and translation of increased quantities of proteins. In particular, it has been demonstrated that in neonatal cardiomyocytes, mechanical deformation can activate the phospholipase C signaling pathway. Cardiomyocytes and fibroblasts possess G protein-coupled $AT_1$ receptors for angiotensin (Ang) II that activate this pathway. Binding of Ang II to its cognate receptor produces dissociation of a specific heterotrimeric G protein, Gq, which dissociates to a GTP-bound $G\alpha q$ subunit and a $\beta\gamma$ subunit. $G\alpha q$ subsequently activates its effector enzyme, phosphoinositide-specific phospholipase $C_\beta$, which is intimately associated with the cytoplasmic face of the plasma membrane and hydrolyzes the membrane lipid phosphatidyl inositol-4–5 bisphosphate. Two biologically active intracellular second messengers diacylglycerol (DAG) and inositol triphosphate ($IP_3$), are generated by this catalytic process. DAG activates the serine threonine kinase PKC, while $IP_3$ binds to its receptor on the endoplasmic reticulum (figure 3). Protein kinase C

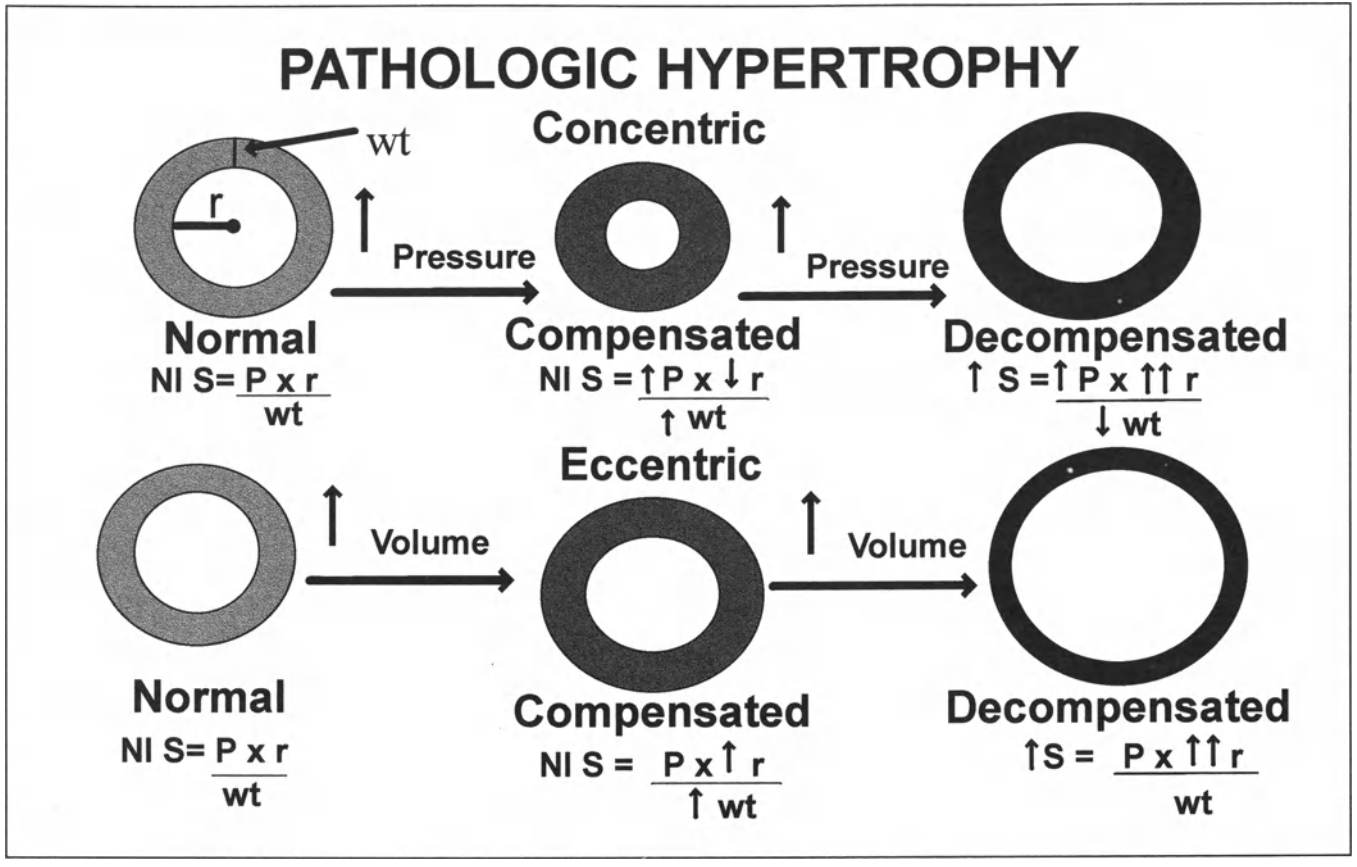


**Figure 1.** Schematic diagram of the continuum between compensated and decompensated cardiac hypertrophy in response to augmented external cardiac work from pressure or volume overload. S = wall stress, r = radius of the left ventricle, wt = wall thickness.

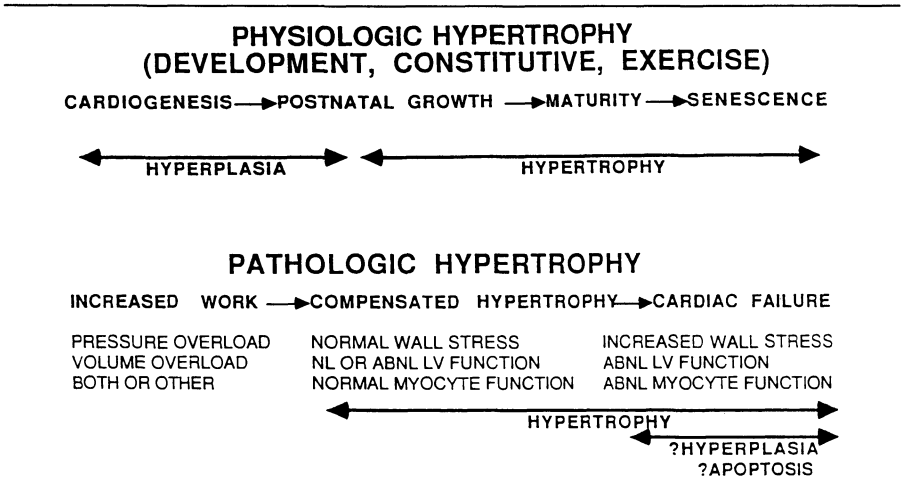


**Figure 2.** Schematic diagram which depicts the relationship among organ level physiological hypertrophy, pathologic compensated and decompensated hypertrophy and cellular hyperplasia, hypertrophy and apoptosis. (Reproduced with permission of author and publisher, from Walsh and Dorn, Hurst's the Heart, 9th Ed, chapter 6.

(PKC) is known to be a potent mediator of transcriptional regulation and hypertrophy in multiple cell types, and inositol trisphosphate has been demonstrated to contribute to cellular calcium homeostasis by the release of calcium from the endoplasmic reticulum after binding to its specific receptor. The cardiomyocyte sarcoplasmic reticulum contains $IP_3$ receptors, but the role of this polyphosphate in cardiac excitation-contraction coupling is unclear. It does appear than the ryanodine receptor is the principal intracellular calcium receptor responsible for binding of calcium, with resultant calcium-induced calcium release since it is over twentyfold more abundant than the $IP_3$ receptor. Interestingly, however, the $IP_3$ receptor appears to be upregulated in human cardiomyopathic congestive heart failure.

Although valuable information regarding mechanotransduction and hypertrophy has been learned from the study of neonatal myocytes, there is little information regarding the mechanisms wherein this process occurs in the adult left ventricle (LV). Specifically, we have been interested in whether and to what extent pathophysiological levels of mechanical stretch can activate the phospholipase C cell-signaling pathway in the adult heart by an Ang II-dependent mechanism.

## MECHANOTRANSDUCTION IN THE ADULT LEFT VENTRICLE

In order to examine this issue, we studied isolated buffer-perfused isovolumically contracting adult guinea pig hearts at physiological and pathophysiological levels of minimum left ventricular (LV) diastolic pressures [3]. Specifically, LV diastolic pressure was manipulated between 5 mmHg and 25 mmHg by augmenting the left ventricular balloon volume. The elevated level of minimum diastolic pressure that

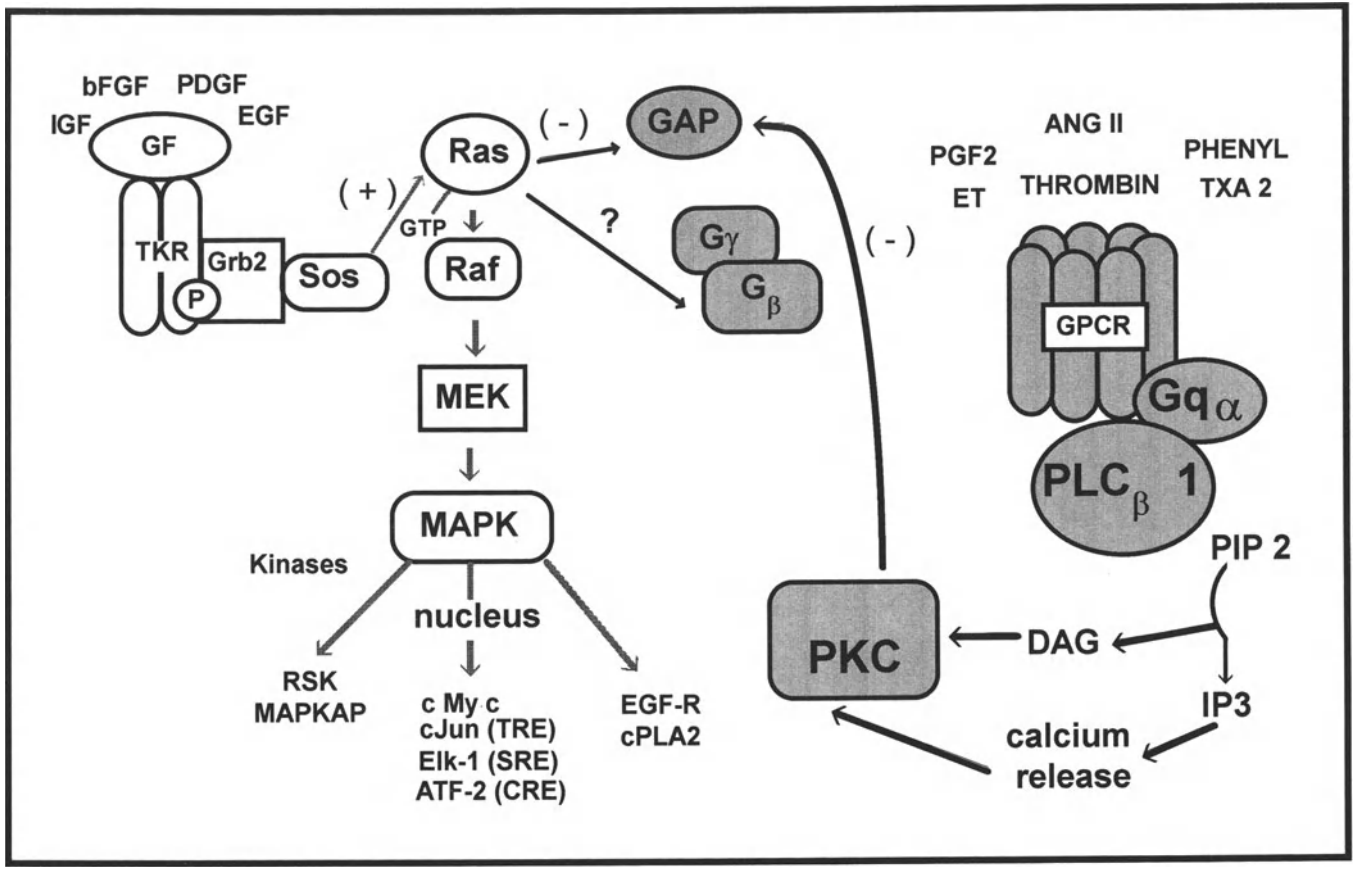


**Figure 3.** Extracellular and intracellular pathways common to phospholipase C and tyrosine kinase-mediated signal transduction in the cardiomyocyte. Angiotensin II, endothelin, norepinephrine, and prostaglandin F1α are known to be elevated in plasma of heart failure patients. Each of these peptides binds to its cognate G protein-coupled receptor (GPCR) and activates phospholipase C via the action of Gαq. Growth factors (GF) bind to tyrosine kinase receptors (TKR) and have been shown to produce hypertrophy in neonatal cardiomyocytes via mitogen-activated protein kinase (MAPK) activation. There is less evidence that this pathway is activated in the adult heart.

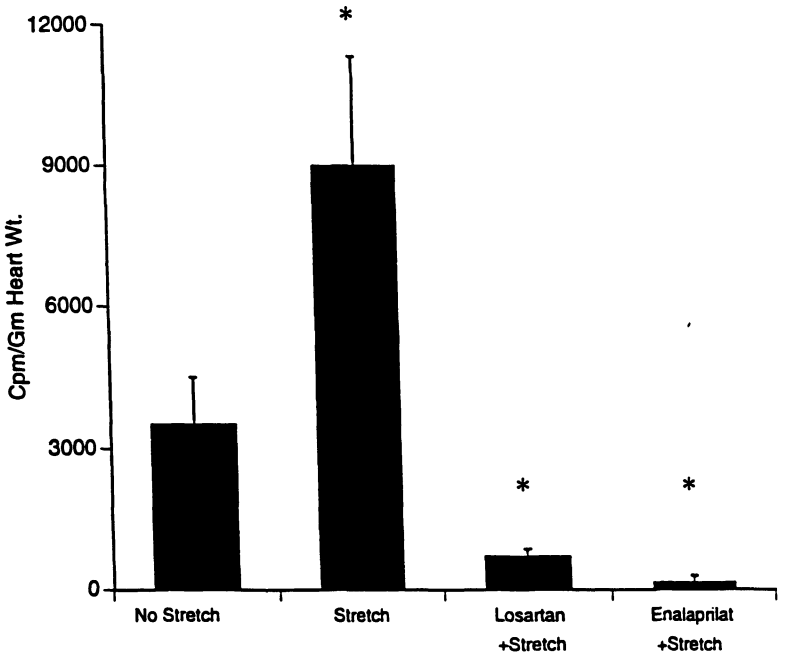


**Figure 4.** Quantitative inositol phosphate accumulation in control hearts (n = 10), hearts in which the LV was stretched for 30 minutes (n = 7), and hearts stretched for 30 minutes in the presence of 1 Mmol/l losartan (n = 3) or 1 Mmol/l enalapril at (n = 3). (Reproduced with permission of author and publisher from Paul et al., Circ Res 81:643–650, 1997.)

we chose is commonly present in human heart failure. Using this approach, we examined the effects of mechanical deformation of the LV on the phospholipase C-signaling pathway by quantitative immunoblotting of PKC isoforms and HPLC analysis of inositol phosphate production in the presence and absence of $AT_1$ receptor blockade and angiotensin-converting enzyme (ACE) inhibition using losartin and enalapril, respectively.

The results indicated that Ang II, phorbol ester stimulation, and pathophysiological mechanical deformation each stimulate phosphatidylinositol hydrolysis (figure 4) and translocate PKC (figure 5) in LV myocardium. Both angiotensin receptor inhibition with losartin and ACE inhibition with enalaprilat completely abolished inositol phosphate accumulation but attenuated PKC translocation. To our knowledge, these were the first data in the adult heart that demonstrated an ex vivo localized production of Ang II and resultant coupling to downstream signal transduction. Finally, we observed that PKC activation by phorbol ester stimulation produced a negative inotropic effect in the isovolumically contracting buffer-perfused guinea pig LV.

These results in the isolated adult heart differed in some respects to prior studies in neonatal cardiomyocytes. Sadoshima and colleagues [4] demonstrated the impor-

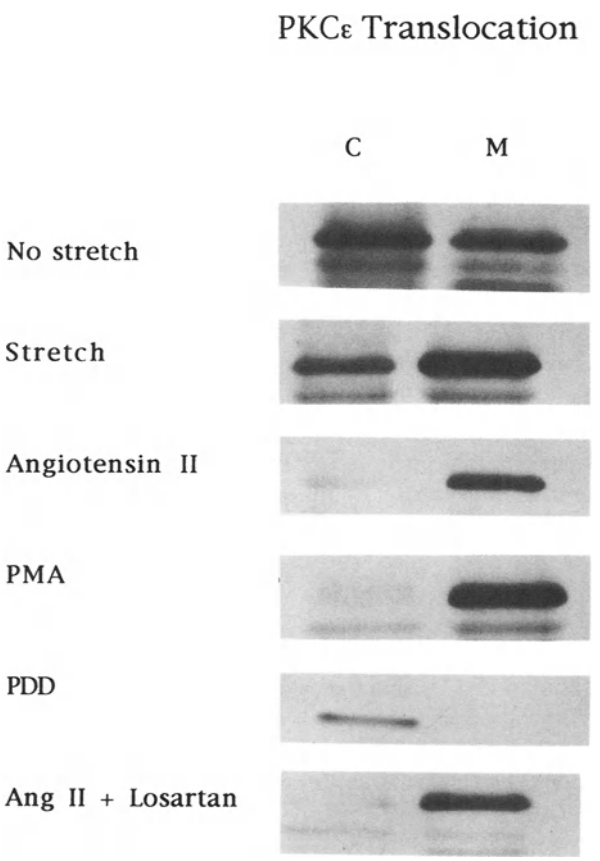


**Figure 5.** Immunoblots of PKC translocation from the cytosol (C) to the particulate fraction (M) in buffer-perfused isovolumically contracting guinea pig hearts. Blots are as follows from top to bottom: absence of stretch (no stretch); 25 mmHg minimum diastolic pressure (stretch); 10 Mmol Ang II; phorbol ester (PMA); inactive congener of phorbol ester (PDD); and Ang II plus losartan. (Reproduced with permission from author and publisher from Paul et al., Circ Res 81:643–650, 1997.)

tance of Ang II in stretch-induced production of the hypertrophy phenotype in cultured neonatal cardiomyocytes. The addition of Ang II under these conditions augmented protein synthesis and induced immediate early genes and growth factors. $AT_1$, but not $AT_2$, receptor blockade prevented these actions. Static stretch of these neonatal cells reproduced these events, whereas stretch-induced hypertrophy was prevented by $AT_1$ receptor blockade. Additional studies that employed immunoelectron microscopy suggested that this effect was mediated by an autocrine action of Ang II. In the adult heart, it also appears that pathophysiological distension of the LV activates phospholipase C-mediated signal transduction. However, $AT_1$ receptor blockade completely inhibits $IP_3$ accumulation, but not activation of PKC.

Our results in the adult LV more closely mirror the observations found by Komuro and colleagues [5], who also employed static stretch of cultured neonatal cardiomyocytes to examine the role of stretch-induced Ang II-mediated activation of phospholipase C. They found that static stretch increased inositol phosphate levels and that translocation of PKC was attenuated but not blocked by $AT_1$ inhibition. Our studies in the adult guinea pig heart demonstrated stretch-induced PKC translocation. However, the incomplete inhibition of PKC activation by $AT_1$ receptor blockade suggests the presence of Ang II-independent processes for stretch-mediated signal transduction in mature myocardium. It is possible that other Gq-coupled receptors such as the endothelin or $\alpha_1$-adrenergic receptor are activated by stretch in the adult heart. Alternatively, it is conceivable that stretch may stimulate phospholipase D-mediated hydrolysis of phosphatidylcholine. The resultant formation of phosphatidic acid and its metabolism to DAG may activate PKC.

Stretch-induced isoform translocation was PKC isoform-specific in the adult normal guinea pig whole heart homogenate. We immunologically identified PKC $\alpha$, $\varepsilon$, and $\zeta$ isoforms under these conditions. The PKC $\alpha$ and $\zeta$ isoforms that we detected in total protein extracts most likely originated from nonmuscle cells in the heart or from the atria. We found that stretch-mediated phospholipase C hydrolysis selectively induced translocation of PKC $\varepsilon$, but not $\alpha$ or $\zeta$, from the cytosol to the particulate fraction.

The ability of phorbol esters to translocate PKC from the cytosolic to the membrane fraction of various homogenates has been used as an indicator of PKC activation. A number of laboratories have demonstrated that phorbol esters may modulate contractile function of neonatal and adult rat cardiomyocytes and perfused hearts. We demonstrated that phorbol ester produced significant negative inotropy in the adult isovolumically contracting guinea pig LV (figure 6). The mechanisms by which phorbol ester-induced PKC activation may contribute to contractile depression of the heart include decreases in myofilament calcium sensitivity, alterations in the L-type calcium current, or altered cardiomyocyte calcium cycling. In particular, there is evidence that the PKC-dependent phosphorylation of troponin I and C may lead to a decrease in myofilament sensitivity to calcium and to reduction of myofibrillar actin-myosin ATPase activity and could contribute to a negative inotropic response [6,7]. Given these findings, it is conceivable that enhanced constitutive and/or stretch-activated phospholipas C hydrolysis could contribute to altered function of the hypertrophied and failing heart. In order to examine the effects of in vivo activation of phospholipase C devoid of the complex combinatorial alterations that occur in conventional animal models, a transgenic analysis of the role of cardiac-specific G$\alpha$q and PKC-$\beta$ overexpression was performed.

## TRANSGENIC ANALYSIS OF CARDIAC GαQ OVEREXPRESSION

The potential deleterious effects of activation of phospholipase C signal transduction was suggested by a variety of studies which employed cultured neonatal rat cardiomyocytes and which demonstrated that $\alpha$-adrenergic receptor stimulation,

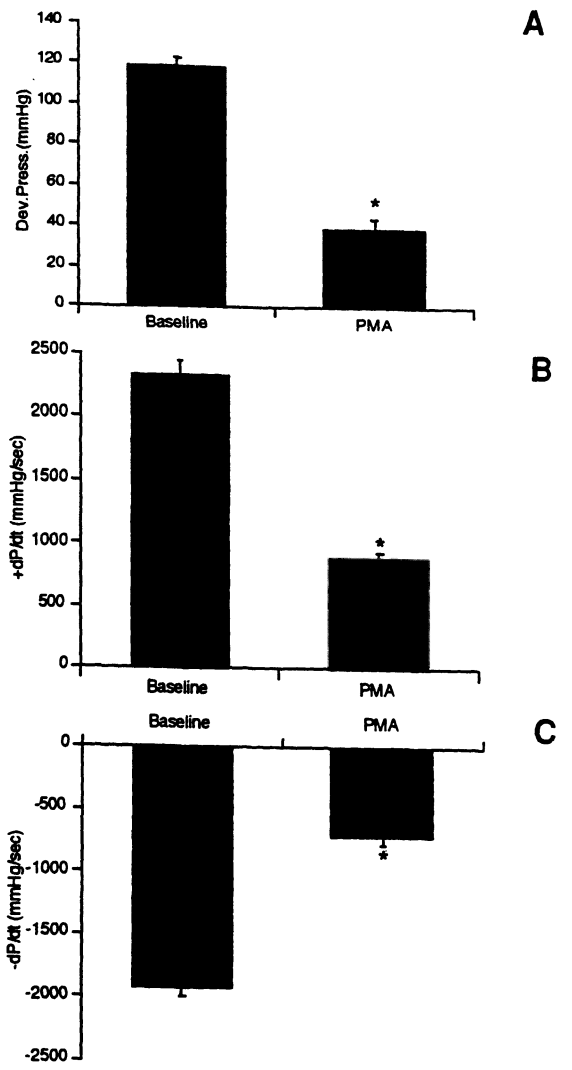


**Figure 6.** Group data for isolated heart mechanics of hearts at baseline and after stimulation for 30 minutes with phorbol ester (PMA, 100 mmol), showing a decrease in developed pressure (Dev. Press.) (*A*), maximum dP/dt (rate of contraction); and (*B, C*) minimum dP/dt (rate of relaxation). (Reproduced with permission of author and publisher from Paul et al., Circ Res 81:643–650, 1997.)

endothelin, or $PGF_{\alpha}$ stimulate hypertrophy in vitro [8–11]. In addition, transgenic overexpression of a constitutively activated mutant $\alpha_1$-adrenergic receptor produced mild cardiac hypertrophy [12]. D'Angelo and colleagues, therefore, transgenically overexpressed the wild type mouse $G\alpha q$ protein in a postnatal cardiac-specific manner using the $\alpha$-myosin heavy-chain promoter [13]. Generation of three

different mouse lines with variable copy numbers of the transgene demonstrated a dose-dependent effect on production of cardiac hypertrophy, recapitulation of the fetal gene program, and mortality. Interestingly, cardiac overexpression of Gαq in vivo failed to increase MAPK phosphorylation in contrast to in vitro studies in neonatal cardiomyocytes (figure 3). The ε isoform of PKC was, however, activated with translocation to the particulate fraction of cardiac homogenates. Importantly, echocardiographic and invasive high-fidelity micromanometer hemodynamic studies demonstrated contractile depression associated with the cardiac hypertrophy. Higher level expression of Gαq resulted in frank congestive heart failure and increased mortality.

## TRANSGENIC ANALYSIS OF CARDIAC PKC-β OVEREXPRESSION

The induction of cardiac hypertrophy and failure by cardiac-specific overexpression of Gαq and resultant activation of phospholipase $C_{\beta 1}$ may occur as a consequence of PKC activation, $IP_3$-mediated modulation of calcium homeostasis, or both arms of this bifurcated cell signaling pathway. Therefore, Wakasaki and colleagues produced postnatal cardiac-specific overexpression of PKC-$\beta_2$ to directly test the hypothesis that sustained activation of PKC can cause cardiomyopathic disease [14]. The rationale for the selection of PKC-$\beta_2$ was that this isoform is preferentially activated in aorta, retina, renal glomeruli, and hearts of diabetic animals. Furthermore, oral administration of a PKC-β-specific inhibitor ameliorated some of the early eye and kidney dysfunction in diabetic rats. Transgenic mouse lines that overexpressed PKC-$\beta_2$ again demonstrated dose-dependent cardiomyopathic changes at the gross morphological, ultrastructural, and functional levels. Specifically, transthoracic echocardiography demonstrated substantial increases in LV mass and decreases in fractional shortening, but no difference in systemic arterial pressure by tail cuff determination. Oral administration of the selective PKC-β isoform inhibitor beginning at age three weeks for a two-month period largely reversed the changes in cardiac mass geometry, function, and histology compared to nontransgenic litter mates. This study represented the first successful overexpression of a PKC isoform in the cardiovascular system and unambiguously demonstrates that activation of protein kinase can produce cardiac hypertrophy, contractile depression, and heart failure in vivo.

## THERAPEUTIC IMPLICATIONS

ACE inhibitors have been widely utilized in early and late human congestive heart failure of diverse etiologies. These agents have been shown to improve subjective and objective functional capacity and to modestly improve survival relative to diuretic and digoxin treatment in patients who are in NYHA functional class 4. Importantly, however, the mortality of patients who have advanced heart failure and are treated with ACE inhibitors remains at approximately 40% at four years. More recently highly specific $AT_1$ receptor blockers have been introduced or are under development for the treatment of hypertension and heart failure. Blockade of the

angiotensin receptor itself avoids the side effects associated with concomitant elevation of bradykinin levels that occur as a consequence of ACE inhibition. In addition, in contrast to rodents, nonhuman primates and man generate angiotensin by a predominant ACE-independent pathway involving a highly specific chymase [15]. Whether and to what extent $AT_1$ receptor blockade is clinically superior to ACE inhibition for the management of hypertension and heart failure because of more complete Ang II inhibition is currently under investigation.

Neurohormonal activation in early and late heart failure involves elevated plasma levels of endothelin, norepinephrine, angiotensin, and PGF-1α. Activation of the phospholipase C signal transduction pathway is the common mechanism whereby these peptides produce their biochemical effects. Pharmacological antagonism of each of these molecules has been accomplished, and such agents are individually utilized in patients with heart failure. Our transgenic analyses of the in vivo effects of overexpression of Gαq and isoform-selective PKC accumulation suggest the possibility of novel therapeutic strategies for congestive heart failure. Specifically, it may be possible to pharmacologically inhibit Gαq or to maintain it in its heterotrimeric form or to produce isoform-specific PKC inhibitors that produce salutory effects in human congestive heart failure syndromes. Preliminary data from our laboratory suggests that transcriptionally mediated isoform-specific elevation of PKC occurs in human congestive heart failure. Furthermore, ongoing studies suggest that isoform-specific PKC inhibition can improve cardiomyocyte function in experimental heart failure in part by increasing calcium sensitivity of myofilament regulatory and contractile proteins.

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## D. ANGIOTENSIN BLOCKADE AND REMODELING OF HEART IN MYOCARDIAL INFARCTION

# ROLE OF ANGIOTENSIN II RECEPTOR BLOCKADE DURING REMODELING AFTER MYOCARDIAL INFARCTION

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***Summary.*** Increased levels of angiotensin (Ang) II in myocardial infarction (MI), left ventricular (LV) volume overload, and heart failure promote vasoconstriction, increased LV impedance, ischemic injury, ischemia-reperfusion (IR) injury, and LV remodeling with hypertrophy and fibrosis. One strategy for achieving cardioprotection is to decrease Ang II receptor stimulation using angiotensin-converting enzyme (ACE) inhibitors. Another strategy for achieving cardioprotection is by selective blockade of Ang II type 1 ($AT_1$) receptors or type 2 ($AT_2$) receptors using selective antagonists. Functionality has been associated mainly with $AT_1$ receptors, and $AT_1$ receptor blockade is emerging as a new class of agents for therapy of hypertension, LV hypertrophy, and congestive heart failure. The role of $AT_1$ blockade in limiting remodeling after MI has been controversial. Our recent results using the isolated rat working heart model suggest functional roles for both $AT_1$ and $AT_2$ receptor blockade in recovery from IR injury. Furthermore, chronic treatment with $AT_1$ blockade in dogs limited early LV remodeling. Thus, Ang II receptor antagonists are emerging as new cardioprotective agents, alone or in combination with ACE inhibitors.

## INTRODUCTION

Left ventricular (LV) remodeling after myocardial infarction (MI) is now becoming recognized as the principal mechanism for cardiac enlargement and heart failure, two leading causes of death and disability worldwide [1,2]. It is associated with progressive structural changes that include disruption of the supporting collagen matrix, regional dilation of the infarct zone (IZ) followed by global LV dilation, left ventricular hypertrophy (LVH) of the noninfarct zone (NIZ), and further global LV dysfunction [3]. In parallel, it is associated with healing, which involves growth of

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the NIZ and repair of the IZ with collagenous scar. Remodeling and healing are both time-dependent processes. While most infarct healing is completed over 3 weeks in rats, 6 weeks in dogs, and 3–6 months in humans [3–5], remodeling can continue for years depending on the substrate [6]. The magnitude of the problem is increasing because as post-MI survivors increase with improved therapies, the number at risk of remodeling and its consequences also increase.

Over the last decade, the role of angiotensin (Ang) II in myocardial injury, growth, healing, remodeling, and dysfunction after MI has also been recognized. Neurohumoral activation and LV volume overload after MI lead to overexpression of the renin-angiotensin system (RAS) and increased circulating and local Ang II [7]. This increase in Ang II promotes LV remodeling, LVH, fibrosis, and collagen deposition [8]. Increased Ang II also increases ischemia-reperfusion (IR) injury [9,10]. Cardioprotection against injurious effects of Ang II has therefore become an important therapeutic goal. One approach has been to decrease Ang II receptor stimulation by using angiotensin-converting enzyme (ACE) inhibitors. A new strategy is to use a new class of agents, the Ang II antagonists [8]. Selective blockade of the Ang II type 1 ($AT_1$) receptors offers the potential to block the vasoconstrictor and growth-promoting effects of Ang II in essential hypertension, LVH, and congestive heart failure.

This paper focuses on the potential role of Ang II receptor blockade in limiting LV remodeling after MI, with or without reperfusion.

## REPERFUSION AND REMODELING

Over the last decade, widespread use of thrombolytic agents, angioplasty, or bypass surgery has increased the number of survivors, with flow restored to ischemic myocardium. The algorithm "salvage of ischemic myocardium → preserve structure and shape → improve systolic squeeze → improve outcome and survival" has been tested in many studies. However, "mismatch" occurs between restoration of flow and recovery of mechanical dysfunction, despite the absence of irreversible damage [11]. This mismatch has become a major clinical problem after successful reperfusion. Thus, reperfusion after 2 hours of occlusion limits early remodeling, without reducing MI size [12]. It results in persistent postischemic LV dysfunction, or "stunning" [13], and does not guarantee that function will subsequently improve [14–17]. Reperfusion within 2 hours of the onset of acute MI is ideal [14] but is frequently not feasible [18]. Consequences of IR injury include lethal arrhythmias, lethal cell injury, and sublethal stunning [9]. The algorithm "reperfusion → decrease infarct size → limit remodeling → improve function" often fails. The survivors with persistent postischemic LV dysfunction remain at risk for progressive LV remodeling and its consequences [1,2,14]. These include infarct expansion and thinning, LV aneurysm, dilation, rupture, failure, LVH, volume overload, arrhythmias, and death. Reperfusion also damages the extracellular collagen matrix [19,20]. Adjunctive therapies that limit or prevent ischemia-reperfusion (IR) injury, speed functional recovery, protect collagen matrix, and limit further remodeling are therefore needed [3].

Main mechanisms proposed for stunning (table 1) include oxygen-derived free radical generation, oxidative stress, uncoupling of excitation-contraction (due to sarcoplasmic reticulum dysfunction), calcium overload, impaired metabolism (decreased mitochondrial energy production versus impaired energy utilization by myofibrils), impaired sympathetic neural responsiveness, decreased sensitivity of myofibrils to calcium, impaired perfusion and damage to the extracellular collagen matrix, and possibly myocardial hibernation.

## ACE INHIBITION VERSUS ANGIOTENSIN II ANTAGONISTS

Decrease in Ang II receptor stimulation by ACE inhibitors has been shown to effectively limit LV dilation, LVH, and heart failure after MI [21–29]. Despite overall benefits on the balance [30–32], ACE inhibitors also inhibit fibroblast proliferation and collagen deposition [33–39], which tend to promote IZ remodeling [3,37]. Their use during IR is also controversial [9,10]. In addition, ACE inhibitors inhibit bradykininase, thereby increasing bradykinin (BK) [40], which acts via mediators, such as prostacyclin ($PGI_2$) and nitric oxide (NO), to amplify antitrophic effects [41,42], limit LVH, and lower IZ collagen. Selective antagonists of Ang II type 1 ($AT_1$) and type 2 ($AT_2$) receptors are now available [8,43,44]. We have postulated that $AT_1$ blockade, which lacks the BK contribution, should result in less decrease in IZ collagen than ACE inhibition. Although $AT_1$ and $AT_2$ receptors are expressed in human and rat hearts [45], clear demonstration of $AT_2$ functionality in myocardium has been scarce [8] until very recently [46,47]. Recent evidence suggests that signal transduction pathways for $AT_1$ and $AT_2$ receptors (on fibroblasts) via Ang II coupling to protein kinase C (PKC) might be a key step in protection against IR injury [46,48,49], but their role in IR has not been studied.

Although early studies favor ACE inhibitors for limiting remodeling [21–24], and subsequent trials after MI have shown a further small decrease in mortality below 7% in selected patients [25–28], negative results of the CONSENSUS II trial [29], the

**Table 1.** Mechanisms for myocardial stunning

| Mechanisms |
|---|
| • Generation of oxygen-derived free radicals |
| • Reduction in antioxidant reserve |
| • Uncoupling of excitation-contraction |
| • Calcium overload |
| • Impaired metabolism |
| • Decreased mitochondrial energy production |
| • Impaired energy utilization by myofibrils |
| • Impaired sympathetic neural responsiveness |
| • Decreased sensitivity of myofibrils to calcium |
| • Impaired perfusion |
| • Damage to extracellular collagen matrix |
| • Myocardial hibernation |

lack of early separation of survival curves in other studies with ACE inhibitors [25–28], and the persistent LV dysfunction after early captopril in rats [34] could be explained by negative effects on IZ collagen, collagen matrix, and IZ remodeling [30–32]. It is possible that results might be better if these undesirable effects could be avoided. In addition, ACE inhibitors do not entirely block actions of Ang II on the receptors and vary in their ability to block tissue ACE. Selective Ang II blockade is therefore promising.

We recently made four pertinent observations: (1) chronic ACE inhibitors decrease IZ collagen, which can increase IZ remodeling post-MI [37,38]; (2) NO donors accelerate functional recovery and limit remodeling after IR [15]; (3) acute $AT_2$ blockade (PD123,319) during acute IR in rats enhances recovery of LV function, whereas $AT_1$ blockade (losartan) prevents recovery of function [47,50,51]; and (4) chronic treatment with $AT_1$ blockade (L-158,809) in dogs [52] limits IZ expansion.

## ANGIOTENSIN II BLOCKADE IN IR INJURY

Ang II elicits several physiological effects which increase IR injury [9]. Mechanisms include vasoconstriction [53], ventricular fibrillation [54], facilitation of norepinephrine release [55], and stimulation of phospholipase C and/or $A_2$ [56]. Increased Ang II after MI promotes myocyte hypertrophy and fibrosis [8]. We postulated that cardioprotection can be achieved by decreasing Ang II receptor stimulation by selectively blocking Ang II receptors with $AT_1$ and $AT_2$ antagonists. It is well established that Ang II mediates its effects via these receptor subtypes [7], albeit mainly $AT_1$ [8]. Although chronic administration of an $AT_1$ antagonist reduces LVH [57] and ischemic injury [58], effects of $AT_1$ or $AT_2$ blockade during acute IR had not been reported. Since both $AT_1$ and $AT_2$ receptors are expressed in human and rat hearts [45], with few interspecies differences in drug affinity [59], the isolated rat heart is a suitable model. We have therefore used the isolated working rat heart model in our studies of recovery after IR injury.

## CARDIOPROTECTIVE POTENTIAL OF ACUTE $AT_1$ AND $AT_2$ ANTAGONISTS DURING IR

Whether early cardioprotection involves Ang II (decrease in circulating and/or cardiac Ang II) or non-Ang II mechanisms is an unresolved issue. Although $AT_1$ antagonists, when given chronically, reduced the deleterious consequences of IR injury [58], the cardioprotective efficacy of acutely administered $AT_1$ or $AT_2$ antagonists had not been investigated. One belief has been that all known effects of Ang II are mediated via the $AT_1$ receptor [8], so that specific $AT_1$ receptor blockade would completely block vasoconstrictor and growth promoting effects of Ang II. The fact that Ang II levels rise significantly after $AT_1$ blockade [8,43,44] suggested that the free Ang II might preferentially bind to $AT_2$ receptors and mediate beneficial effects. Since $AT_2$ receptors are cell proliferation regulators, free Ang II in the presence of $AT_1$ blockade would potentiate its therapeutic effects.

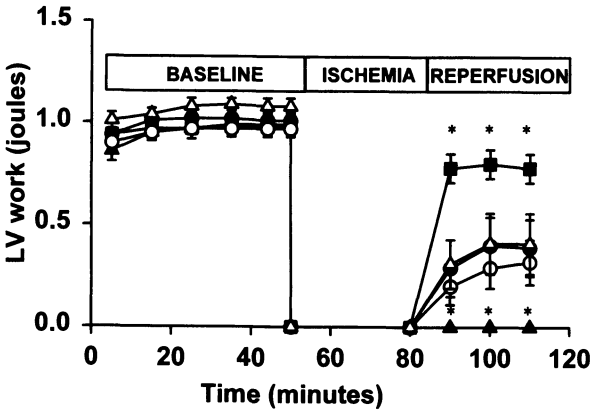


**Figure 1.** Effect of $AT_1$ antagonism on recovery of left ventricular (LV) work after ischemia-reperfusion: ● = Control; ■ = CHA, $N^6$-Cylohexyladenosine (0.5 μmol/l); ▲ = Losartan (1 μmol/l); ○ = Ang II (1 nmol/l); and Δ = Losartan + Ang II. *P < 0.05 vs controls.

We first assessed the contribution of endogenous cardiac Ang II to the postischemic recovery of myocardial function with the $AT_1$ antagonist losartan (DuP 753) using the isolated rat working heart. Rat hearts, paced at 300 bpm, were perfused with Kreb's buffer at 37°C containing 1.2 mmol/l palmitate prebound to 3% BSA, 11 mmol/l [$^3H/^{14}C$] glucose, and 100 μU/ml insulin. Proton ($H^+$) production from glucose metabolism was calculated from the rates of glycolysis and glucose oxidation. The hearts underwent an aerobic perfusion period (50 min), followed by 30 min of global no-flow ischemia in the presence or absence of losartan (1 μmol/l) or $N^6$-cyclohexyladenosine (CHA, 0.5 μmol/l), a selective adenosine $A_1$ receptor agonist shown to be cardioprotective. During reperfusion, the recoveries of LV work and cardiac efficiency relative to controls were enhanced ($P < 0.05$) by CHA, but were depressed ($P > 0.05$) by losartan. Postischemic proton production from glucose metabolism was reduced ($P < 0.05$) by CHA but remained unaltered by losartan (figure 1). The lack of cardioprotective efficacy of losartan, under these conditions, suggested that myocardial recovery from ischemia might be dependent, at least in part, on endogenous cardiac Ang II production. In addition, the fact that acute $AT_1$ blockade with losartan (DuP753) was deleterious in IR [50] suggests that $AT_1$ agonism may also offer a novel approach to therapy.

Second, we evaluated the effects of acute $AT_1$ and $AT_2$ antagonists on recovery of mechanical function following 30 minutes of global, no-flow ischemia in the same model [47]. In control hearts, there was incomplete recovery of LV minute work and cardiac efficiency during reperfusion, the values being 51% and 61% of preischemic levels, respectively. The $AT_2$ antagonist PD123,319 (0.3 μmol/l), administered prior to ischemia, improved recovery of LV work and efficiency compared to control hearts, the values being 82% and 98% of preischemic levels, respectively. In contrast, the $AT_1$ antagonist losartan (1 μmol/l) prevented recovery

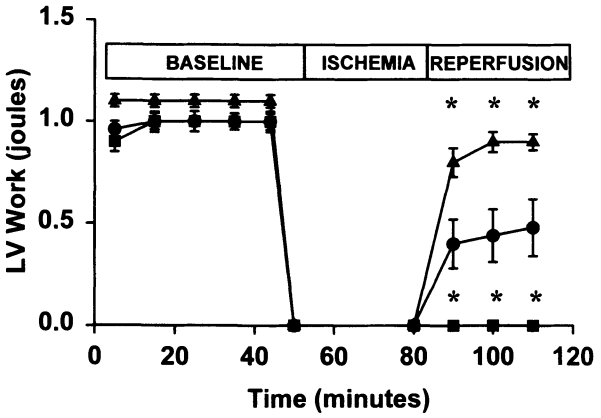


**Figure 2.** Effect of $AT_2$ antagonism on recovery of left ventricular (LV) work after ischemia-reperfusion. Time courses of recovery of LV work from 30 min of global, no-flow ischemia are shown for control hearts (n = 8) and hearts treated 5 min before the onset of ischemia with either PD-123,319 (0.3 μmol/l; n = 7) or losartan (1 μmol/l; n = 7). ● = Control; ▲ = PD-123,319; ■ = Losartan. *P < 0.01 compared to controls.

of LV work and depressed efficiency, the values being 0% and 1% of preischemic levels, respectively. Neither antagonist affected coronary vascular conductance. In summary, we showed that acute treatment with the $AT_2$ antagonist PD123,319 during IR, in the isolated working rat heart, was cardioprotective (figure 2), while the $AT_1$ antagonist losartan was not protective under the conditions and in the window used [47]. The overall results suggested that adjunctive $AT_2$ antagonists and $AT_1$ agonists may offer novel approaches for the treatment of mechanical dysfunction after ischemia-reperfusion.

### $AT_2$ RECEPTOR FUNCTIONALITY

The importance of $AT_2$ receptors and their functionality in the cardiovascular system was questioned [43] before the results of certain studies [47,50,51]. Increasing evidence suggests that $AT_2$ receptors mediate responses to Ang II in adult tissues besides those related to development [60–63]. We demonstrated that, compared to controls, short-term treatment with the selective $AT_2$ antagonist PD123,319, unlike treatment with the selective $AT_1$ receptor antagonist losartan, was cardioprotective and enhanced postischemic recovery of mechanical function. This represented the first demonstration of $AT_2$ receptor involvement in the pathogenesis of IR injury [47].

We believe that the effects of $AT_1$ receptor antagonists can differ in different models. There is increasing evidence indicating that effects of $AT_1$ antagonists depend on the experimental situation and the specific window in which they are tested. For example, with in vivo dog models of infarction, cardioprotection was found with the selective $AT_1$ antagonist (L-158,809) applied one hour after infarction [52] but not when $AT_1$ antagonism (DuP 532) was applied 24 hours after

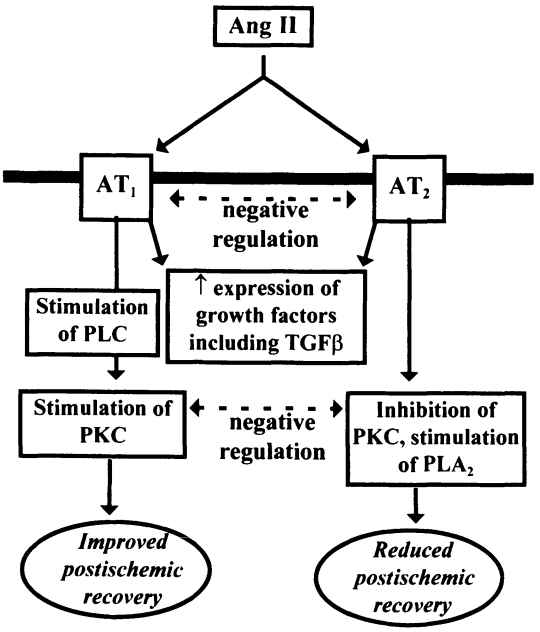


**Figure 3.** Schematic of $AT_1$ and $AT_2$ receptor interaction. PKC = protein kinase C; $PLA_2$ = phospholipase $A_2$; PLC = phospholipase C; TGFβ = transforming growth factor β.

infarction [64]. In rats, $AT_1$ antagonism with TCV 116 applied over 4 weeks prior to in vitro assessment of IR injury (Langendorff) was cardioprotective [58], whereas losartan treatment immediately prior to IR (working heart), to our surprise, was not [47]. It is possible that chronic pretreatment with Ang II receptor antagonists, as reported with TCV116 [58], is associated with an upregulation of receptor populations, thereby influencing functional recovery after IR.

Receptor studies have recently shown that the ratio of $AT_2$ to $AT_1$ was 1:3 in purified adult rat cardiomyocytes [65]. Although measurement of receptor number and type alone does not tell us about receptor functionality, the presence of $AT_2$ receptors on myocytes suggests that $AT_2$ antagonism could have functional consequences. It is also highly likely that receptors interact. Thus, $AT_1$ and $AT_2$ receptor interaction, cross-talk, or negative cooperativity (figure 3) is a distinct possibility. This could explain different acute and chronic effects of IR injury. There is evidence for $AT_1$ downregulation in heart failure and during ACE inhibition [45].

It is important to note that in our study [47] using the isolated working rat heart, the perfusate contained free fatty acids prebound to bovine serum albumin (BSA). In studies using high free concentrations of losartan without BSA, $AT_2$ receptor antagonism might offset $AT_1$-mediated deleterious effects of losartan and result in protection.

### LIMITATION OF EARLY LV REMODELING WITH THE POTENT NOVEL ANG II $AT_1$ RECEPTOR ANTAGONIST L-158,809 AFTER ACUTE MI IN THE DOG

Whether Ang II receptor blockers can limit post-MI remodeling as seen with ACE inhibitors in the dog was controversial. Because sustained $AT_1$ receptor blockade with chronically administered losartan is thought to be less effective in the dog than rat models of heart failure, we had to verify whether the more potent novel $AT_1$ blocker (L-158,809: Merck) produces significant blockade of the Ang II pressor response in our dog model [52]. Dogs with anterior MI were given intravenous infusions of L-158,809 (0.1 mg/kg bolus followed by 0.6 μg/kg/min, $n = 6$) or saline or vehicle ($n = 5$) between 1 hour and 48 hours after left anterior descending coronary ligation. Continuous infusion of the $AT_1$ antagonist produced near 100% blockade of the Ang II pressure response. It also produced sustained LV unloading and limited early LV remodeling (or infarct expansion) with less percent increase in LV volume (2% versus 35%, $p < 0.001$) and infarct expansion index (4% versus 18%, $p < 0.001$) and better global ejection fraction (51% versus 38%, $p < 0.05$). The global LV shape index (short-axis/long-axis length) increased in controls but remained unchanged with $AT_1$ blockade. In vivo LV mass did not change in either group over the 48 hours. These results indicated that the novel Ang II type 1 receptor antagonist L-158,809 produces significant $AT_1$ receptor blockade after canine MI and effectively reduces LV loading and limits early remodeling.

### ACE INHIBITION AND INFARCT COLLAGEN DURING POST-MI REMODELING

The roles of the supporting collagen matrix in the NIZ [66–68] and extracellular collagen in the IZ [3,5] in protection against LV remodeling are now recognized. Conversion of fibroblasts to myofibroblasts may also have a protective role. Therapies that disrupt the collagen matrix, decrease IZ collagen, or interfere with myofibroblast formation can be expected to promote remodeling [2,3]. Interestingly, $AT_1$ and $AT_2$ receptors are found mainly on fibroblasts [7]. Inhibition of fibroblast proliferation, collagen deposition, and fibrosis with ACE inhibitors might promote IZ remodeling and act as a "double-edged sword". We showed that ACE inhibitors decrease IZ collagen in dogs [37–39], and even low dose (2.5 mg BID) enalapril decreased IZ collagen and made the scars flatter [37]. Many others documented decreases in NIZ collagen with ACE inhibitors in the rat but did not measure IZ collagen [33–36,57]. Early ACE inhibition can promote IZ expansion by inhibiting collagen formation and reducing tensile strength and myocyte tethering [69].

Since reperfusion can damage collagen matrix during MI [70], the potential for harm might be even more when reperfusion is combined with early ACE inhibition. Reperfusion also accelerates healing in rats [71] and injures collagen matrix in dogs [20]. Collagen matrix disruption plus inhibition of IZ collagen with the combination could promote more regional and global LV dilation. Clinically, ACE inhibitors (e.g., captopril) given early after reperfusion produced certain benefits [2,72], suggesting that, on the balance, there is more good than harm.

Since ACE inhibitors also inhibit bradykininase [43,44], they increase BK, which

induces NO and $PGI_2$ release, thereby contributing to vasodilator and antitrophic effects of ACE inhibition via BK. If all effects of Ang II are mediated via $AT_1$ receptors [43,44], collagen deposition would be mainly $AT_1$-mediated and $AT_1$ blockade would be expected to inhibit its deposition. However, high Ang II levels during $AT_1$ blockade could mediate fibrosis and other side effects by increased signal transduction via the normally nonfunctional $AT_2$ receptors [43,44].

Since evidence for $AT_2$ functionality is now appearing [45,47,51], classical receptor theory suggests that high Ang II levels during $AT_1$ blockade would downregulate $AT_2$ receptors. Because of the additional BK contribution to hemodynamic and antitrophic effects of ACE inhibitors, one conclusion is that the ACE inhibitors should have greater antimyocyte hypertrophy and antifibroblast effects than selective $AT_1$ receptor blockade.

Recent evidence emphasizes cellular mechanisms in remodeling and Ang II antagonist effects. Increased tissue ACE after rat MI [73] is inhibited by enalapril and the $AT_1$ antagonist losartan [57]. The antitrophic effect of ACE inhibition is explained by inhibition of Ang II acting via $AT_1$ (and possibly $AT_2$) receptors and growth promoting factors [74–76]. Blockade of the antitrophic effect of the ACE inhibitor ramipril by the BK antagonist HOE-140 in hypertensive rats [77] supports the view that the antitrophic effect of ACE inhibition is related to local increase in BK. This effect might be mediated via BK-induced release of NO [40,42] and prostacyclin ($PGI_2$) [40]. Recently, HOE-140 blocked the decrease in LV mass produced by ramipril after DC shock necrosis in dogs [78], supporting the ACE/BK mechanism. It is not clear why $AT_1$ blockade and ACE inhibition produce similar inhibition of LV mass in rat MI [56], but not in the dog [77]. Species differences are probably involved [5]. One should bear in mind this factor when extrapolating findings in rats or dogs to humans.

## CONCLUSION

Selective Ang II receptor antagonists are powerful tools for probing the mechanisms by which Ang II promotes vasoconstriction, increased impedance, ischemic injury, myocyte growth, fibrosis, and LV remodeling. In addition, use of $AT_1$ and $AT_2$ antagonists allowed us to detect functional role(s) for $AT_1$ and $AT_2$ receptor blockade in cardioprotection against IR injury and for $AT_1$ blockade in early remodeling after MI. Whether long-term $AT_1$ blockade after MI, alone or in combination with ACE inhibition, produces more effective limitation of LV remodeling and preservation of function after MI remains to be determined.

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# LOCAL ANGIOTENSIN II AND TISSUE REPAIR POST-MYOCARDIAL INFARCTION

YAO SUN

*D. Angiotensin Blockade and Remodeling of Heart in Myocardial Infarction*

***Summary.*** At sites of tissue repair, phenotypically transformed fibroblast-like cells, myofibroblasts, are responsible for fibrous tissue formation. In vivo studies of injured rat cardiac tissues have shown that myofibroblasts (myoFb) express membrane-bound angiotensin-converting enzyme (ACE) and angiotensin (Ang) II receptors. In vitro, these cells have been shown to generate Ang I and II peptides. Locally generated Ang II acts in an autocrine/paracrine manner to regulate myofibroblast collagen turnover in injured tissue.

## INTRODUCTION

Tissue repair involves the appearance of granulation tissue that subsequently is replaced by fibrous tissue that primarily consists of type I and III fibrillar collagens. In the presence of parenchymal cell loss, a *replacement fibrosis* (or scarring) preserves tissue structural integrity. Fibrosis leads to as an abnormal increase in tissue collagen concentration that can adversely affect organ function.

The principal cells responsible for fibrous tissue formation at the sites of repair are phenotypically transformed fibroblast-like cells having distinctive morphological features and phenotypical characteristics. These cells are termed myoFb because they express α-smooth muscle actin microfilaments and are contractile [1]. They are abundant at sites of tissue repair [1,2]. Interstitial fibroblasts are responsible for normal collagen turnover and are considered a source of myoFb. Signals responsible for this transformation in cell phenotype are under investigation. MyoFb have cell-cell and cell-matrix connections that provide for a contractile assembly [1]. This contractile assembly contributes to scar tissue remodeling during later stages of repair (e.g., infarct scar thinning). Fibrous tissue contraction is induced by various sub-

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stances, including angiotensin (Ang) II and endothelin-1 ($ET_1$) [1,3]. The source of these peptides has been uncertain. Could they arise from cells within granulation tissue, or are they derived from the circulation? Recent in vivo and in vitro studies indicate that fibroblast-like cells are metabolically active—activity that extends beyond their synthesis and degradation of collagen. This includes their ability to generate substances such as Ang II, which in an autocrine/paracrine manner, influences collagen turnover and scar tissue contraction. It therefore is no longer tenable to consider metabolic activity of fibroblast-like cells as confined solely to the secretion of matrix components. The purpose of this manuscript will be to address the elaboration of Ang II by myoFb at sites of repair in the infarcted heart and the contribution of Ang II to fibrous tissue formation at these sites.

## MYOCARDIAL REPAIR POSTINFARCTION

Myocardial infarction in rats was created by left coronary artery ligation. Repair of infarcted rat heart included (1) extensive myocardial infarction (MI) of the left ventricular free wall; (2) noninfarcted sites remote to MI; (3) opening of the perietal pericardium and manual handling of the heart; and (4) placement of a foreign body (silk suture) in the myocardium. Type I collagen gene expression was determined by in situ hybridization; its fibrillar collagen composition by the collagen-specific stain picrosirius red; and its cellular elements using hematoxylin and eosin and/or specific immunohistochemistry for detection of cell phenotype (vide infra).

### Infarct site

Type I makes up about 80% of total collagen and is, therefore, the most important collagen in the repairing tissue. Type I collagen gene expression is normally low in the myocardium of both ventricles. It is increased (tenfold) at the site of infarction on day 7 (figure 1, panel A) and is gradually reduced, therefore, but is still much higher (fivefold) at day 28 than normal myocardium. Microscopic evidence of early fibrillar collagen formation is seen at the site of MI on day 7 (figure 1, panel B). A fibrillar assembly of collagen that borders on necrotic tissue representing early scar formation is seen on day 14. Continued collagen accumulation is evident at days 28 and 56 [4]. Necrotic cells have been completely replaced by fibrous tissue on day 28, and it is at this time that thinning of the infarct scar begins to become more advanced at week 8. Detailed aspects of scar remodeling has been reported by others [5].

### Remote sites

Increased fibrous tissue, evidenced by hydroxyproline assay and histochemistry, is observed by day 14 at remote sites in hearts with extensive MI. This, likewise, has been observed in the human myocardium [6]. At these remote sites, microscopic scars follow myocyte necrosis and appear in the right ventricular free wall and septum. Interstitial collagen formation is observed at these sites in the absence of myocyte necrosis. A perivascular fibrosis of intramyocardial coronary arteries is also

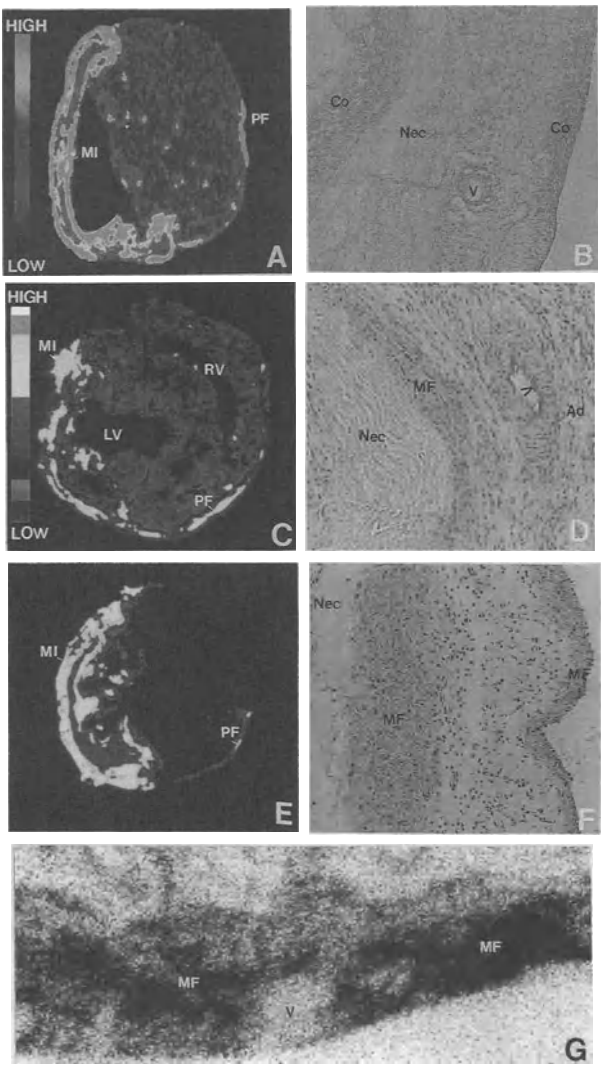


**Figure 1.** In the infarcted rat heart, type I collagen mRNA expression is markedly increased at the site of MI and pericardial fibrosis (PF) (panel *A*). Collagen (Co) starts to accumulate surrounding necrotic myocytes (Nec) (panel *B*) and gradually replace necrotic tissue. ACE binding density is markedly increased at the site of MI and pericardial fibrosis (panel *C*). Cells expressing ACE at the site of MI are fibroblast-like cells surrounding necrotic tissue, endothelial cells of blood vessels (arrowhead), macrophages, and fibroblast-like cells in adventitia (Ad) (panel *D*). These ACE containing fibroblast-like cells also express α-smooth muscle actin and, therefore, myoFb (MF) (panel *F*, brown). Marked increase in Ang II receptor-binding density is colocalized with high ACE and fibrosis (panel *E*). Panel G is a overlapped picture of immunohistochemical α-smooth muscle actin labeling (brown) and autoradiographic Ang II receptor binding (silver grains) at the site of MI. MF express high Ang II receptors, while blood vessels (V) contain a low amount of Ang II receptors.

seen at these sites. Finally, an endocardial fibrosis of the left ventricular aspect of the interventricular septum represents a structural remodeling of the heart by fibrous tissue at a site remote to MI [4]. In rats with or without MI, fibrosis of the visceral pericardium and myocardium surrounding the silk ligature is evident at postoperative week 2. Markedly increased type I collagen mRNA is seen at sites of endocardial fibrosis, pericardial fibrosis (figure 1, panel A), and microscopic scars in the right ventricle and septum.

## CELLS EXPRESSING COLLAGEN AT SITES OF INJURY

### Infarct site

Cells responsible for collagen gene expression at the site of MI were identified by in situ hybridization [7] and found to be fibroblast-like cells, not cardiac myocytes, endothelial cells, or vascular smooth muscle cells. These fibroblast-like cells, together with macrophages, surround necrotic myocytes. These cells were identified as myoFb by immunohistochemistry [8]. At the site of MI, myoFb started to appear at day 3 post-MI and became abundant and extensive thereafter (figure 1, panel F). Unlike skin, where myoFb appear and then disappear by day 28 following injury [9], myoFb remain at the site of infarction for years [10].

MyoFb impart fibrous tissue with contractile activity. Substances that promote contraction include Ang II and $ET_1$. These findings suggest diastolic dysfunction, often seen in the infarcted ventricle, could be a fibrocontractive disorder.

### Remote sites

In situ hybridization localized fibroblast-like cells as expressing type I and III collagen mRNA at these sites [7]. MyoFb were identified by $\alpha$-smooth muscle labeling in the fibrosed visceral pericardium, endocardium, and the site of foreign-body fibrosis surrounding silk suture. In the study of Sun et al. [8], where microscopic scars replaced lost myocytes in the noninfarcted right ventricle, myoFb were also observed. Cleutjens et al. [7] did not find such scars remote to the MI, and in this case, interstitial fibroblasts ($\alpha$-smooth muscle actin-negative) were involved with interstitial fibrosis. Differences in experimental preparation of the infarct model may explain these disparate findings. Factors responsible for the appearance of myoFb at or remote to MI remain uncertain. TGF-$\beta_1$, perhaps released by necrotic myocytes or macrophages involved in repair, could be implicated. Added to a wound healing chamber implanted subcutaneously, TGF-$\beta_1$ leads to the appearance of myoFb in subsequent granulation tissue that surrounds the chamber; exogenous administration of TGF-$\beta_1$ to cultured, serum-deprived skin fibroblasts is associated with their transformation to myoFb.

## ACE AND ANG II RECEPTORS IN REPAIRING CARDIAC TISSUE POSTINFARCTION

### Normal heart

In vitro quantitative autoradiography demonstrates the presence of ACE binding ($^{125}$I-351A) throughout the myocardium of each ventricle and atria in the normal rat

heart. Binding density, however, is low at these sites. High-density ACE binding, on the other hand, is present in heart valve leaflets and adventitia of intramural coronary arteries [11]. In the normal rat heart, Ang II receptors are present in low amounts. High-density binding is found in normal valve leaflets, where they are predominantly of the $AT_1$ subtype [12]. An $AT_1$ but not an $AT_2$ receptor antagonist abrogated these responses [13]. The presence of high-density ACE and Ang II receptor binding at sites where collagen turnover is expected to be high suggests Ang II, generated within connective tissue, is normally involved in fibrogenesis via Ang II receptor-ligand binding. Keeley et al. [14] found that 5 weeks of enalapril administration (nonpressor dose) in four-week-old rats retarded collagen formation in the right and left ventricles, aorta and, superior mesenteric artery. Heart valves were not examined.

### Infarct site

Three days after MI, ACE and Ang II receptor-binding density in the infarcted left ventricle were unchanged compared to normal myocardium. One week post-MI, both ACE and Ang II receptor-binding density was markedly increased at the site of MI (figure 1, panels C and E) [4,15]. Such high ACE and Ang II receptor binding in the infarcted ventricle was also seen on week 2, 4, and 8.

### Noninfarct site

One week post-MI, marked increase in ACE and Ang II receptor binding was seen at sites of fibrosis remote to infarction, including pericardial fibrosis (figure 1, panels C and E), endocardial fibrosis, myocardial foreign body fibrosis, perivascular fibrosis, and microscopic scars [4,15]. ACE and Ang II receptor binding remained high at these sites of repair for at least 8 weeks. Each fibrous tissue site was, therefore, coincident with high-density ACE and Ang II receptor binding. Displacement studies using either at $AT_1$ receptor antagonist (losartan) or an $AT_2$ receptor antagonist (PD123177) demonstrated dominant $AT_1$ receptor binding at these sites [15]. Furthermore, ACE expression at sites of repair was shown to not be regulated by circulating Ang II or ALDO [16].

## CELLS EXPRESSING ACE AND ANGII RECEPTORS IN VIVO

Monoclonal antibody to ACE has been used to identify cells expressing this ectoenzyme at the site of MI. Positively-labeled cells include endothelial cells, found in blood vessels (figure 1, panel D) that appear in granulation tissue as part of neovascularization; macrophages; and myoFb (figure 1, panel D) [4,8]. At remote sites, myoFb alone express ACE [8]. Each site was likewise coincident with high-density autoradiographic Ang II receptor binding, predominantly of the $AT_1$ subtype. Predominant cells expressing $AT_1$ receptors at these sites were subsequently identified, by emulsion autoradiography and immunolabeling, as myoFb (figure 1, panel G) [17]. Smooth muscle cells of blood vessels express low $AT_1$ receptors (figure 1, panel G) [17].

## PHARMACOLOGICAL INTERVENTIONS AND CONNECTIVE TISSUE FORMATION

In vivo studies, using pharmacological agents that interfere with Ang II generation (i.e., ACE inhibitors) or which bind to $AT_1$ receptors, support a role for locally generated Ang II in regulating collagen turnover at sites of tissue repair in the heart and related structures.

### Infarct site.

Following experimental MI, elevations in circulating Ang II and ALDO are not observed [16]. Administration of an ACE inhibitor, either captopril or enalapril, for 6 weeks reduced infarct size (percent of epicardial circumference of the left ventricle occupied by scar) and infarct area (planimetered scar area) [18]. Similar findings have recently been reported for an $AT_1$ receptor antagonist (losartan), suggesting locally produced Ang II contributes to fibrogenesis [19].

### Remote sites

The ACE inhibitor captopril or perindopril, initiated at the time of MI, prevents fibrosis at remote sites [20]. A similar response was observed for losartan [19], implicating locally produced Ang II in fibrogenesis at these sites. Autoradiographic ACE binding density at remote sites was attenuated by losartan, suggesting either the number of myoFb or their metabolic activity/cell was reduced [20].

## CONCLUSIONS

Experimental evidence gathered to date indicates that myoFb are the predominant cell responsible for collagen formation at sites of repair in the rat heart and related structures. These phenotypically transformed fibroblast-like cells are not normal residents of ventricular tissue. They appear on day 4 at sites of injury following an inflammatory cell response that consists primarily of macrophages. MyoFb express high density of ACE and $AT_1$ receptors, suggesting locally generated Ang II may have an autocrine function in regulating myoFb function. Macrophages also express ACE, and their generation of Ang II induces macrophages to express TGF-$\beta_1$, which in turn is responsible for subsequent fibroblast switch to myoFb. Ang II generated by myoFb likewise promotes TGF-$\beta$ gene expression. Together, Ang II and TGF-$\beta_1$ promote myoFb transcription of type I and III collagens and tissue inhibitor to matrix metalloproteinase. Collectively these latter responses beget fibrosis.

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# LOSARTAN PRETREATMENT INHIBITS AN EARLY ACTIVATION OF MATRIX METALLOPROTEINASES IN ACUTE MYOCARDIAL INFARCTION

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***Summary.*** To elucidate the role of matrix metalloproteinase in acute myocardial infarction (MI), we examined collagen degradation by zymography (collagenolytic and gelatinolytic activities). MI was found to be induced by a surgical occlusion of the left coronary artery in the rat, and then the findings were compared with those in sham-operated rats, as a control specimens. Experimental samples were taken from infarcted tissue specimens of the left ventricle (LV) after occlusion. The collagenolytic activities were assessed by zymography and denatured $^{3}$H-collagen type I. Zymography showed a significant amount of MMP-1 (interstitial collagenase), MMP-2 (gelatinase) and MMP-9 (neutrophil gelatinase) in the infarcted lesion from 6 to 48 hours after a coronary artery ligation. Myeloperoxidase activity significantly increased in infarcted lesions at 24 hours after the induction of MI. The collagenolytic activity determined by denatured $^{3}$H-collagen type I also increased in the infarcted lesion 24 hours after MI. The pretreatment with losartan (20 mg/kg) significantly decreased the collagen concentration and inhibited both the MMP-2 and MMP-9 activities. Losartan thus appears to have a beneficial effect on the infarcted myocardium in the early remodeling process after MI by preventing the formation of angiotensin II.

## INTRODUCTION

Left ventricular myocardial infarction (MI) induces morphological and molecular alterations in both the infarcted tissue and in regions of the heart remote to the infarction, and these changes are collectively referred to as ventricular remodeling [1]. The remodeling process of infarcted cardiac muscle includes a disruption of fibrillar collagen, which can occur with increased collagenolytic activity in infarcted cardiac muscle [2] and can therefore have multiadverse consequences on the archi-

*N.S. Dhalla, P. Zahradka, I. Dixon and R. Beamish (eds.). Angiotensin II Receptor Blockade: Physiological and Clinical Implications.* 

tecture and function of the myocardium [3]. The role of structural stromal proteins, i.e., the collagens and glycoproteins, in the remodeling of both scar tissue and viable cardiac tissue has gained renewed recognition during recent years [4,5]. The total tissue collagen content after infarction was assessed using the model of MI [6], and at 1, 2, and 3 hours from the time of induced infarction, the cardiac infarct zones were found to contain 75%, 65%, and 50% of the total collagen in the noninfarcted left ventricle, respectively [6]. The cardiac interstitial collagenase, elastase, and cathepsin G activity significantly increased in this tissue compared with that of the noninfarcted control values, and these findings thus suggest that the increased activities of both collagenase and other types of neutralproteinase may be responsible for the rapid degradation of collagen in heart tissue after MI [6]. Similarly, it has also been reported that there is a 73% increase in collagenase activity in the crude tissue homogenates of a stunned myocardium [7].

Matrix metalloproteinases (MMPs) are a group of zinc enzymes that are responsible for the degradation of extracellular matrix components including fibronectin, collagen, elastin, proteoglycans, and laminin in normal embryogenesis, inflammation, wound healing, and tumor invasion [8,9]. The extracellular pathways of interstitial collagen degradation are initially mediated by either interstitial collagenase (MMP-1) or gelatinase (MMP-2), with cleavage at a specific site in the collagen molecule, which thus renders it susceptible to other neutral proteinase (e.g., gelatinase) in the extracellular space. However, little has so far been reported on either degradation or MMP activation in vivo.

The cardiac remodeling process can be reduced by the prolonged administration of an effective angiotensin-converting enzyme (ACE) inhibitor [10,11]. Angiotensin type 1 receptor ($AT_1$) blockade attenuates the degree of viable ventricular fibrosis after MI [12,13]. Although the increased deposition of extracellular matrix components (fibrosis) has been documented in the viable myocardium after MI, the pathological significance of matrix remodeling still remains to be elucidated. The present study was therefore undertaken to determine whether or not $AT_1$ blockade is effective in degradating extracellular matrix (ECM) in acute MI as well as whether or not $AT_1$ blockade can modify ECM remodeling in the chronic phase of MI.

## MATERIALS AND METHODS

### Experimental infarction

Male Wistar rats weighing 250 to 300 g were obtained from Charles River Inc. (Atsugi, Kanagawa, Japan) and housed in our animal facility for at least 3 days before operation. A coronary artery ligation was performed as previously described by us [12]. Briefly, the rats were anesthetized with 50 mg/kg of pentobarbital i.p. and intubated for mechanical respiratory control. The left coronary artery was then ligated 2–3 mm distal to its aortic origin with a 4-0 silk suture, and the heart was repositioned within the chest. The mortality rate within the first 48 hours was about 30%. In the sham-operated animals, the suture was tied loosely so as not to obstruct

the coronary flow. All animals were allowed free access to standard rat chow. Drinking water was provided ad libitum. To study the early remodeling of the collagen matrix after MI, fourteen rats were treated with the $AT_1$ receptor antagonist losartan (Banyu Pharmaceutical Co., Ltd., Japan) dissolved in drinking water at a dose of 20 mg/kg, 30 minutes before performing the coronary artery ligation. To study the chronic phase of MI, the $AT_1$ receptor antagonist E-4177, 3-[(2′-carboxybiphenyl-4-yl) methyl]-2cyclopropyl-7-methyl 3H-imidazol[4,5-b] pyridine (Eisai Co., Ltd., Japan), was introduced in rats. Twenty-two rats were treated with E-4177 dissolved in drinking water at a dose of 10 mg/kg per day from the second day after ligation of the coronary artery. At a predetermined time after the left coronary artery ligation, the hearts were quickly removed, rinsed, and perfused with Evan's blue dye to distinguish between the infarcted lesions and the noninfarcted lesions of the left ventricle (LV). The infarcted lesion was dissected from the noninfarcted lesion, which was located in the interventricular septum (IVS). Both the infarcted and noninfarcted LV lesions were then used for biochemical and morphological studies.

**Collagen concentration of the myocardium**

The myocardial collagen concentration was measured by determining the hydroxyproline concentration [14] of the LV partially modified by us [15]. After drying the heart for 24 hours, the specimens were then hydrolyzed in a 6N hydrogen chloride solution at 100°C. After resolution in a buffer at pH 7.0, p-dimethylamino-benzaldehyde (Ehrlich's reagent) was added to form a complex with hydroxyproline. The concentration of hydroxyproline was then measured by a spectrophotometric analysis at a wavelength of 558 nm. The collagen concentration was estimated by multiplying the hydroxyproline content by a factor of 8.2. The concentration of collagen was expressed as milligrams of collagen per gram dry weight.

**Gelatin-Zymography**

Frozen tissue was washed three to four times with cold saline and then was incubated in 2 ml of extraction buffer [0.05 M Tris-Cl, pH 7.5, 0.01 M CaCl2, 0.02% NaN3, 0.01% Triton X-100, pH 5.0] per 25 mg wet weight at 4°C, with continuous agitation for 24 h. This step was repeated with fresh buffer. The extraction buffer was collected and the pH raised to 7.5 by the addition of 1 mol/l Tris (pH8.0). MMP were activated with trypsin, plasmin, and serine protease at 37°C. Trypsin was inhibited with a tenfold excess PMSF prior to being loaded into the gel. The MMP activity in the gel was measured using the procedure of Tyagi et al. [16]. Type I gelatin was added to the standardize Laemmli acrylamide polymerizetion mixture at a final concentration of 1 mg/ml, under nonreducing conditions. The tissue extract was mixed and loaded immediately into wells of a 4% acrylamide Laemmli stacking gel on a cast vertical gel. The gels were rinsed and incubated overnight at 37°C in a substrate buffer. After incubation, the gels were

stained in 0.05% coomassie blue R-250 in acetic acid:methanol:water (1:4:5), destained in the same solvent, scanned for lytic activity, and photographed. In each gel, a reference sample was used to normalize the scanned lytic activities.

### Collagenolytic activity

To remove the plasma protease inhibitors from the myocardial samples, the heart was perfused with 500 ml of chilled potassium phosphate buffer (pH 7.3–7.4). Tissue samples were separated into either infarcted or noninfacted portions and then frozen on dry ice and stored at −70°C. The preparation of the samples and protease assays was performed as previously described with some modifications [17]. The tissue gelatinolytic activity was determined, and then collagenolytic activity was determined by the method of Hibbs et al. [18]. The proteolytic activity of the extract was examined using either serine protease trypsin (7 μg/ml) or plasmin (15 μg/ml). One hundred mg of denatured $^{3}$H-labeled type I collagen was incubated in a reaction mixture containing 0.02 M Tris-Cl, pH 7.6, 0.005 M $CaCl_2$, and 0.02% $NaN_3$ in a final volume of 150 ml at 37°C. Type I collagen was a heat-denatured mixture. At the termination of the assay, the samples were cooled to 4°C, and then the unchanged and large molecular weight fragments were precipitated by the addition of trichloroacetic acid to a final concentration of 15% (w/v). After centrifugation at 10,000 × g for 5 min, an aliquot of the supernatant was counted using a liquid scintillation counter.

### Myeloperoxidase assay

The myeloperoxidase (MPO) activity in the myocardium was assayed to quantitatively determine the neutrophil uptake. The MPO was extracted from the LV tissue, and the content was measured according to the method of Goldblum et al. [19], with some modifications. The LV tissue was suspended in hexadecyltrimethylammonium bromide (HTAB, Sigma) at 5 ml/g tissue in 50 mM phosphate buffer, adjusted to pH 6.0 and homogenized on ice for 30 seconds with a Polytron. The homogenate was centrifuged at 40,000 g for 10 minutes. The initial supernatant was then discarded. The pellet was resuspended in HTAB. The sample was freeze-thawed, followed by homogenization and centrifugation. The supernatant MPO activity was assayed by mixing a 0.1 ml aliquot of the sample with 2.9 ml of 50 mM PBS containing 0.0005% hydrogen peroxide and 0.167 mg/mL s-dianisidine hydrochloride (Sigma). The change in absorbance was measured at 460 nm for 3 minutes using a spectrophotometer. The MPO activity was thereafter expressed as the change in absorbance per minute per gram of tissue.

### Statistical analysis

The grouped results are expressed as the mean ± SEM. A one-way analysis of variance (ANOVA) showed significant differences among the groups. The groups were then compared using the unpaired t test with the Bonferroni correction for multiple comparisons. Statistical significance was set at $p < 0.05$.

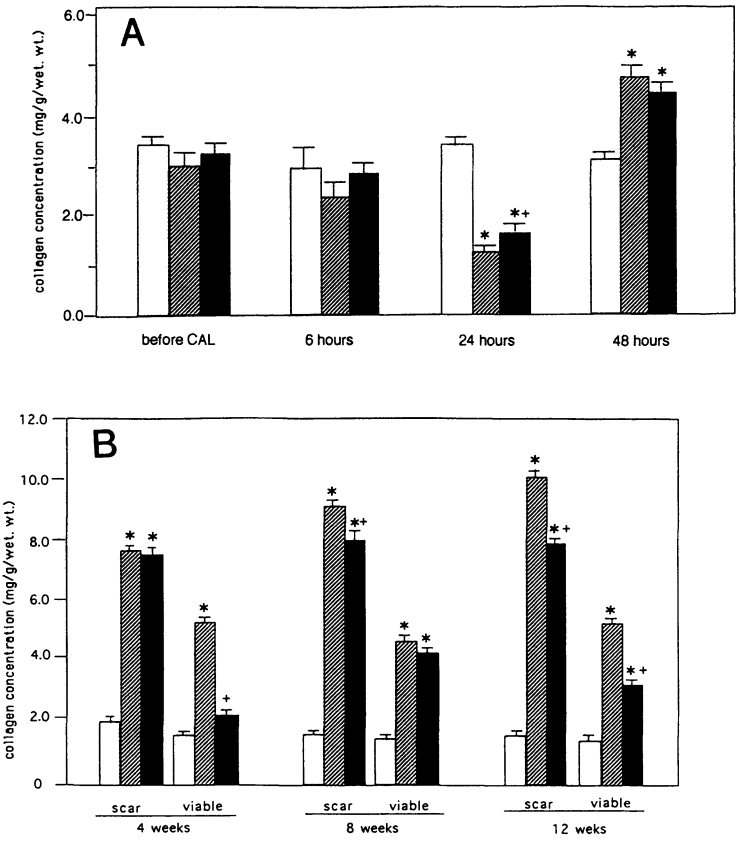


**Figure 1.** The collagen concentrations at an early phase (*A*) and the chronic phase (*B*) of myocardial infarction (MI) in rats. At the early phase of MI, tissue samples were taken from infacted areas of the left ventricle. The open, dashed, and solid columms represent the values from the sham-operated, untreated infact, and losartan-treated myocardium, respectively. At the chronic phase of MI, samples were taken from either the scar or viable myocardium. *$p < 0.05$ compared with the sham-operated rats, †$p < 0.05$ compared with the untreated respective myocardium.

## RESULTS AND DISCUSSION

### Collagen degradation after MI

The collagen concentration in the myocardium significantly decreased in the infarcted tissue at 24 hours after coronary artery ligation compared with that at 0, 3, and 6 hours (figure 1A). However, at 48 hours after coronary ligation, this concentration was higher than the previously reported values. When losartan was administered to rats 30 minutes before occlusion, the collagen loss in infarcted lesions was inhibited only at 24 hours after coronary artery occlusion, and not at any other times. Thus, the pretreatment with losartan has a beneficial effect for collagen loss in acute MI. Within 24 hours of experimental MI, the rapid collagen breakdown

has been already noted. Takahashi S et al. [17] reported decreases as high as 50% in the collagen content of the infarcted area 3 hours postinfarction. These findings suggest that the increased activities of collagenase and other neutral proteinases may thus be responsible for the rapid degradation of collagen in the heart tissue after MI. Similarly, it has also been reported that there is a 73% increase in the collagenase activity in the crude tissue homogenates from the stunned myocardium [7]. Thus, the loss of collagen in the early phase after infarction is probably mediated by an increased degradation of structural proteins. It has previously been postulated that the acute loss of cardiac matrix may lead to myocytes slippage, chamber dilation, wall thinning, and might thus even rupture the myocardium [20]. The present study suggests losartan to be effective in the partial attenuation of collagen protein deposition in infarcted hearts. The treatment of experimental animals with losartan was associated with a partial regression of both cardiac hypertrophy and interstitial fibrosis, which thus indicates the effective delivery of losartan.

The collagen concentration was examined in the chronic phase of MI. Figure 1B shows the collagen concentration in the heart tissue from the sham-operated rats, and rats either untreated or treated with E-4177. This concentration in the untreated MI group significantly increased in both the viable and scar tissue specimens at 4, 8, and 12 weeks postoperatively compared with the concentrations in the sham-operated group. The administration of E-4177 significantly decreased the collagen concentration in the viable LV at all three stages of healing compared with the concentration in the untreated MI group. This agent also decreased the collagen concentration in scar tissue at 8 and 12 weeks after MI. Similar observations have also been reported by other investigators, who described that the myocardial collagen content significantly increased in the viable tissue at 7, 14, 21, and 35 days after induction of MI [21]. These effects are usually ascribed to the prevention of angiotensin (Ang) II formation because the renin-Ang II system is activated after acute MI. In experimental and clinical studies of congestive heart failure, the administration of low doses of $AT_1$ receptor antagonist, losartan, produced vasodilatation [22] and improved the cardiac pump performance [23]. The cardiac fibrosis of the cardiac interstitum, thus, constitutes a series of events occurring in the chronic phase of MI, and this collective fibrosis is now becoming recognized as a hallmark of this disease.

**Collagenase activity in MI**

The cardiac MMP activity was detected by the appearance of a lytic band in the gelatin-containing SDS-PAGE (figure 2A). We thus included serine proteinase inhibitors, PMSF, and leupeptin to show that the lytic bands are not caused by serine proteinase. Using zymography, a major lytic band was observed to correspond to MMP-1 (54kDa), MMP-2 (Gelatinase A; 72kDa), and MMP-9 (Gelatinase B; 92kDa) in infarcted tissue. The MMP-1 cleave native fibrillar collagen helices [24] at unique sites in the native triple helix at 3/4 from the N-terminal end, generating 3/4 and 1/4 collagen fragments, also called gelatins [25]. These gelatins quickly

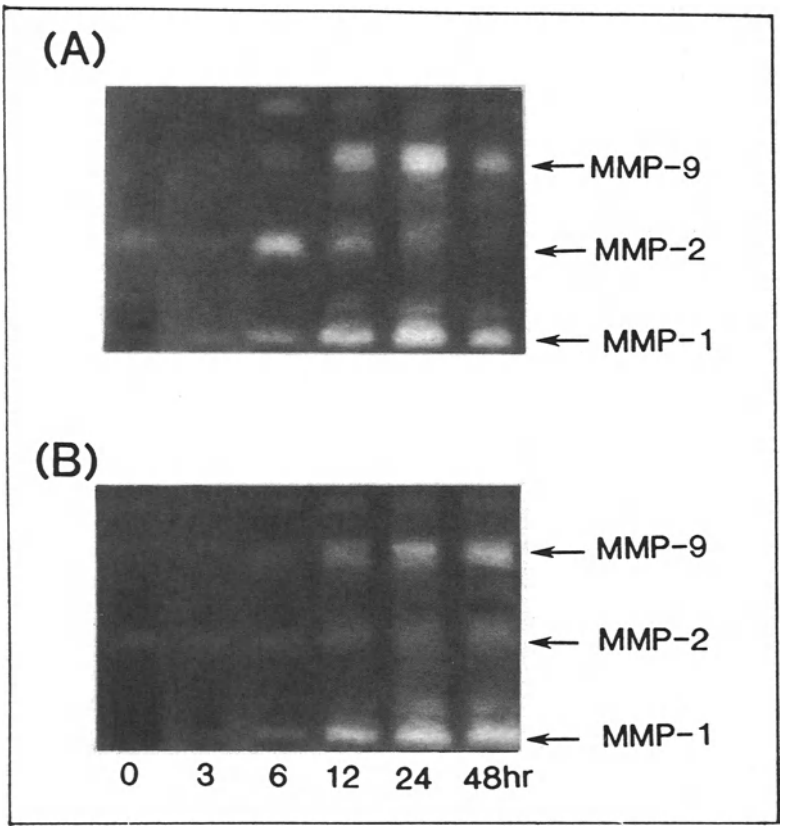


**Figure 2.** The detection of gelatin-zymography in the infarcted lesion isolated from either the untreated (*A*) or losartan treated (*B*) rat hearts from 0 to 48 hours after coronary artery ligation. Each lytic-band is detected with interstitial collagenase (MMP-1, 54kDa) and gelatinase (MMP-2, 72kDa and MMP-9, 92kDa).

unfold their triple helix conformation because of thermal degradation, and then further degradate into amino acid oligopeptides by MMP-2 and MMP-9 [25]. There is a modest but significant increase in the MMP-2 activity at 6 hours and in the MMP-9 activity at 12 hours in infarcted tissue after the induction of MI. These findings can thus account for the concomitant increase and contribution to collagenlytic activity of gelatinase. No MMP-9 activity (neutrophil collagenase; 65KDa) was observed. In pretreatment with losartan, zymographies showed that the activities of MMP-2 and MMP-9 both decreased in infarcted lesions at 6 and 24 hours, respectively, after the induction of MI (figure 2A). Other investigators [17] observed morphological evidence of fibrillar collagen degradation between cardiac myocytes soon after the induction of myocardial ischemia (one hour to one day).

This was accompanied by a decrease in the hydroxyproline content from one to three hours later. One day after infarction, the hydroxyproline content in involved tissue was reduced in the present study. However, since the degradated collagen was not removed from the heart, this assay could not discriminate between the intact and cleaved collagen fragments. Our findings seem to suggest that the MMP-1 activity rises from 6 hours after infarction, and, therefore, these enzymes are not likely to be involved in the early degradation of fibrillar collagen. MMP-1 alone is not able to completely degradate insoluble collagen, and, thus, a multienzyme system must be active to fully degradate collagen. In the present study, MMP-2 and MMP-9 were activated in infarcted tissues at 6 and 12 hours, respectively. This may contribute to the degradation process, where inflammatory cells, including macrophages and PMN leukocytes, invade infarcted tissue soon after myocyte necrosis. Cannon et al. [26] have demonstrated that the collagen content could be preserved within 24 hour of infarction by making rats leukopenic by irradiation. These findings suggest that protease was thus produced by inflammatory cells, including PMN leukocytes and macrophages [27]. The collagenase and the MPO activities were also determined in the infarced lesions at 24 hours after a coronary ligation. Both activities significantly increased in infarcted lesions compared with the nonoperated controls, sham-operated and interventricular septum (figure 3A). The collagenase increased at 24 hours to the maximum level in the infarct myocardium. Similarly, we also assessed the MPO activity in the infarct lesions after coronary ligation (figure 3B). The MPO activity, as well as the collagenase activity, decreased in the infarcted lesions when treated with losartan at 24 hours after the induction of MI. Thus, the loss of cardiac collagen in the early phase after infarction is probably mediated by the increased degradation of structural proteins. The acute loss of cardiac matrix may thus lead to myocyte slippage, chamber dilation, wall thinning, and even a rupture of the myocardium.

After rats were treated with 20 mg of losartan, both of the MMP-2 and MMP-9 activities significantly decreased (figure 2B). The precise mechanism for the decreased MMP activities by losartan after the induction of MI is unclear. A number of cytokines and growth factors have also been shown to induce or stimulate the synthesis of MMPs, including IL-1, PDGF, and TNF-$\alpha$, whereas TGF-$\beta$, heparin and corticosteroids all have an inhibitory effect [28]. Several reports have revealed that both ACE inhibition and cardiac angiotensin receptor blockade treatment are effective in the partial attenuation of collagen protein deposition in infarcted heart [10,21,29]. It is still not clear as to whether or not the stimulation of collagen synthesis by Ang II is due to either an increase in the transcription of the collagen gene or a decrease in the degradation of newly synthesized collagen [30]. However, Ang II can directly stimulate the proliferative growth of neonatal rat cardiac fibroblasts in culture [31,32]. Our results in the present study may thus account for the fact that losartan reduces the Ang II levels through Ang II generated locally or produced in the peripheral circulation. Therefore, both the MMP-2 and the MMP-9 activities may be directly or indirectly inhibited through such cytokines as TGF-$\beta$.

In conclusion, the early activation of collagenase and gelatinase activities may be

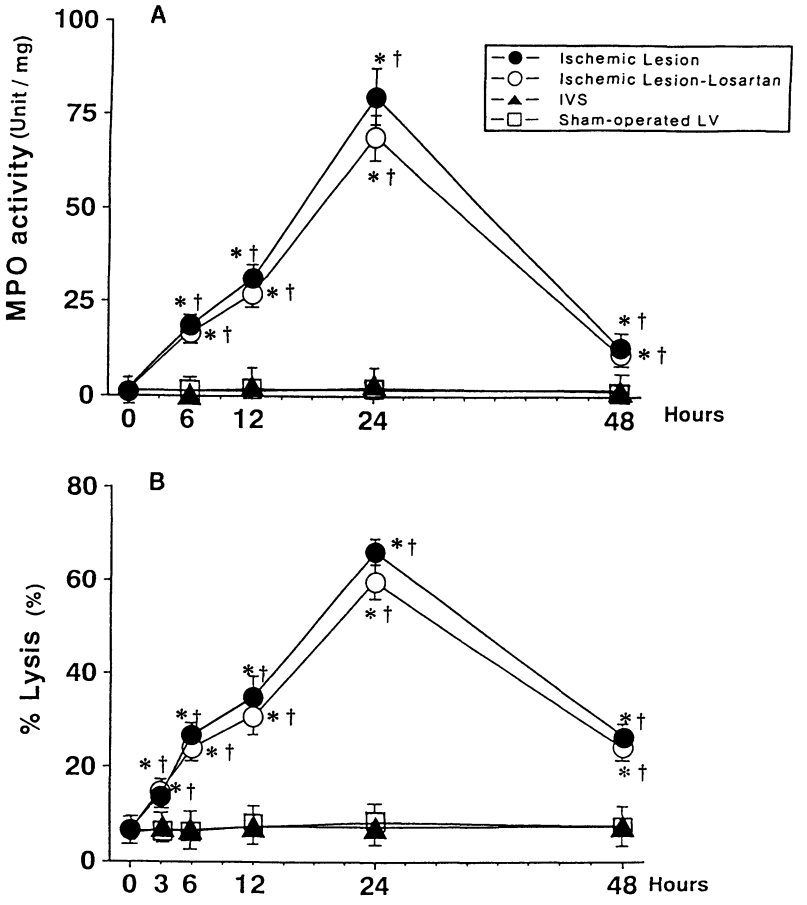


**Figure 3.** The myeloperoxidase (*A*) and the collagenolytic (*B*) activities in heart tissue specimens from the LV in sham-operated rats, the interventricular septum of MI rats, untreated infarcted lesions, and infarcted lesions treated with losartan. The myeloperoxidase activity represents the % activity per 100 mg protein. The collagenolytic activity represents the % lysis for the amount of $^{3}H$-labeled in the incubation medium. *$p < 0.05$ compared with the data from the sham-operated rats or the interventricular septum of the MI rats.

responsible for a disruption of the intercellular struts during LV remodeling postMI. An $AT_1$ receptor antagonist, losartan, is thus considered to have a beneficial effect on the infarcted myocardium in the remodeling process by preventing the formation of Ang II.

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# THE ROLE OF ANGIOTENSIN II IN POST-TRANSLATIONAL REGULATION OF FIBRILLAR COLLAGENS IN FIBROSED AND FAILING RAT HEART

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***Summary.*** An explanation of the molecular mechanisms that trigger the development of pathological cardiac fibrosis, myocyte hypertrophy, and heart failure associated with common ailments such as chronic postinfarction has been sought at the benchtop and clinic for the past forty years and is a current topic of intensive investigative activity. Among the information garnered from these studies is that (1) growth factors including angiotensin (Ang) II are involved in modulation of gene products specifically expressed by cardiac fibroblasts in vitro and (2) their enhanced presence has been associated with myocardial stress, inappropriate cardiac growth, and fibrosis in vivo. Although increased deposition of collagen proteins has been described after myocardial infarction (MI), little is known of (1) time-dependent transcriptional alteration of specific cardiac collagen subtypes or (2) the degradative mechanisms for cardiac collagens in right and left ventricular myocardium remote to large left ventricular infarction. We have investigated collagen mRNA abundance and the deposition of specific collagen subtypes in noninfarcted left and right rat heart muscle at different times after MI. We have also assessed the activity of different myocardial matrix metalloproteinases (MMPs) using zymography to gain some information about degradative pathways for collagen. Furthermore, we assessed passive compliance properties of the right ventricle in experimental hearts. Finally, the efficacy of an angiotensin-converting enzyme (ACE) inhibitor (ramipril) and that of an Ang II receptor type I antagonist (losartan) was compared in experimental animals. We observed that the mRNA abundance of types I and III collagen were increased 3 days after myocardial infarction in both viable left and uninfarcted right ventricular tissues and were maintained at relatively high levels throughout the duration of our studies. Stiffness of the right ventricular myocardium was significantly increased in the 56 days experimental group when compared with that of control values; this was positively correlated with increased immunoreactive collagens in surviving right (and left) cardiac tissue

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of 14, 28, and 56 day experimental groups. The elevation of fibrillar collagen mRNA abundance in noninfarcted muscle from ventricular chambers was not normalized by treatments with either ramipril or losartan. MMP activity was increased in viable left ventricle at 14, 28, and 56 days and at 14 days in the right ventricle in experimental animals when compared with controls. Finally, we observed that $AT_1$ receptor blockade in infarcted hearts was associated with normalization of elevated cardiac 4-prolyl hydroxylase protein expression compared with nontreated post-MI rats. Thus, increased expression of collagen types I and III occurs in noninfarcted heart after MI, followed thereafter by deposition of collagen proteins. Increased MMP activity is present in chronic experimental hearts; MMP activation may be important in remodeling of the noninfarcted cardiac stroma. Because losartan treatment was not associated with any normalization of elevated collagen mRNA abundance, a clear causal relationship between the upregulation of fibrillar collagen mRNA abundance and the $AT_1$ receptor was not apparent. On the other hand, the reduction of cardiac fibrosis mediated by ACE inhibition and losartan treatment may reside at the posttranslational level in cardiac collagen metabolism.

## INTRODUCTION

Myocardial stress is characterized by the specific response of, and maladaptive cardiac growth is regulated by a number of hormones including angiotensin (Ang) II. These ligands initiate various intracellular signaling pathways via specific membrane-bound receptors. Over the past few years, a number of studies support the hypothesis that the renin-angiotensin system (RAS) is important in the onset of pathological myocyte hypertrophy, cardiac fibrosis, and subsequent development of congestive heart failure (CHF) [1]. A complete catalogue of the activated proteins and factors associated with binding of Ang II to their receptors remains incomplete. Abnormal signaling by Ang II may stimulate cardiac fibroblasts to participate in excessive deposition of extracellular matrix components associated with the hypertrophy of cardiac myocytes, resulting in the development of CHF. The purpose of the present paper is to provide a review of the effects of Ang II on the myocardial intersitium by (1) describing postreceptor events after the initiation of Ang II signaling, (2) summarizing the role of Ang II in the development of cardiac fibrosis and heart failure and (3) presenting recent data from our laboratory to address the nature of Ang II-mediated regulation of fibrillar collagen expression in post-myocardial infarction (MI) rat heart.

## ANGIOTENSIN II: GENERATION OF PEPTIDE AND RECEPTOR ACTIVATION IN HEART

Classically, RAS components include renin, angiotensinogen, angiotensin-converting enzyme (ACE), as well as Ang I and II. The liver is a major source of angiotensinogen, which is a relatively large plasma protein and is the only known natural substrate of renin which is produced by the juxtaglomerular apparatus of the kidney [2]. Renin cleavage of angiotensinogen releases the Ang I decapeptide, and this inactive precursor is immediately converted to active Ang II (an octapeptide) by ACE. ACE is well characterized as the synthetic product of endothelial cells lining the vessels of the arterial and venous trees of the cardiovascular system. During the

past several years, the existence of a local or tissue RAS system in the heart has gained considerable attention [3]. It has since been demonstrated that Ang II is generated and released by cardiac myocytes [4] and cardiac fibroblasts [5]. For the purpose of this discussion, local cardiac RAS may be defined as the ability of the heart to express most of the RAS components to generate Ang II [3]. Although the heart may use circulating renin for in situ synthesis of Ang II, the importance of cardiac synthesis of this component may not be crucial for local generation of Ang II.

Whether generated locally or not, Ang II influences the myocardium in both a direct and indirect manner. The direct actions of Ang II on the cardiovascular system comprise potent vasoconstriction, positive cardiac inotropism, and positive cardiac chronotropism [6]. Indirect actions of Ang II on the heart include increased cardiac loading from activation of the sympathetic system and stimulation of aldosterone synthesis [6]. Cellular responses induced by Ang II in cardiac (and other) tissues are dependent on the balanced activation of different Ang II receptors. Biochemical, molecular, pharmacological, and functional studies have revealed the presence of two main subgroups, which are further divided into multiple receptor subtypes [7]. Studies on binding affinities for plasma membrane receptors to nonpeptide antagonists such as losartan and PD 123177 have defined the existence of $AT_1$ and $AT_2$ receptors, respectively [8]. To date, the vast majority of known physiological functions mediated by Ang II within the cardiovascular system are carried out by Ang II binding to the $AT_1$ receptor [9]. A corollary of this finding is that an overwhelming expression of this receptor subtype (vs. $AT_2$ receptor) is present in adult cardiovascular tissue.

The tertiary structure of the $AT_1$ receptor is that of a "typical" seven-transmembrane domain membrane receptor protein. The $AT_1$ group of Ang II receptors is further subdivided into $AT_{1A}$ and $AT_{1B}$ classes [7]. Among them, the $AT_{1A}$ and $AT_{1B}$ isoforms contain 22 different amino acids, yet maintain similar binding profiles for Ang II and nonpeptide, as well as peptide $AT_1$ receptor antagonist(s), including losartan [7]. $AT_{1A}$ subtype is localized mainly in vascular smooth muscle cells, hypothalamic tissue, lung, kidney, adrenal, fetal pituitary, and liver tissues [7,10]. The $AT_{1A}$ receptor subtype is known to be transcriptionally inducible and may be influenced by diverse stimuli, including tissue culture conditions; receptor numbers are also known to be variable with the stage of cardiac development as well as in the face of various pathological stimuli [11]. In the cardiovascular system, the $AT_{1A}$ receptor is constitutively expressed in all developmental stages [12]. The $AT_{1B}$ receptor has been described in the zona glomerulosa of the adrenal medulla, uterine, anterior pituitary, and renal tissues [7,10].

The $AT_2$ receptor shares the seven transmembrane domain receptor protein configuration and only 32% homology with the $AT_1$ receptor [13]. Early studies have provided evidence that, unlike the $AT_1$ receptor, $AT_2$ receptors lack functional coupling to any trimeric G proteins [14]. Recently this evidence has been met with the results of Zhang et al. which indicate that $AT_2$ receptors may bind several $G_\alpha$ subunits in whole fetal tissue [15]. Furthermore, $AT_2$ receptors have been further

subdivided into classes $AT_{2A}$ and $AT_{2B}$ based on distinct pharmacological characteristics which include differential binding of trimeric G protein [7]. Steinberg found that while the $AT_{2B}$ receptor protein did not couple to G proteins, the $AT_{2A}$ receptor subtype was observed to complex with them [7]. The work of Zhang et al. supports the suggestion that the selective interaction, or lack thereof, between the G protein subunits and $AT_2$ receptor subtypes may confer specificity in the cellular response that is dependent on the prevailing receptor expression patterns [15]. In general, neonatal and adult cardiac cells seem to express relatively low levels of $AT_2$ receptors. For example, both neonatal and adult cardiac fibroblasts are characterized by the presence of very low levels of the $AT_2$ receptor [16–17]. Expression of $AT_{2A}$ receptor is widespread in the brain, whereas the $AT_{2B}$ receptors appear in abundance in adrenal medullary and uterine tissues [7]. An important difference between $AT_1$ and $AT_2$ receptor classes is the ease in design and utilization of specific nonpeptide antagonist agents for use in their characterization [7]. Furthermore, unlike $AT_1$ receptors, $AT_2$ receptors do not undergo ligand-mediated endocytosis upon complexing with Ang II [2].

In spite of the intense scrutiny paid to the investigation of the function of the $AT_2$ receptor, our understanding of the precise role of this receptor in the cardiovascular system is far from clear. Nonetheless, some lines of evidence point to multiple putative functions in various tissues. Activation of $AT_2$ receptors has been shown to induce G protein-dependent apoptotic remodeling via the dephosphorylation of mitogen-activated protein kinase (MAPK) [18]. Attenuation of MAPK protein tyrosine phosphatase 1 by vanadate or by antisense oligonucleotides will inhibit MAPK dephosphorylation; their application has been shown in PC12W and R3T3 cells to block $AT_2$ receptor-mediated apoptosis [18]. These results suggest that MAPK phosphatase 1 is involved in $AT_2$-mediated apoptosis [18]. Stoll et al. have shown that the $AT_2$ receptor mediates the inhibition of cellular proliferation in coronary endothelial cells [19]. This novel finding is supported by a study using gene transfer methods that demonstrate that the $AT_2$ receptor also has an antiproliferative effect on the neointima after vascular injury; that is, the overexpression of $AT_2$ receptor in carotid artery attenuates neointimal formation [20]. Mouse $AT_2$ receptor knock-out studies conducted by Ichiki et al. raise the possibility that $AT_2$ receptor function is important in maintenance of normal cardiovascular and central nervous system function, but that this receptor subtype is not required for embryonic development [21,22]. Loss of $AT_2$ receptor function is associated with increased systolic blood pressure, depressed body temperature, impaired dypsogenic response to water deprivation, and a reduction in spontaneous movements [22]. The expression of $AT_2$ receptor is known to be elevated in tissue undergoing wound repair, as well as in vascular injury and cardiac hypertrophy associated with myocardial infarction [20,23]. In view of some of these findings, it has been suggested that $AT_2$ receptor actions may oppose some of the functions mediated by the $AT_1$ subtype receptor i.e., in blood pressure regulation, so that in homeostasis, the overall response to Ang II is a balanced activation of downstream effector pathways. A caveat of this hypothesis stipulates that $AT_2$

receptor expression in the cardiovascular system is usually less than that of $AT_1$ receptor.

## CARDIAC ANGIOTENSIN II—MEDIATED SIGNALING

Ang II activation of the $AT_1$ receptor is characterized by the activation of a heterotrimer of G proteins and this ligand-receptor complex is known to undergo immediate endocytosis [9]. Experiments utilizing both adult and neonatal cardiac fibroblasts have demonstrated that $AT_1$ receptor activation is associated with stimulation of phospholipase C β (PLCβ) [24]. It is suggested that the $G_{q/11\alpha}$ is associated with the activation of PLC [25]. Activation of $G_{q/11\alpha}$ (via the $AT_1$ receptor) facilitates PLCβ-mediated cleavage of phosphoinositol 4,5-bisphosphate (PIP-2) to inositol 1,4,5-triphosphate ($IP_3$) and 1,2 diacylglycerol (DAG). $IP_3$ and DAG are well-known intracellular mediators of a unique series of signaling pathways that may function in parallel. The release of $IP_3$ leads to a rapid rise in intracellular $Ca^{2+}$ in the cytosol of myocytes; this phenomenon is independent of the external $Ca^{2+}$ concentration and culminates in an acute positive inotropic effect in heart [26,27]. On the other hand, DAG binding is known to activate a membrane-associated protein kinase C (PKC)-dependent pathway [28], which has been postulated to follow one of several downstream sequences [24]. MAPK is thought to play a pivotal role in coordinating external stimuli with nuclear events, and this cascade is implicated in the induction of myocardial protein synthesis and is associated with the development of cardiac hypertrophy [29]. Characterized as cytosolic serine/threonine kinases, MAPKs are triggered by a number of growth stimuli in addition to Ang II, including endothelin-1 [30]. Ang II rapidly induces tyrosine kinase proteins, which are immunologically related to MAPK 42- and 44-kD, as well as increases the activity in the downstream kinase, PSK, in both cardiac myocytes and fibroblasts [29]. Maximal stimulation of cardiac fibroblasts is associated with an initial peak of elevated MAPK activity (2–5 minutes), followed by a smaller sustained plateau of activity (up to 3 hours), and both phases of MAPK activation require $AT_1$ receptor binding [31]. In adult cardiac fibroblasts, sustained elevation of MAPK activity is necessary for Ang II induction of increased DNA synthesis and cell proliferation [31]. While it is suggested that activation of MAPK is a critical component for mitogenic events in cardiac fibroblast cells, the relatively small increase in DNA synthesis suggests that MAPK may work jointly with other factors for the occurrence of optimal Ang II-mediated cardiac tissue remodeling. In this respect, concerted chelation of $Ca^{2+}$ and downregulation of PKC have been found to interfere with Ang II induction of MAPK [24,32]. Ang II-mediated activation of MAPK both in cardiac myocytes and fibroblasts is known to induce expression of early response genes such as Egr-1, c-fos, c-jun or c-myc [24,26]. Since the c-fos gene promoter contains a nucleotide sequence designated the serum response element (SRE), it may bind factors such as $p62^{TCF}$, which is the substrate for MAPK phosphorylation [24]. Furthermore, in neonatal cardiac myocytes and fibroblasts, $AT_1$ activation has been also associated with stimulation of tyrosine kinase and

ribosomal S6 protein kinase (RSK) [33,34]. Finally, Ang II has been shown to activate soluble tyrosine kinases belonging to the Janus kinase (JAK) family in vascular smooth muscle cells [35]. Activated JAK proteins will specifically phosphorylate a family of proteins known as "signal transducers and activators of transcription" (STAT). Hence, Ang II leads to the translocation of STAT protein into the nucleus [36]. In cultured neonatal cardiac fibroblasts, Ang II induces STAT protein phosphorylation, translocation of STAT into the nucleus, and initiation of gene transcription [37]. Therefore, it is apparent that Ang II may rapidly stimulate the growth of myocardial cells involved in cardiac remodeling by the activation of several systems.

Desensitization of the $AT_1$ receptor has been suggested to function as an autoregulatory mechanism to modulate the potent effects of Ang II in the heart. This may occur via covalent modification of the $AT_1$ receptor protein, as well as by internalization and recycling of this protein [2]. The hypothesis that desensitization is initiated by the phosphorylation of the $AT_1$ receptor by specific kinases requires further investigation. On the other hand, internalization of the $AT_1$ receptor has been suggested to be critical for long-term regulation of the $AT_1$ receptor density [9]. Using vascular smooth muscle cells, studies have shown that Ang II-induced internalization of the $AT_1$ receptor may autoregulate the transcription of the $AT_1$ receptor gene [38].

## CARDIAC FIBROSIS AND HEART FAILURE

In cardiac fibroblasts, the $AT_1$ receptor participates in the induction of extracellular matrix (ECM) protein component synthesis and gene expression-mediating mitogenic responses [39,40]. We and others have shown that in vivo administration of Ang II to experimental animals is associated with increased steady-state cardiac collagen and fibronectin [41,42]. Experimental studies and clinical trials undertaking the investigation of ACE inhibition and $AT_1$ blockade provide evidence that their use is associated with normalization of cardiac fibroblast growth and deposition of myocardial ECM mediated by fibroblasts [43–46]. In this respect, chronic treatment of infarcted heart with either ACE inhibitors or $AT_1$ receptor antagonists is effective in partial attenuation of collagen protein deposition, which is characteristic of these hearts [44]. The factor(s) responsible for persisting interstitial fibrosis remain obscure. It may be argued that ACE inhibitor treatment may potentiate bradykinin in the heart by inhibition of the kininase II enzyme (i.e., ACE); however, the relevance of bradykinin in this role is brought into question by results of a comparative study whereby enalapril (an ACE inhibitor) and losartan treatment were associated with equal attenuation of myocardial fibrosis in the noninfarcted left ventricle of experimental rats [44]. Thus, it seems that the efficacy of ACE inhibition lies in the ability of these agents to suppress Ang II in the stimulation of cardiac fibroblasts in post-MI heart. The critical role of the cardiac $AT_1$ receptor in the stimulation of cardiac fibrosis is underscored by results of different studies. A study of infarcted tissue in post-MI rats demonstrated that fibrosis is marked by an increase in locally produced

Ang II via increased ACE activity and $AT_1$ receptor density [47]. These investigators also reported that these changes were localized to fibroblasts and myofibroblasts, which appeared at the site of infarction 1 week from induction of damage [48]. Furthermore, a 4.2-fold and 3.2-fold increase in $AT_{1A}$ and $AT_2$ mRNA levels, respectively, were found in infarcted regions, while a ~ twofold increase in these mRNAs for both $AT_{1a}$ and $AT_2$ receptors were observed in noninfarcted regions of the myocardium in the 7-day post-MI group [23].

Although small increases in interstitial fibrillar collagens may be beneficial for optimization of active relengthening of myocytes and function of the heart as a suction pump, excessive collagen accumulation contributes to increased myocardial stiffness resulting in a degeneration of cardiac function that is consistent with the development of congestive heart failure [49,50]. Hence, altered synthesis of the ECM may play a major role in the development of heart failure.

## CARDIAC COLLAGEN METABOLISM AFTER MYOCARDIAL INFRACTION

Chronic myocardial infarction induces morphological and molecular alterations in the infarcted and noninfarcted regions of the heart and these changes are collectively referred to as ventricular remodeling [51]. These alteration are accompanied by the occurrence of significant left and right heart growth with attendant pulmonary and liver congestion [52]. Relatively little information is available with regard to the role of nonmyocytes in the process of remodeling of viable cardiac tissue after myocardial infarction [52–54]. Fibrillar collagen types I and III are widely expressed in many organs, including the heart. These molecules aggregate to form myocyte-myocyte and myocyte-vessel struts which impart tensile strength to the myocardium and thereby mediate passive stiffness of both left and right ventricular chambers. Cardiac fibroblasts account for the synthesis of fibrillar collagen types I and III in the heart, and unlike terminally differentiated cardiac myocytes, may proliferate under certain pathological conditions [55]. It appears that phenotypically transformed fibroblast-like cells, i.e., myofibroblasts, which express fibrillar collagens, $AT_1$ receptors, ACE, and α-smooth muscle actin are the primary contributors in mediating wound healing in the heart [48,56]. It is known that altered synthesis of this matrix may play a major role in the development of heart failure [57,58]. Furthermore, fibroblasts and/or myofibroblasts are mitotically active in heart failure, and the active DNA-synthesizing cells in the surviving myocardium of infarcted rat heart have been identified as nonmyocytes [46]. Results of either clinical or experimental studies from this lab [59] and others [46,60–62] provide evidence for the appearance of total collagen protein in the left ventricle remote to the infarct site. Nevertheless, Sirius red dye staining or 4-hydroxyproline measurement provides little information on the relative contribution of specific collagen subtypes in cardiac fibrosis. Furthermore, very little is known about the passive compliance properties of right ventricular muscle in this experimental model, although these changes may herald the loss of normal diastolic function.

There is limited information about the alteration of cardiac collagen mRNA abundance and characterization of the collagen subtypes in the hypertrophied

noninfarcted tissues in this model of heart failure. Knowlton et al. (1992) studied acute changes (1–7 days) in fibronectin isoform and fibrillar collagen expression specifically within the infarcted regions in rabbit heart (reparative fibrosis) and noted a rapid increase in total fibronectin and collagen steady-state mRNA abundance after 1 and 2 days, respectively [63]. We have undertaken studies to resolve the expression of collagen mRNA in both ventricles remote to the infarct. Immunohistochemical studies were carried out to determine the pattern of deposition for specific collagen types I and III after myocardial infarction, and a comparison of mRNA and protein expression of specific fibrillar collagens in noninfarcted left and right heart is offered. To study the role of $AT_1$ receptor activation in the expression and deposition of different collagens, we investigated the effects of losartan on steady-state collagen mRNA abundance. Myocardial metalloproteinase (MMP) activities in sham control and experimental hearts were also investigated to assess the status of matrix removal in noninfarcted cardiac tissue.

## MATERIALS AND METHODS

### Experimental model

All experimental protocols for animal studies were approved by an appointed Animal Care Committee located at the University of Manitoba, Canada, following guidelines established by the Medical Research Council of Canada. Myocardial infarction was produced in male Sprague-Dawley rats (weighing 200–250 g) by surgical occlusion of the left coronary artery as described previously, with minor modifications [52]. In short, after isofluorane anesthesia, the thorax was opened by cutting the third and fourth ribs, and the heart was extruded through the intercostal space. The left coronary artery was ligated about 2–3 mm from the origin with a suture of 6-0 silk, and the heart was repositioned in the chest. Closure of the wound was accomplished by the use of a purse-string suture. Throughout the operation, ventilation of the lungs was maintained by positive-pressure inhalation of 95% $O_2$ and 5% $CO_2$ mixed with isofluorane. The advantage of isofluorane over other aromatic anesthetics is that no excessive fluid secretion in the respiratory tract occurs with its use, and therefore the animals did not suffer from respiratory distress during the operation. Sham-operated animals were treated similarly, except that the coronary suture was not tied. The mortality of all animals operated upon in this fashion was about 45% within 48 hr. Only animals with large infarcts ($\geq$40% of the left ventricular free wall) were used in this study. Because we were concerned with alterations in myocardium remote to the infarct site, including the right ventricle, and since this chamber only becomes hypertrophied after relatively large infarction in this model [52,64], determination of the average infarct size under our conditions was necessary. Briefly, a group of $n = 7$ animals were subjected to coronary ligation surgery as described above, and the percentage of infarcted left ventricle was estimated 4 weeks after coronary ligation by planimetric techniques, as previously described [46].

### RNA extraction

Myocardial total RNA was isolated from viable left and right ventricle by the method of Chomczynski and Sacchi [65] at 3, 7, 14, 28, and 56 days after coronary occlusion. Briefly, animals were sacrificed and the heart was rapidly excised. The atria were removed and the viable left ventricle and right ventricle were washed twice with a solution containing 10 mM 3-[N-morpholino] propanesulfonic acid (MOPS) and 10 mM sodium ethylenediaminetetraacetate (EDTA). Left and right ventricular tissue were immediately frozen and maintained at −196°C in liquid nitrogen until use. The frozen samples were then ground with mortar and pestle while immersed in liquid nitrogen. Powdered samples were suspended in solution D (4 M guanidine isothiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% N-laurylsarcosine, 0.1 M 2-mercaptoethanol) and subjected to polytron homogenization. Tissue homogenates were subjected to phenol and chloroform extraction. RNA pellets were dissolved in diethyl pyrocarbonate (DEPC) treated water, and the concentration of nucleonic acid was calculated from the absorbance at 260 nm prior to size fractionation.

### Northern blot analysis

Twenty μg of total RNA was electrophoresed in a 1.2% agarose/formaldehyde gel, and the fractionated RNA was transferred to a 0.45 mm positive charge-modified nylon membrane (Zeta-Probe membrane, Bio-Rad). The RNA were covalently cross-linked to the matrix using UV radiation (UV Stratalinker 2400, Stratagene). Using an INNOVA 4000 incubator (New Brunswick Scientific) oscillating at a rate of 60 rotations per minute, blots were prehybridized at 43°C for 6–16 hours. Each membrane was hybridized with $^{32}$P-labeled cDNA probes (specific activity $>10^9$ cpm per μg DNA) at 43°C for 16–20 hr. cDNA probes were labeled by a random primer DNA labeling system using Klenow fragment. Filters were exposed to x-ray film (Kodak X-OMAT) at −80°C with intensifying screens. cDNA fragment for human procollagen type α1(I) (Hf 677) [66], human type α1(III) (Hf 934) [67], and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [68] were obtained from the American Type Culture Collection. Rat 18S rRNA (5′-ACGGTATCAGATCGTCTTCGAACC-3′) [69] was synthesized using the Beckman Oligo 1000 DNA synthesizer, with amidites and solutions supplied by Glen Research (Sterling, VA, USA). Results of autoradiographs from Northern blot analysis were quantified by densitometry (Bio-Rad imaging densitometer GS 670). The signals of specific mRNAs were normalized to those of GAPDH mRNA and 18S ribosomal RNA to normalize for differences in loading and/or transfer of mRNA.

### Immunohistochemistry

A total of 36 rats were used in this assay; 4 sham and 5 post-MI rats were included in groups timed at 7, 14, 28, and 56 days after surgery. After anesthetic administra-

tion with a ketamine: xylazine mixture, the animal was sacrificed by decapitation. Hearts were rapidly excised from the thoracic cavity and immediately immersed in a 1 M phosphate buffered saline (PBS) solution. The atria were removed and the viable left ventricle remote to infarction zone and right ventricle were washed twice, immersed in OCT compound (Miles Inc.), and stored frozen at −80°C. Serial cryostat sections (7 μm thick) of the left and right ventricles were mounted on gelatin coated slides, prefixed in 1% paraformaldehyde, and allowed to air-dry. At least 6 sections from each ventricle of each group (sham and experimental) were processed, and representative sections were chosen. Immunohistochemical staining was performed by the indirect immunofluorescence technique [70]. In brief, after rinsing in PBS, the tissue section was incubated with the primary antibody, against either collagen I or collagen III. Goat polyclonal anti-types I and III collagen (Southern Biotechnology Associates Inc, Alabama, Ga., USA) at a 0.4 mg/mL concentration were diluted 1:100 with 1% BSA in PBS and applied as the primary antibodies. The cross-reactions of the antibody for collagen type I (or III) with collagen type III (or I) is less than 10%, as indicated by the manufacturer. After incubation overnight at 4°C, the sections were subsequently washed three times (5 minutes each) in PBS and incubated with biotinylated anti-goat IgG secondary antibody (Amersham, Canada) for 90 minutes. The tissues were rinsed again three times in PBS and treated for 90 minutes with Texas Red-labeled streptavidin and FITC-labeled streptavidin (Amersham, Canada) for collagens I and III, respectively. Finally, the slides were mounted with Vectashield (Vector Laboratories, California) and coverslipped. The tissue sections were examined under a Nikon Labophot microscope equipped with epifluorescence optics and appropriate filters, and the results were recorded by photography on Kodak TMAX 400 black and white film.

**Determination of cardiac total collagen**

Viable left and right ventricles from sham-operated and MI groups were separated, cut into small pieces, dried, and weighed. Tissue samples were digested in 6 M HCl for 16 hr at 105°C. Cardiac hydroxyproline was measured according to the method of Chiariello et al., [71] and modified by Pelouch et al., [59]. A stock solution containing 40 mM of 4-hydroxyproline in 1 mM HCl was used as standard. Collagen concentration was calculated multiplying hydroxyproline levels by the factor 7.46, assuming that interstitial collagen contains an average of 13.4% hydroxyproline. The data are expressed as μg collagen per mg dry tissue [71].

**Zymography: detection of cardiac matrix metalloproteinase activity**

Viable left and right ventricular muscle were ground with mortar and pestle, while immersed in liquid nitrogen. Powdered tissue (50 mg) was suspended in 1 mL in phosphate-buffered saline (pH 7.4) containing 100 μg/ml phenylmethylsulfonyl fluoride (PMSF) and 2 μg/ml leupeptin and incubated at 4°C with continuous agitation for 20 hr to extract metalloproteinases. The sample was then centrifuged at

10,000 rpm at 4°C for 10 min. The resulting supernatant was used for total protein assay and zymographic analysis. Total protein was determined by using the bicinchoninic acid protein assay kit (Sigma) [72]. Myocardial matrix metalloproteinase (MMP) activity was detected by zymography [73]. Gelatin (final concentration 1 mg/mL) was added to a 7.5% standard sodium dodecyl sulfate-polyacrylamide gel eletrophoresis (SDS-PAGE). Gelatin was used as a substrate because it is readily cleaved by connective tissue-degrading enzymes and is easily incorporated into polyacrylamide gels. Thirty μg of protein was loaded per lane without reduction and boiling to maintain the activity of MMP, and samples were run at 15 mA/gel. After electrophoresis, gels were washed two times (15 min/wash) in 25 mM glycine (pH 8.3) containing 2.5% Triton X-100 with gentle shaking at room temperature to eliminate SDS. Gels were rinsed and incubated at 37°C for 18 hr in substrate buffer (50 mM Tris-HCl, pH 8.0, 5 mM $Ca_2Cl$). After incubation, gels were stained in 0.05% coomassie blue R-250 for 30 min and then destained in acetic acid and methanol. Gels were then dried and scanned using a CCD camera densitometer (Bio-Rad imaging densitometer GS 670) for relative lytic activity.

**Passive pressure-volume relationship in right ventricle**

Myocardial stiffness was determined according to the methods described by Raya et al., [74]. Briefly, rat hearts from both sham-operated and infarcted groups were subjected to Langendorff perfusion with Krebs-Henseleit solution. A latex balloon, attached to a stiff plastic tube, was inserted into the right ventricle via the tricuspid valve. The other end of the tube was connected to a pressure transducer and a 1 mL syringe via a three-way stopcock. After 15 min equilibration, the heart was arrested by perfusion with a 15 mM potassium Krebs-Henseleit solution. The volume of the balloon was adjusted to reduce the pressure to −5 mmHg. Steady-state right ventricular pressure was recorded during increments in balloon volume (10 μl) until the pressure increased to 50 mmHg. The data was computed in real time and analyzed using a dedicated IBM PC with AcqKnowledge (version 3.0) software (Biopac, Harvard Apparatus Canada).

**Administration of losartan and ACE inhibitor to experimental animals**

In some experiments, losartan ($AT_1$ receptor antagonist) or ramipril (ACE inhibitor) were administered to experimental animals with large MI. All treatments were initiated one day following coronary occlusion and were continued for 7–28 days post-MI. Ramipril was dissolved into saline at a concentration of 1 mg/ml. It was administered to animals at a dosage of 1 mg/kg/day in conscious animals and was given by gavage once a day for 7 days [75,76]. For comparative purposes, age-matched animals with large MI and sham-operated controls were administered vehicle by daily gavage (0.9% saline). Losartan (15 mg/kg/day) was administered by implanting osmotic minipump to experimental animals for 7, 14, and 28 days [60]. To achieve 28 days treatment, two 14-day osmotic minipumps (model 2002) were implanted consecutively. As above, age-matched animals with large MI, as well as

sham-operated control animals, were administered vehicle. The animals were decapitated after treatment with either losartan or ramipril, and left and right ventricular tissues were subsequently used to assess fibrillar collagen gene expression.

### ELISA for cardiac 4-prolyl hydroxylase

Viable left ventricular tissues (n = 36) were frozen in liquid nitrogen and ground to powder, followed by homogenization in 10 mM Tris-HCl buffer, pH 7.8, with 0.1 M NaCl, 0.1 M Glycine, 0.1% Triton X-100, 20 mM EDTA, 10 mM N-ethylmaleimide, 1 mM PMSF, 1 mM P-hydroxymercuribenzoic acid, and 1 mM dithiothreitol (DTT). The homogenized samples were centrifuged at 20,000 × g at 4°C for 30 min, and the supernatants were transferred to fresh tubes for ELISA, using the kit supplied by Fuji Chemical Industries, Ltd. (Toyama, Japan) [77]. Two monoclonal antibodies were employed; the first was a capture antibody in solid phase while the secondary antibody was linked to horseradish peroxidase. Samples were diluted 1:20 in distilled water prior to quantification of proteins by the BCA method (Sigma, St. Louis, Mo., USA) [72].

### Statistical analysis

All values are expressed as mean ± S.E.M. The differences between control and experimental sample at each time point were calculated using the student's t-test. The Northern blot data in each figure was expressed as percentage of control according to the method of Fisher and Periasamy [78]. One-way analysis of variance was used for multigroup comparisons (SigmaStat, version 1.0). Significant differences among groups were defined by a probability of less than 0.05.

## RESULTS

### Infarction size and cardiac hypertrophy

Transmural infarct size in the five surviving animals used for this aspect of the study was 42% ± 3% of the total left ventricular circumference. Experimental animals were characterized by the presence of large MI which was comparable to values reported earlier which was associated with development of time-dependent right and left ventricular hypertrophy (table 1) [52,79,80]. Early (3 days) after induction of MI, neither right ventricle wet weight (RVW) nor left ventricle wet weight (LVW) was significantly different from control ventricles. It is pointed out that at these relatively early time points, scar formation in the infarcted left chamber is known to be incomplete, and thus the process of thinning of the necrotic free wall had not yet completely evolved. In the 7, 14, 28, and 56 day experimental groups, a significant increase in the mass of right and viable left ventricular tissue was noted by all indices (RVW, LVW, RV/BW, LV/BW) when compared to values from sham-operated control hearts. Thus the incidence of hypertrophy for right and left ventricular chambers noted in this study were comparable to our previous findings [52]. ACE inhibitor therapy (7 days) was associated with a significant reduction of LVW (0.50 ± 0.02 g in ramipril-treated animals vs. 0.64 ± 0.02 g in nontreated MI

**Table 1.** Cardiac hypertrophy and transmural scar weight in experimental rats at 3, 7, 14, 28, and 56 days after induction of myocardial infarction

| | 3-day | | 7-day | | 14-day | | 28-day | | 56-day | |
|---|---|---|---|---|---|---|---|---|---|---|
| Parameters | Sham | MI | Sham | MI | Sham | MI | Sham | MI | Sham | MI |
| BW, g | 246 ± 8.4 | 219 ± 11* | 280 ± 7.0 | 269 ± 9.6 | 354 ± 5.7 | 340 ± 5.5 | 409 ± 13 | 415 ± 13 | 507 ± 9.4 | 509 ± 12 |
| LVW, g | 0.57 ± 0.01 | 0.56 ± 0.03 | 0.64 ± 0.02 | 0.66 ± 0.03 | 0.79 ± 0.01 | 0.86 ± 0.02* | 0.78 ± 0.02 | 0.87 ± 0.02* | 0.89 ± 0.03 | 0.99 ± 0.03* |
| Scar, g | ... | 0.21 ± 0.03 | ... | 0.21 ± 0.01 | ... | 0.25 ± 0.03 | ... | 0.28 ± 0.03 | ... | 0.29 ± 0.03 |
| RVW, g | 0.18 ± 0.01 | 0.19 ± 0.01 | 0.19 ± 0.01 | 0.22 ± 0.02* | 0.25 ± 0.01 | 0.30 ± 0.02* | 0.23 ± 0.01 | 0.31 ± 0.03* | 0.23 ± 0.01 | 0.48 ± 0.05* |
| LV/BW, mg/g | 2.02 ± 0.30 | 2.57 ± 0.09 | 2.27 ± 0.04 | 2.45 ± 0.04* | 2.22 ± 0.06 | 2.53 ± 0.05* | 1.92 ± 0.04 | 2.09 ± 0.04* | 1.75 ± 0.06 | 1.95 ± 0.04* |
| RV/BW, mg/g | 0.74 ± 0.04 | 0.89 ± 0.08 | 0.68 ± 0.04 | 0.82 ± 0.03* | 0.71 ± 0.04 | 0.88 ± 0.03* | 0.57 ± 0.02 | 0.76 ± 0.08* | 0.45 ± 0.03 | 0.94 ± 0.11* |

Note: MI indicates experimental animals with large left ventricular myocardial infarction; sham, noninfarcted age-matched control animals; BW, body weight; LVW, left ventricle wet weight; RVW, right ventricle wet weight. Results are mean ± SEM of 8–10 experiments. *$P < 0.05$ vs, sham-operated control at each time point.

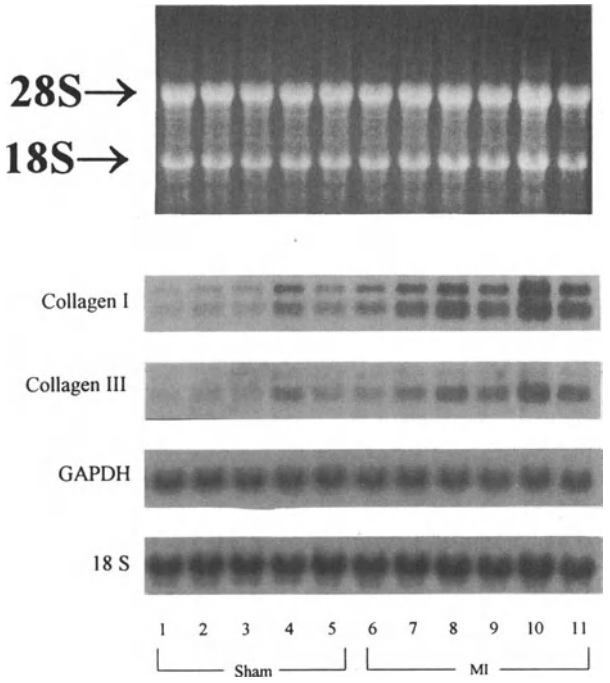


**Figure 1.** *A*, representative agarose gel stained with ethidium bromide to visualize the 28S and 18S rRNA bands in total RNA samples extracted from cardiac ventricular tissues. *B*, Autoradiograph from Northern blot analysis wherein each lane was loaded with 20μg left ventricular total RNA extracted from noninfarcted control animals (sham, lanes 1–5) and animals 14 days after myocardial infarction (MI, lanes 6–11). The control group was age-matched. Hybridization of fractionated total RNA with cDNA probes for procollagen α1(I), procollagen α1(III) (i.e., collagen types I and III, respectively), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and 18S rRNA indicate relative steady-state mRNA levels for each gene tested.

rats) and RVW (0.16 ± 0.01g in ramipril-treated animals vs. 0.19 ± 0.02g nontreated MI rats) in experimental animals. Regression of left and right ventricular hypertrophy was also found in experimental animals treated with losartan for 28 days when compared with nontreated MI groups.

### Cardiac collagen mRNA abundance

We addressed mRNA abundance changes in the myocardium at several points very early after the induction of infarction (3, 7, and 14 days), prior to the development of overt heart failure. For comparative purposes, we also assayed collagen mRNA expression at later stages of heart failure (28 and 56 days). Verification of the integrity of total fractionated RNA samples are provided by visualization of the 28S and 18S rRNA bands in a representative photograph of an agarose gel stained with ethidium bromide (figure 1, upper panel). Specific hybridization of cDNA probes

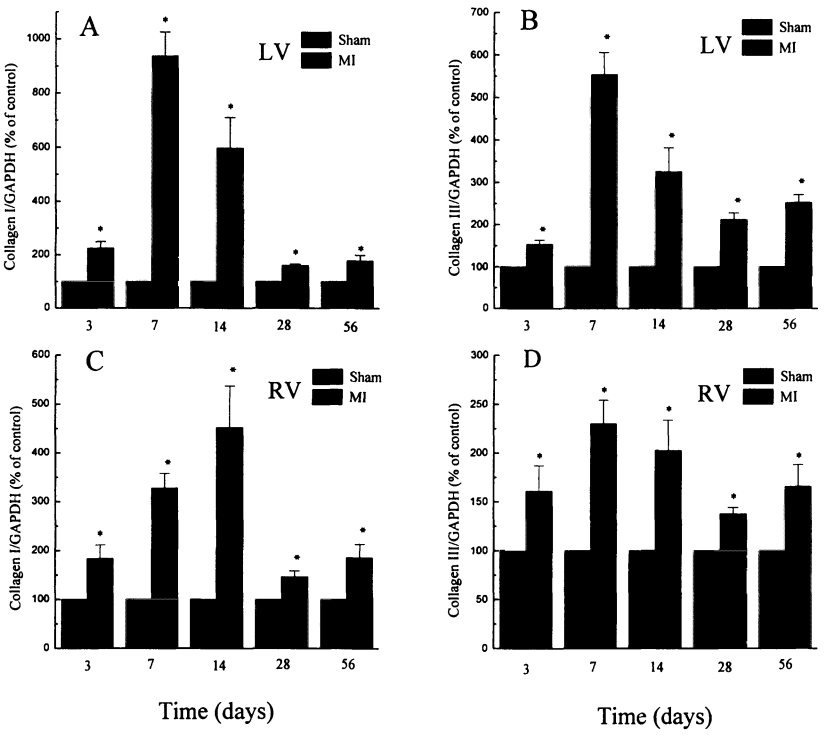


**Figure 2.** Estimation of the relative steady-state abundance of viable left (LV) and right ventricular (RV) collagen types I and III mRNAs at different times after myocardial infarction (MI = experimental animals) or in noninfarcted controls (sham). *A*, Collagen type I/GAPDH signal ratio in LV; *B*, Collagen type III/GAPDH signal ratio in LV; *C*, Collagen type I/GAPDH signal ratio in RV; and *D*, Collagen type III/GAPDH signal ratio in RV. The data were expressed in arbitrary densitometric units, normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) autoradiographic band intensity, and noted as a percent value of control expression levels (%). The data depicted is the mean ± SEM. of 4–8 experiments. *$P < 0.05$ for each experimental group value vs. age-matched sham operated values.

revealed characteristic mRNA bands, and these are shown in autoradiographs of representative blots probed with cDNAs of collagen types I and III, GAPDH, and an oligonucleotide specific for 18S rRNA (figure 1, bottom panel). In the viable left ventricle, collagen type I mRNA abundance was increased significantly when compared with sham-operated animal at 3 days after MI. Furthermore, collagen type I mRNA expression was increased ~9-fold in the experimental samples 7 days after induction of MI. The mRNA abundance for this collagen sub-type remained significantly elevated over control levels at all time periods within the current experimental design (14, 28, and 56 days) (figure 2A). Similarly, in the right ventricle, collagen type I gene mRNA abundance was found to be significantly elevated when compared with samples of sham-operated right ventricular total

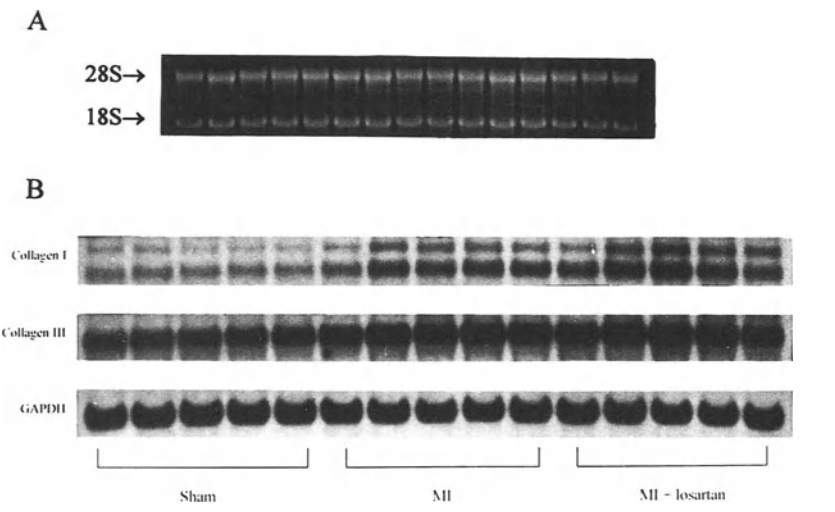


**Figure 3.** *A*, A representative agarose gel stained with ethidium bromide to visualize the 28S and 18S rRNA bands in total RNA samples extracted from viable left ventricular tissues at 4 weeks after MI. *B*, Autoradiograph from Northern blot analysis wherein each lane was loaded with 20 μg total RNA extracted from sham animals (lanes 1–5), myocardial infarction (MI, lanes 6–10), and MI treated with losartan (lanes 11–15) for 4 weeks after MI. All losartan treatment regimens were initiated one day following coronary occlusion. Hybridization of fractionated total RNA with cDNA probes for collagen types I and III and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) indicate relative steady-state mRNA levels for each gene tested.

RNA at 3 days. Right ventricular collagen type I mRNA expression peaked in the experimental animals (~2.3-fold vs. control level) at 14 days after MI. The right ventricular collagen type I/GAPDH ratio remained elevated in the 28 and 56 day experimental groups, and this was comparable to the pattern of collagen type I expression in the left ventricle after coronary ligation (figure 2C). Ventricular collagen type III expression was elevated in both left and right ventricles at all times after the induction of infarction; however, the relative increases in expression of collagen type III mRNA were less dramatic when compared to the increases in collagen type I mRNA abundance (figure 2B and D).

**Effect of losartan on collagen mRNA abundance**

As described above, fibrillar collagen mRNA expression was significantly increased in right and left ventricular myocardium at four weeks after induction of MI. For this reason, and because we wished to investigate the effect of chronic $AT_1$ blockade on mRNA expression, we chose to treat experimental animals with losartan for 4 weeks immediately after infarction. Northern blot analysis (figure 3) revealed that left ventricular fibrillar collagen (types I and III) mRNA expression in treated experimental animals was not different from that observed in the untreated post-MI

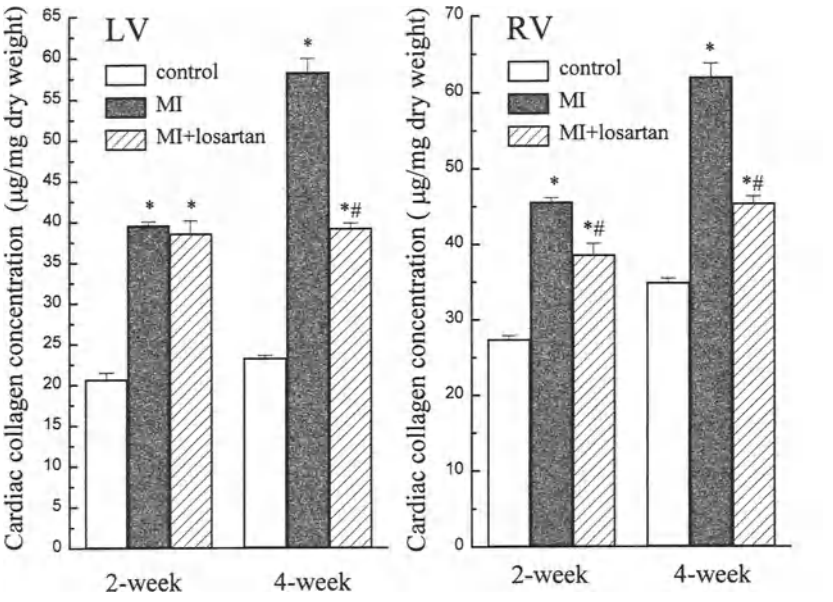


**Figure 4.** Effect of losartan (15 mg/kg/day) on total collagen concentration in viable left (LV) and right ventricular tissues (RV) at different times after myocardial infarction (MI). All losartan treatment (2 and 4 weeks) regimens were initiated one day following surgical coronary occlusion. Data shown is the mean ± SEM of 8–10 experiments and were expressed as μg/mg dry weight. *P < 0.05 and #P < 0.05 vs. sham-operated control and nontreated post-MI heart samples, respectively.

group. In other words, both treated and untreated experimental groups were found to have significantly elevated left ventricular fibrillar collagen mRNA expression in comparison to control values.

### Total collagen protein (4-hydroxyproline) in post-MI hearts and the effect of losartan

Total cardiac collagen concentration (determined by biochemical analysis of cardiac 4-hydroxyproline concentration) was significantly increased in viable left and right ventricles at 14 and 28 days post-MI (figure 4). We previously have observed that viable left and right ventricles remote to infarction are marked by progressive accumulation of immunoreactive fibrillar collagens at days 14, 28, and 56 after MI (data not shown). Two-week losartan treatment had no effect on cardiac collagen protein concentration in the left ventricle; a significant decrease was seen in treated right ventricular tissue when compared with values from nontreated experimental animals (figure 4). On the other hand, 4-week losartan treatment was associated with a significant decrease in collagen protein deposition in both the left and right ventricles when compared with nontreated post-MI rat hearts.

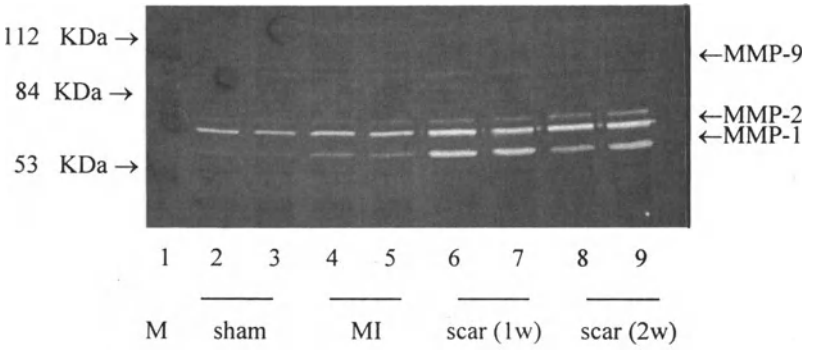


**Figure 5.** Representative zymography showing matrix metalloproteinase (MMP) activity in viable left ventricle (7-days post-MI) and scar tissues (7- and 14-days post-MI). Lane 1 is a low molecular weight marker, lanes 2–3 represent samples from sham-operated animals, lanes 4–5 are from viable left ventricular tissue, lanes 6–7 are 7-day scar samples, and lanes 8–9 represent 14-day scar. MMP-1 (54kDa), MMP-2 (72kDa), and MMP-9 (92kDa) are indicated by differences in motility through the gel.

### Cardiac matrix metalloproteinase activity in surviving post-MI heart and scar

Cardiac MMP activity was detected by the appearance of a lytic band in gelatin-containing SDS-PAGE gels (representative zymogram, figure 5). All assays were conducted in the presence of serine proteinase inhibitors, PMSF, and leupeptin to rule out the possible nonspecific lysis of gelatin proteins bands by serine proteinases. The specificity of this method was verified by use of 1, 10 phenanthroline, which abolished all specific gelatinolytic activity. A major lytic band from viable tissue corresponded to MMP-2 (gelatinase A, 72kDa), and two lytic bands MMP-1 (54kDa) and MMP-9 (92kDa) were observed in the scar tissues (figure 5) [81] A significant increase in MMP-2 activity in viable (noninfarcted) left ventricle was observed at days 7, 14, 28, and 56 after MI. On the other hand, MMP-1 activity was not significantly altered in viable post-MI tissue, but was markedly increased in the scars of experimental animals.

### Right ventricular passive pressure-volume relation

To test whether the presence of increased collagen concentration in the interstitium of the right ventricle after left ventricular infarction is indeed accompanied by decreased right ventricular tissue compliance, we obtained data to construct passive pressure-volume curves obtained from right ventricles of 56-day sham-operated and age-matched experimental animals (figure 6). The right ventricular pressure-volume curve from 56-day experimental animals was characterized by a significant leftward shift. This increment of right ventricular chamber stiffness corresponds well with significantly increased myocardial 4-hydroxyproline concentration levels in animals with moderate heart failure [59].

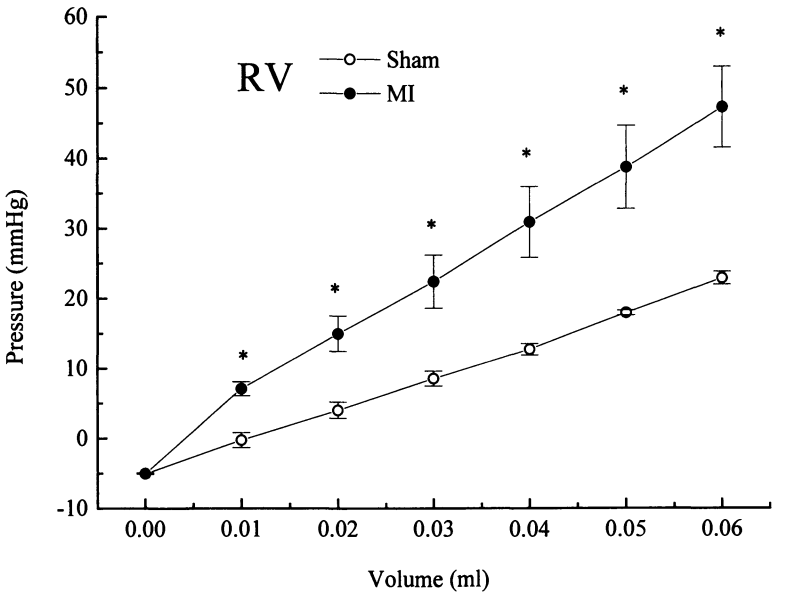


**Figure 6.** Right ventricular pressure-volume relation in control (• = sham) rats and in 56-day experimental (o = post-MI) animals. The data depicted is the mean ± SEM of 5 experiments wherein *P < 0.05 at each time point, compared to control values.

### Effect of $AT_1$ receptor blockade on cardiac prolyl 4-hydroxylase concentration

A 68.4% and a 68.1% increase in immunoreactive cardiac prolyl 4-hydroxylase concentration was observed in viable left ventricular tissue at 2 and 4 weeks after induction of MI when compared to control values. Treatment of experimental animals with losartan for 2 weeks had no effect on prolyl 4-hydroxylase concentration when compared to values derived from untreated post-MI animals. Treatment of infarcted animals with losartan for 4 weeks was associated with a significant decrease in the immunoreactive 4-hydroxylase concentration in viable tissues (figure 7).

## DISCUSSION

Necrosed tissue in infarcted myocardium undergoes gradual resorption and becomes replaced by scar tissue (reparative fibrosis), and continued cardiac function depends on remodeling of the surviving myocardial tissue [82,83]. However, the process and clinical consequences of the wound healing response in regions of the heart remote to the site of infarction (reactive fibrosis) are unclear [39]. Cardiac remodeling in these hearts is characterized by hypertrophy of cardiac myocytes and hyperplasia of nonmyocytes (cardiac myofibroblasts). In the present study, we examined both synthetic and degradative aspects of collagen metabolism in hypertrophied and

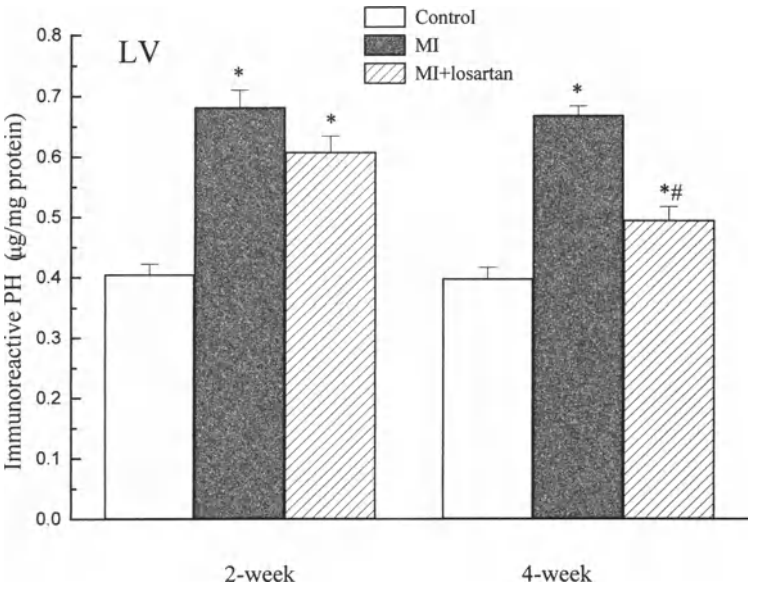


**Figure 7.** Effect of losartan (15 mg/kg/day) on prolyl 4-hydroxylase (PH) concentration in viable left ventricles in (1) sham-operated animals, (2) post-myocardial infarction (post-MI) animals, and (3) post-MI animals treated with losartan for either 2 or 4 weeks. All losartan treatment regimens were initiated one day following coronary occlusion. The data depicted is the mean ± SEM of 6 experiments and were expressed as μg/mg protein. *$P < 0.05$ and #$P < 0.05$ vs. sham-operated control and nontreated post-MI heart samples, respectively.

failing myocardium remote to the site of MI. Right ventricular fibrosis was noted in experimental animals and was associated with significantly increased chamber stiffness in the 56-day experimental group. The time-dependent increment of collagen expression in the right ventricle was similar to that of noninfarcted left ventricular tissue. Furthermore, acutely elevated mRNA expression of cardiac fibrillar collagen species in the prefailure experimental animals is uncoupled from concomitant collagen protein deposition. As collagen mRNA levels remain significantly increased into the chronic postinfarct phase, they are, at that time, associated with cardiac fibrosis. An increase in MMP activity was observed in areas remote to the site of infarction in chronically infarcted animals. As losartan treatment of experimental animals was not associated with normalization of collagen mRNA levels, we suggest that its antifibrotic properties may be effected at the posttranscriptional level.

Our previous immunohistochemical study of post-MI heart demonstrated that both collagen types I and III progressively accumulate in the interstium of experimental viable left and most notably in right ventricles (data not shown). Our determination of 4-hydroxyproline concentration allowed for the quantification of

"absolute" cardiac collagen concentration and highlighted the temporal differences between the onset of increased collagen mRNA abundance and collagen types I and III protein deposition in the noninfarcted heart. The current results also extend a recent report which focused on immunoreactive collagen types I and III in the infarcted zone in rats as well as another that described changes in extracellular matrix in failing human heart [84,85]. Our data support the hypothesis that the entire heart is involved in the wound healing response following MI. Furthermore, a distinct lag period among gene and protein activation was observed in noninfarcted tissues taken from the 3 and 7 day (acute study) experimental animals. The sustained elevation of mRNA abundance of type I and type III collagen seen in the present study suggests that biventricular reactive fibrosis continues into chronic heart failure (8 weeks) and that a progressive rise in collagen concentration occurs. Nonlinearity among collagen gene activation and collagen protein deposition has been reported in aging rats [86], and it is suggested that this may be due to the involvement of complex posttranslation modification processes, i.e., hydroxylation, cleavage and secretion steps in collagen metabolism.

We have previously characterized the development of congestive heart failure in experimental animals with large infarction ($\geq$ 40% of the left ventricular free wall) by noting the progression of cardiac dysfunction and the presence of clinical signs of failure, including the occurrence of cardiac hypertrophy at points up to and including 16 weeks after infarction [52,64]. It is well known that increased interstitial collagen concentration (reactive cardiac fibrosis) results in decreased cardiac compliance [55]. Little information is available in the literature with respect to the appearance of collagen in the right ventricle; nevertheless, the clinical implications of right ventricular fibrosis are considerable in view of the eventual contractile failure of this chamber and subsequent development of systemic congestion in severe stages of heart failure [1]. We observed that total collagen concentration was found to be increased not only in viable left ventricle but also in the right ventricle in 2, 4, and 8 week experimental animals. It is known that excessive deposition of collagen proteins may impair heart function because of morphological and functional separation of myocytes, with subsequent inhibition of electrical coupling of these cells and decreased ventricular compliance [85,87]. Studies using the rat model of chronic infarction have revealed that the apparent left ventricular stiffness exhibited a pattern of biphasic time-dependent changes, increasing up to one day and then progressively decreasing until 22 days [74]. Infarct expansion is associated with increased left ventricular volume, and thus the effects of volume predominate over changes in viable myocardial stiffness in the left ventricle. To avoid this problem, Litwin and coworkers used noninfarcted left ventricular papillary muscle to study compliance and found that stiffness was increased 42 days after MI [88]. In the present study, right ventricular stiffness was significantly increased in the 56-day experimental group. Thus, increased cardiac collagen concentration in the right ventricle was associated with altered passive compliance of this chamber in these animals. Recent studies have provided support for the hypothesis that marked myocardial fibrosis and increased passive stiffness are critical determinants for

the transition from compensated hypertrophy to heart failure in spontaneously hypertensive rats [89]. Our results support the hypothesis that fibrosis is associated with the transition of infarcted heart from prefailure stage (28 days post-MI) to moderate and severe congestive heart failure (56 days post-MI) and that this change is associated with global changes in heart muscle stiffness.

The precise mechanism(s) for increased collagen expression and for the development of right and left ventricular fibrosis after induction of MI is unclear. As we observed similar patterns of collagen mRNA alteration in both left and right ventricles in the early phase post-MI, our results support the hypothesis that diffusible hormones are factors for increased cardiac collagen deposition [39,90], although mechanical stretching of the myocardium has been suggested to play a stimulatory role [91]. Since both left and right ventricular chambers are subject to altered hemodynamic loading at 56 days after MI in this experimental model, we cannot rule out the possibility that hemodynamic loading was involved in the regulation of collagen mRNA and proteins. Several hormones, including Ang II and transforming growth factor-β1 (TGF-β1), may regulate collagen gene expression and, therefore, mediate collagen deposition in the interstitium of heart muscle [92,93]. It was well documented that the local cardiac RAS becomes activated after MI [56,94–98]. Several reports have revealed that both ACE inhibition and cardiac angiotensin receptor blockage ($AT_1$ subtype) treatment are effective in partial attenuation of collagen protein deposition in infarcted hearts [44,46,60]. However, it is not clear whether stimulation of collagen synthesis by angiotensin is due to either an increase in the transcription of collagen genes or a decrease in the degradation of newly synthesized collagen [99]. Our results of losartan (and ramipril) treatment of experimental animals are in agreement with two recent reports [100,101]. In these studies, treatment of rats with delapril or TCV-116 (Ang II type I receptor antagonist) was not associated with regression of increased collagen mRNA abundance associated with different pathological stimuli. Because treatment of experimental animals with losartan was associated with partial regression of both cardiac hypertrophy (data not shown) and interstitial fibrosis (with no deflection in mRNA abundance), it is likely that effective losartan delivery occurred. Similarly, ramipril-treated animals exhibited significantly diminished right and viable left ventricular heart mass vs. controls that had no change in elevated collagen mRNA abundance (see results section—the dose of ramipril used in these studies is in excess of the minimum dose for significant inhibition of cardiac ACE activity, as reported by others [75,76]). Our results with either losartan or ramipril are similar to those of a recent study by Brecher and coworkers who found that trandolapril (ACE inhibitor) treatment was not associated with a reversal of increased fibronectin mRNA levels in phenylephrine-induced cardiac fibrosis in rats [102]. It has been reported that one of a number of posttranslational mechanisms may play an important role in the regulation of collagen deposition in myocardial extracellular matrix [103]. Although the transcriptional control of cardiac fibrillar collagen genes does not appear to be attenuated by ramipril or losartan treatment in this experimental model, it must be stressed that our findings do not rule out angiotensin-mediated modulation of

cardiac collagen synthesis at posttranscriptional levels and/or of degradation of newly synthesized collagens.

Relative cardiac collagen concentration is the product of a dynamic balance between collagen synthetic and degradative pathways. Collagen types I and III can be cleaved by a 54kDa interstitial collagenase (MMP-1) and a 75kDa neutrophil collagenase (MMP-8) [81]. These enzymes cleave fibrillar collagen at a site 3/4 from the N-terminal end, resulting in 1/4 and 3/4 collagen fragments, called gelatins [104]. Our data is in agreement with others who have used the rat model of chronic infarction to address collagen degradation and have shown that MMP-1 activity was increased at day 2, peaked at day 7, and declined thereafter in the infarcted site in the left ventricle experimental animals [104]. As MMP-2 can degrade type IV collagen, increased MMP-2 activity may be responsible for degradation of collagen present in the basement membrane. Furthermore, MMP-2 may degrade gelatin (a breakdown product of fibrillar collagens) to constitutive amino acids. We believe that this process may facilitate remodeling of extracellular matrix in the viable ventricle after infarction, as cardiac myocytes rearrange their spatial orientation in three dimensions.

Hydroxylation of collagen monomers is a crucial step in the biosynthesis and secretion of mature collagens from fibroblast endoplasmic reticulum by facilitation of the self-assembly of the α-chains to trimeric form. Thus, the increase in immunoreactive prolyl 4-hydroxylase in viable heart muscle may be a major mechanism for increased deposition of collagen in these experimental hearts. Our data demonstrated that losartan treatment was associated with attenuation of the increase of prolyl 4-hydroxylase usually seen in these hearts and that this trend is positively correlated with the inhibition of total collagen protein in treated experimental hearts. The hypothesis that the antifibrotic effect of losartan is mediated via inhibition of the expression of prolyl 4-hydroxylase enzyme in surviving muscle from experimental heart is supported by these results. This study also provides an indication that angiotensin may modulate posttranslational regulation of cardiac collagen in mammalian hearts with chronic experimental MI.

In summary, the results of this study indicate that prefailure and moderate congestive heart failure stages after induction of large left ventricular MI are associated with (1) an elevation of mRNA levels of cardiac fibrillar collagen species in right and left ventricular myocardium within 3 days of induction of left MI (a relatively early time point not associated with fibrosis of noninfarcted tissue per se; (2) marked right ventricular fibrosis in 56-day experimental animals with attendant increased chamber stiffness; (3) significantly increased fibrillar collagen mRNA abundance that is maintained in the left and right ventricular chambers throughout the course of the study, wherein the onset of fibrosis (increased deposition of collagen types I and III) occurs at two weeks; (4) an increase in MMP-2 activity in noninfarcted left and right ventricles in experimental animals. The latter finding suggests that net degradation of collagen may be increased in experimental hearts, and this finding supports the hypothesis that increased deposition of collagen protein is a result of elevated synthesis of collagen, rather than a decrease in collagen

(and collagen fragment) removal. Furthermore, we found that as $AT_1$ receptor antagonism was not associated with regression of increased fibrillar collagen mRNA abundance in either right or left ventricular tissues in experimental animals, the upregulation of collagen mRNA transcripts may be not directly mediated by the $AT_1$ receptor; and finally we found that increased protein expression of 4-prolyl hydroxylase in experimental hearts is blocked with losartan treatment. Thus, the reduction of fibrosis by losartan may be effected at nontranscriptional sites in the synthetic pathway, and it is clear that the whole heart, i.e., both infarcted and noninfarcted tissue, becomes involved in wound healing. It is likely that this global cardiac wound healing response is not completed in conjunction with maturation of scar tissue, i.e., at 3–4 weeks, but is sustained in the chronic phase of MI.

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# CHARACTERISTICS AND MECHANISMS OF ANGIOTENSIN II-RELATED MYOCARDIAL DAMAGE

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***Summary.*** Chronic, pathophysiological elevations of angiotensin (Ang) II cause myocyte necrosis and coronary vascular damage. These adverse effects are mediated by the angiotensin II type 1 ($AT_1$) receptor and are, therefore, preventable with $AT_1$ receptor blockade. Additionally, the intracellular signaling cascade stimulated by elevations in Ang II results in an $AT_1$ receptor-mediated catecholamine release, and the myocardial damage can also be attenuated by β1-adrenergic receptor blockade. The restriction of myocardial damage to the first 3 days of Ang II infusion is due to subsequent downregulation of the β1-adrenergic receptor population. Discontinuation of the Ang II infusion results in a return to normal β1-adrenergic receptor density, which makes the myocardium susceptible once again to subsequent elevations of Ang II. This Ang II-related myocardial damage could play an important role in the pathogenesis of heart failure post-myocardial infarction or in other cardiac disease states.

An elevation in plasma angiotensin (Ang) II levels induces many physiological actions including stimulation of arginine vasopressin release, constriction of peripheral arterioles, constriction of glomerular efferent arterioles, increased sodium reabsorption in the kidney, stimulation of aldosterone release, and increased release of norepinephrine from the adrenal medulla and sympathetic nerves. All of these physiological consequences are elicited via the Ang II type 1 ($AT_1$) receptor and result in a compensatory elevation in blood pressure. Accordingly, in heart failure, plasma Ang II is elevated in what is thought to be a compensatory response to maintain adequate organ and tissue perfusion. However, if the increase in Ang II is abnormal, myocyte necrosis and coronary vascular damage are known to occur. In fact, hearts from patients who died because of heart failure secondary to renovascular

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hypertension have been found to have extensive myocardial damage similar to that observed following an experimental elevation in Ang II [1]. Thus, it is conceivable that pathophysiological levels of Ang II may contribute to the pathogenesis of heart failure. The adverse effects that Ang II exerts on the myocardium are also mediated via the $AT_1$ receptor in that the accompanying myocardial damage can be prevented with an $AT_1$ receptor antagonist. In this report, the characteristics and mechanisms of Ang II-induced myocardial damage and the role of the $AT_1$ receptor in causing this damage will be reviewed.

## HISTOLOGICAL CHARACTERISTICS OF ANGIOTENSIN II-MEDIATED MYOCARDIAL DAMAGE

Pathophysiological Ang II levels cause myocyte necrosis and damage to the coronary arterioles. The first report of Ang II causing myocardial damage was published in 1971 by Gavras and colleagues [2]. They infused rabbits with 0.9–1.8 μg/kg/min of Ang II intravenously for 72 hours to determine whether it caused renal tubular necrosis. In addition to renal damage, they found widespread, focal "myocardial infarction". In a follow-up paper in 1975, these "myocardial lesions", as they are now termed, were described as extensive, frequently confluent, multifocal myocardial necrosis [1]. This article is also noteworthy for its description of similar myocardial lesions at death in five patients who had elevated Ang II levels.

In experimental rat models of renovascular hypertension or chronic Ang II infusion (150 to 200 ng/min), the plasma levels of Ang II increase to 57 ± 22 and 71 ± 6 pg/ml, respectively [3]. These values are consistent with the range of 28–155 pg/ml reported for humans with heart failure [4,5] and can therefore be considered to be pathophysiological. In these models, the focal areas of Ang II-induced myocardial damage are found in both the right and left ventricles, with the number of sites per ventricle ranging from 4 to 23 [6]. However, because of its smaller size, the right ventricle generally exhibits a greater percent of damage (0.8 to 15.4%) than the left ventricle (0.4 to 4.9%). The necrotic sites are randomly distributed in the epi-, mid-, and endomyocardial regions, with a tendency for many to be concentrically oriented around arterioles [7].

Ang II-related myocyte damage typically presents as multifocal myocyte necrosis involving small groups of myocytes (figure 1). These necrotic foci are characterized by (1) the loss of definition of the linear arrangement of myofibrils and cross striations; (2) the appearance of dense, eosinophilic, transverse bands in the sarcoplasm with translocating mitochondria between them; (3) subsequent shrinkage and pyknosis of the myocyte nucleus; and (4) a progressive loss of nuclear basophilia with the eventual disappearance of the nucleus [8]. The necrosis is followed by a granulomatous inflammatory infiltrate consisting of neutrophils, lymphocytes, and macrophages, which are responsible for the removal of the damaged myocytes from the area. Concurrent with the inflammatory response and phagocytosis, fibroblasts migrate into the damaged area to begin the wound healing or reparative

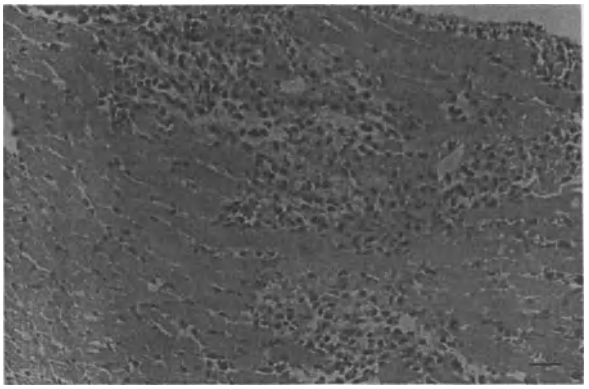

**Figure 1.** Representative photomicrograph of an area of angiotensin II-induced myocyte necrosis with granulomatous infiltrate. This heart was exposed to chronic angiotensin II infusion for 2 days and represents the acute phase of angiotensin II-induced myocyte necrosis with active phagocytosis of the necrotic myocardium. (Scale bar = 20 μm). (Reproduced with permission from Kabour et al. [6]).

process. After a replacement fibronectin/collagen network has been established, myofibroblast transformation occurs to promote collagen fiber cross-linking and contraction of the scar. The period from onset of myocyte necrosis to the final stages of wound healing takes about 14 days.

The other facet of the myocardial damage observed with elevated Ang II is damage to the coronary arterioles in both ventricles (figure 2). In 1976, Giacomelli et al. [9] found that an acute, intravenous infusion of a pharmacological dose of Ang II in rats damaged the coronary arterioles. This damage was analyzed using transmission electron microscopy and was characterized as swollen endothelial cells, vacuolization in the vascular smooth muscle cells, and a perivascular inflammatory infiltrate. Two years later, Bhan et al. [10] reported that renovascular hypertension, created by unilateral renal artery constriction, caused similar damage to the intramyocardial coronary arterioles. In a subsequent study, Bhan and his co-workers [11] found that 92% of the intramural arterioles which were examined contained ultrastructural lesions following an acute, intravenous infusion of a very high dose (1.7 μg/kg/min) of Ang II. In animal models with experimentally elevated plasma Ang II levels similar to those seen in human heart failure, 20 to 50% of the intramural arterioles were found to be abnormal as assessed by light microscopy. The criteria for assessing vascular damage were abnormally shaped, swollen, or rarefied endothelial cells; vacuolated vascular smooth muscle cells; and/or a perivascular inflammatory infiltrate [12]. This degree of vascular damage may be responsible for the altered coronary vascular permeability [13,14], perivascular fibrosis [15], and decreased coronary reserve [16] that have been reported to occur secondary to elevations in Ang II.

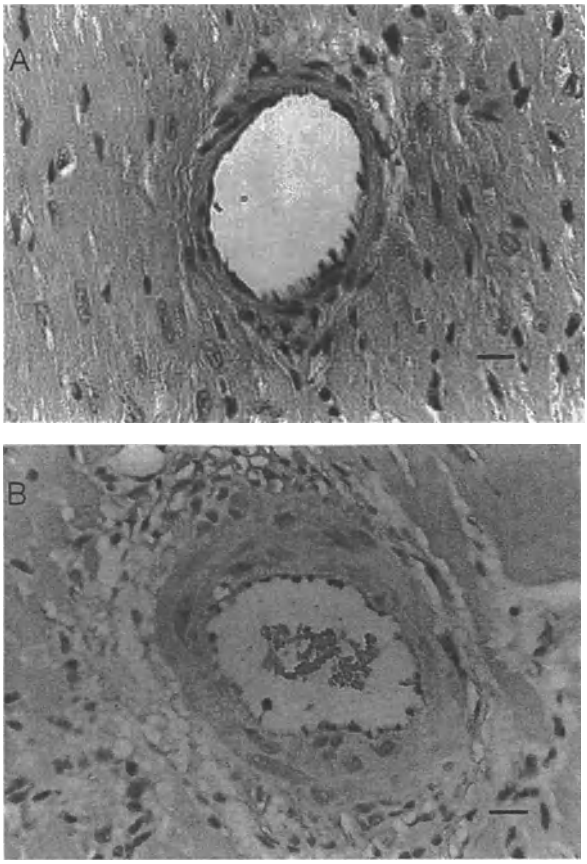


**Figure 2.** Example of normal (panel *A*) and damaged (panel *B*) coronary arteriole. The abnormal endothelial cells and adventitia in panel *B* are characterized by swollen endothelial cells bulging into the lumen of the vessel and the marked thickening of the vascular smooth muscle cell layer. (Scale bars = 20 μm). (Reproduced with permission from Kabour et al. [12]).

## MECHANISMS OF ANGIOTENSIN II-INDUCED MYOCARDIAL DAMAGE

### Involvement of the $AT_1$ receptor

In rats with experimental renovascular hypertension or a chronic infusion of Ang II at a rate of 200 ng/min, myocyte necrosis was found to occur only in the first 2 to 3 days of infusion despite a continued elevation in plasma Ang II [4]. As further confirmation of the acute nature of this response, these rats were injected with $^{[3]}$H-thymidine in order to assess cellular proliferation. The cellular thymidine incorporation was transient, reaching a maximum on day 2 of the Ang II infusion and returning to baseline thereafter, with the dividing cells consisting primarily of fibroblasts.

The fact that Ang II-induced myocardial damage is an acute process suggests that it is receptor-mediated and that the subsequent cardioprotection is the result of receptor downregulation. Subsequent studies in our laboratory have addressed this issue [7,12]. In one study, Ang II levels that were chronically elevated for either 2 days or 9 days were found to cause multifocal myocyte necrosis and coronary vascular damage in both ventricles. The myocardial lesions after 2 days of Ang II infusion were characterized by a granulomatous inflammatory infiltrate and ongoing myocyte necrosis, and were therefore considered to be recent necrosis. However, the myocardial lesions after 9 days of Ang II infusion consisted of multifocal areas of fibrosis containing primarily fibroblasts and a marked increase in the amount of collagen. These lesions were considered to be chronic areas of myocyte necrosis because of the advanced stage of wound healing and absence of inflammatory infiltrate. Other evidence that a receptor regulatory event protects the heart from damage after the first few days of Ang II infusion was provided when Ang II was infused for 2 days, the osmotic pump was removed for 5 days, and the pump was reinserted for 2 more days. In this case, areas of *de novo* myocyte necrosis resulting from the second infusion of Ang II were observed in addition to the areas of chronic fibrosis. The *de novo* myocyte necrosis was presumably due to receptor upregulation which rendered the myocytes vulnerable to the second elevation in Ang II (figure 3). Finally, the fact that the necrosis and coronary vascular damage were completely prevented by $AT_1$ receptor blockade suggests that they are $AT_1$ receptor-mediated events [7].

Cardiac $AT_1$ receptors are found in low concentration on myocytes and vascular smooth muscle cells [17]. $AT_1$ receptor function has been well-characterized in many tissues and, as stated above, is responsible for mediating all the known physiological actions of Ang II in adults. Briefly, Ang II binds to the G protein-coupled $AT_1$ receptor and activates tyrosine kinase, which phosphorylates and stimulates phospholipase C-$\gamma$1 activity. Phopholipase C-$\gamma$1 then hydrolyzes phosphatidylinositol 4,5-bisphosphate to form inositol 1,4,5-trisphosphate and diacylglycerol. Inositol 1,4,5-trisphosphate stimulates the release of calcium from intracellular stores. In cultured rat cardiomyocytes, cytosolic calcium concentration has been shown to increase significantly after addition of Ang II to the culture medium [18]. Thus, this increase in intracellular calcium may ultimately be responsible for the Ang II-induced myocyte necrosis and coronary vascular damage.

As mentioned earlier, elevations in Ang II result in the release of aldosterone and catecholamines and an increase in blood pressure via the $AT_1$ receptor. Accordingly, the ability of the $AT_1$ receptor antagonist to protect the myocardium from pathophysiological levels of Ang II may be the result of the antagonist preventing these hormonal and blood pressure increases. Each of these possibilities will now be considered.

**Elevation in blood pressure**

Ang II-mediated myocardial damage could somehow be the result of the accompanying elevation in blood pressure. Bishop et al. [19] concluded that the sudden

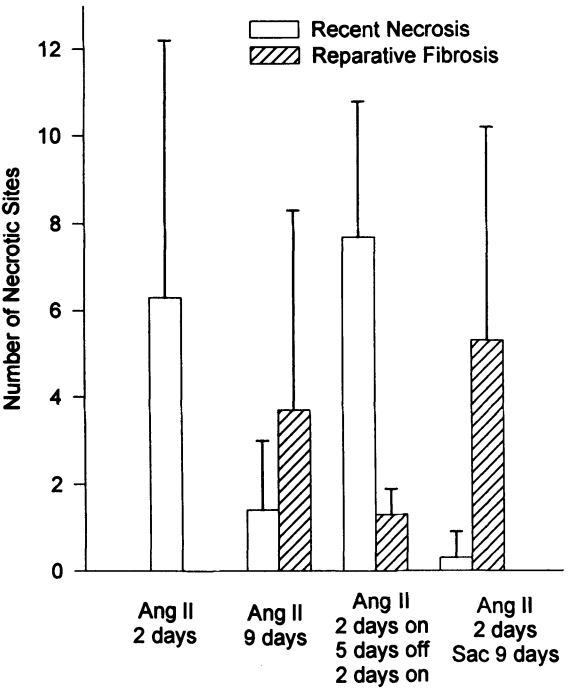


**Figure 3.** The number of necrotic sites per combined LV and RV histological section after angiotensin II infusion (150ng/min) via a subcutaneous osmotic minipump for 2 to 9 days. Solid bars represent the number of necrotic sites consistent with recent or acute necrosis (occurring within the prior 3 days), whereas the hatched bars represent the number of sites of chronic myocyte necrosis or reparative fibrosis. Ang II 2 days = Angiotensin II infusion for 2 days, sacrificed on day 2; Ang II 9 days = Angiotensin II infusion for 9 days, sacrificed on day 9; Ang II 2 days on, 5 days off, 2 days on = Angiotensin II infusion for 2 days, after which the angiotensin II pump was removed for 5 days, followed by an additional 2 day angiotensin II infusion; sacrificed on day 9; Ang II 2 days, Sac 9 days = Angiotensin II infusion for 2 days, after which the angiotensin II pump was removed; sacrificed 7 days later on day 9.

pressure overload induced by thoracic aortic constriction was responsible for the development of multifocal myocyte necrosis that was observed in this model of experimental cardiac hypertrophy. While the necrosis was similar to that seen with Ang II infusion, the state of the renin-angiotensin system was not evaluated, and, therefore, it is not known whether Ang II levels were normal or elevated. Similarly, Bhan et al. [11] demonstrated that the number of damaged small coronary arterioles was significantly reduced when blood pressure was pharmacologically prevented from increasing as Ang II was administered. However, following these two studies, there were several others which strongly suggested that elevated blood pressure was not responsible for Ang II-related myocardial damage. For example, when an angiotensin-converting enzyme (ACE) inhibitor was administered one day prior to the creation of the renovascular hypertension and was maintained throughout the

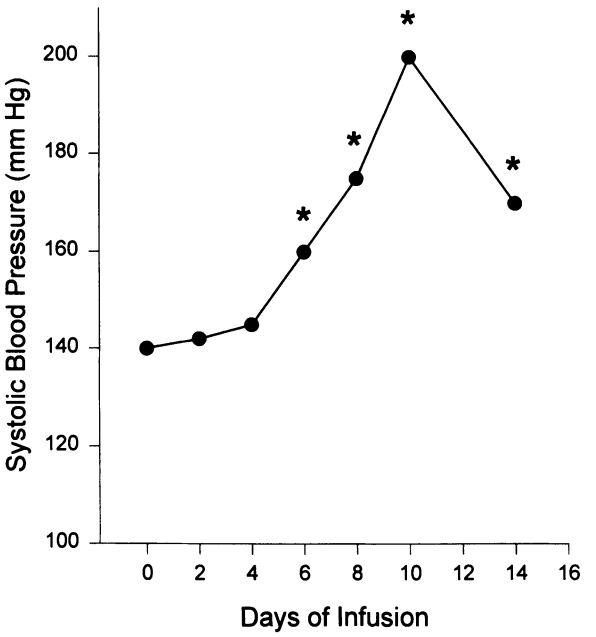


**Figure 4.** The temporal systolic blood pressure response to chronic angiotensin II infusion (150 ng/min) via a subcutaneous osmotic minipump. Systolic blood pressure was measured by the tail cuff method. Note that systolic blood pressure did not increase until after day 4, was not significantly elevated until day 6 of chronic angiotensin II infusion, and remained elevated for the remainder of the infusion period. *$p < 0.05$ vs. Day 0. (Modified with permission from Tan et al. [3]).

study period in doses that did and did not prevent the increase in blood pressure, the myocardial damage was greatly attenuated in both groups [12]. Also, as can be seen in figure 4, systolic blood pressure measured in rats receiving a constant infusion of Ang II does not become significantly elevated until day 6 of the infusion, while most of the Ang II-induced myocardial damage occurs during the first 3 days of Ang II infusion [4]. Finally, hearts from rats subjected to chronic Ang II infusion and concurrently treated with an antihypertensive dose of (ACE) still exhibit significant myocardial damage (figure 5) [12].

### Aldosterone

Stimulation of aldosterone synthesis and secretion is another physiological response to elevated plasma Ang II levels. Shown in figure 6 are the serum aldosterone levels for rats chronically infused with Ang II for 15 days [20]. Serum aldosterone was significantly elevated 1 to 3 days after starting Ang II infusion and remained so throughout the infusion period. The mean aldosterone concentration for the Ang II-infused group over the 14 day period was 245 ± 60 ng/dl. Losartan was able to prevent the Ang II-induced increase in serum aldosterone. The average aldosterone

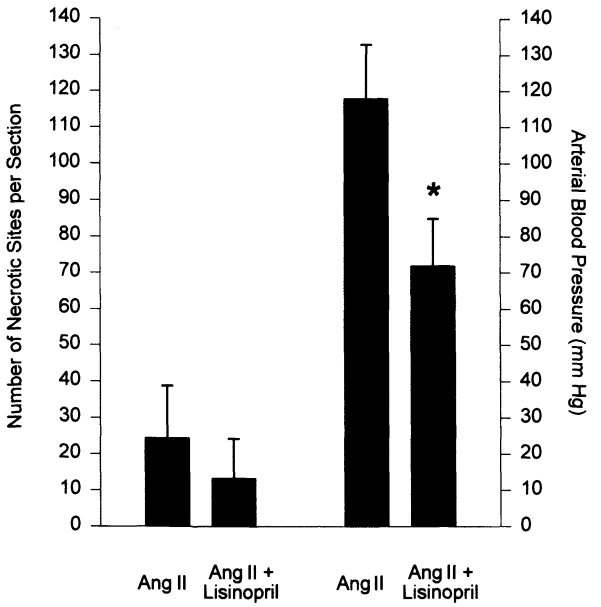


**Figure 5.** The number of necrotic sites per combined LV and RV histological section and blood pressure after 14 days of angiotensin II infusion (150 ng/min) via a subcutaneous osmotic minipump with and without 20 mg/kg/day lisinopril treatment. Lisinopril was given 1 day prior to and throughout the 14 day angiotensin II infusion period. Lisinopril was unable to significantly decrease the number of necrotic sites even though it was able to significantly lower blood pressure. Ang II = angiotensin II infusion for 14 days; Ang II + lisinopril = angiotensin II infusion and lisinopril treatment for 14 days. *$P < 0.05$ vs. Ang II.

value of 8 ± 3 ng/dl for the losartan group was similar to that of the control group (7 ± 4 ng/dl). The marked increase in circulating aldosterone on day 1 of chronic Ang II infusion suggests the possibility that Ang II-related myocardial damage may be the result of increased aldosterone. Moreover, it has been shown that rats undergoing chronic infusion of aldosterone, after the removal of one kidney and receiving 1% sodium chloride drinking water, exhibit myocardial damage similar to that seen with Ang II. However, recently published findings indicate that the myocardial damage resulting from a continuous aldosterone infusion does not occur until after weeks 3 to 4 [21], precluding its role in the Ang II-mediated damage seen within the first 3 to 4 days of elevated Ang II levels.

### Catecholamines

Another possible mechanism for Ang II-related myocardial damage is catecholamine release from the sympathetic nerve terminals in the heart and/or the adrenal medulla. This mechanism is attractive since it has been shown that the patterns of damage which occur with elevated Ang II levels and with elevated norepinephrine levels are similar [1].

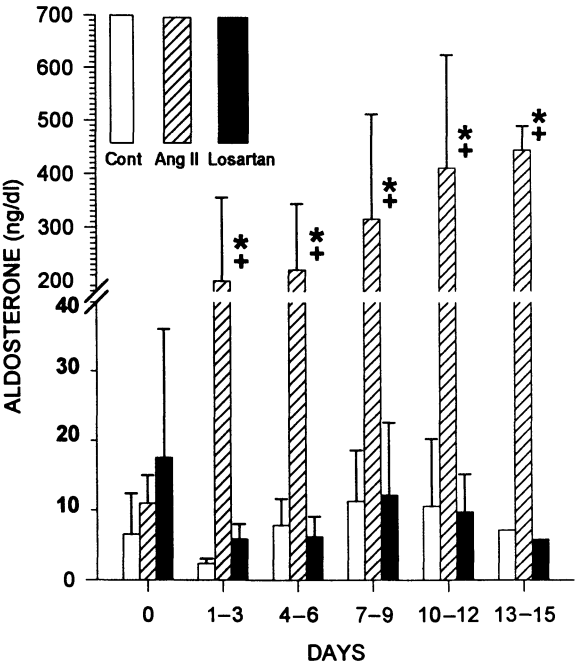


**Figure 6.** Temporal response of serum aldosterone levels to chronic angiotensin II infusion (150 ng/min) via a subcutaneous osmotic minipump. Aldosterone was measured in serum samples taken from conscious, resting rats. Aldosterone was significantly elevated on day 1 of angiotensin II infusion, and levels remained elevated throughout the infusion period. Losartan was able to prevent the angiotensin II-induced aldosterone release. Control samples showed no variation over the angiotensin II infusion period. *P < 0.05 vs. Cont; +P < 0.05 vs. Losartan Cont = Untreated, noninfused control group; Ang II = Angiotensin II infused group, 150 ng/min, subcutaneously; Losartan = Angiotensin II infused group treated with losartan, 7.5 mg/day, intravenously. (Reproduced with permission from Henegar et al. [20]).

The fact that catecholamines can induce myocyte necrosis has been known for some time. Many investigators studying this relation have used the nonspecific β-adrenergic receptor agonist isoproterenol. Infusion of rats with isoproterenol caused myocyte necrosis which could be prevented by the β-adrenergic receptor antagonist propranolol [22]. The damage from isoproterenol was primarily localized to the endocardium and was thought to be due to subendocardial ischemia secondary to an isoproterenol-induced increase in heart rate and contractility (i.e., increased oxygen demand), with an accompanying decrease in blood pressure (i.e., decreased oxygen delivery or supply). In contrast, an endogenous increase in catecholamines is associated with an increase in blood pressure and results in myocardial damage similar to that seen with elevated Ang II [23]. Finally, the toxic effect of catecholamines on cardiac myocytes has been associated with increased intracellular calcium [24], not an ischemic event such as isoproterenol infusion.

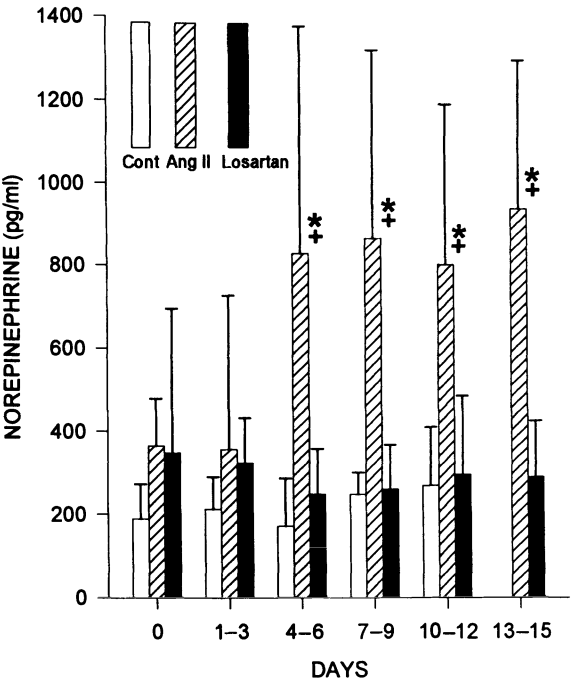


**Figure 7.** Temporal response of serum norepinephrine levels to chronic angiotensin II infusion (150 ng/min) via a subcutaneous osmotic minipump. Norepinephrine was measured in serum samples taken from conscious, resting rats. As can be seen, serum norepinephrine was elevated after 4–6 days of angiotensin II infusion. This angiotensin II-induced norepinephrine release was totally prevented by the $AT_1$ receptor blocker, losartan. Also of note is the lack of variation in the control serum samples over time indicating that the sampling technique was not stress provoking. *$P < 0.05$ vs. Cont; +$P < 0.05$ vs. Losartan Cont = Untreated, noninfused control group; Ang II = Angiotensin II infused group; Losartan = Angiotensin II infusion and losartan (7.5 mg/day, intravenously). (Reproduced with permission from Henegar et al. [20]).

Recently, it has been shown that pathophysiological levels of Ang II stimulate norepinephrine release immediately following acute, intravenous Ang II infusion [25–28]. However, these studies utilized extremely high doses of Ang II administered intravenously. Our laboratory has reported that the β-adrenergic receptor blocker, propranolol, markedly attenuated Ang II-related myocardial damage, suggesting that catecholamines are responsible. The temporal responses of serum norepinephrine concentration for conscious, Ang II-infused rats, with and without the $AT_1$ receptor blocker, losartan, are shown in figure 7 [20]. Noninfused, control rats had little change in their norepinephrine levels over the course of the study. In contrast, the Ang II-infused group had significant increases in serum norepinephrine levels starting on days 4–6 and thereafter through day 15 of the infusion period. Losartan completely prevented this Ang II-related increase in serum norepinephrine. The average norepinephrine value for the losartan group of 282 ± 30 pg/ml was not

significantly different from that of the control group, 257 ± 82 pg/ml ($p = 0.76$). Serum epinephrine levels were variable with 90 of the 105 samples either lying within the normal range for rats (i.e., 111 to 267 pg/ml (20)) or having epinephrine levels below the detectable range of the assay (<10 pg/ml). The 15 remaining epinephrine values were greater than 267 pg/ml. They were randomly distributed with respect to infusion time, occurred in both the control and Ang II infused groups, and did not correlate with norepinephrine levels.

Even though circulating norepinephrine levels were not elevated until day 4 of chronic Ang II infusion, our laboratory has shown that surgical cardiac sympathectomy was effective in preventing the myocardial damage seen in the first 3 days of Ang II infusion [29]. Therefore, the local release of catecholamines is responsible for the Ang II-related myocardial damage seen in this model, and the prevention of Ang II-related myocardial damage by $AT_1$ receptor blockade is the result of an inhibition of this local release of catecholamines.

Recently, Ratajska and colleagues [30] also reported that catecholamines were important in the myocardial damage associated with Ang II infusion. However, they concluded that the source of catecholamines was the adrenal gland. That is, myocyte necrosis associated with a subcutaneous infusion of Ang II, at a rate of 150 ng/min, was markedly attenuated when the Ang II infusion was started 1 week after adrenal medullectomy, suggesting that catecholamines secreted from the adrenal gland were responsible for the damage. In their study, however, blood samples were acquired only at the time of sacrifice. As discussed above, plasma norepinephrine levels are not significantly elevated until day 4 of the Ang II infusion. Thus, without the temporal profile of serum norepinephrine, the conclusion of Ratajska et al. that circulating catecholamines are the direct cause of the acute episode of myocardial damage cannot be substantiated. A more likely explanation for their findings is that the sympathetic stores of norepinephrine were depleted and not replenished during the five-day period between medullectomy and the onset of the Ang II infusion. It is known that a major source of cardiac sympathetic norepinephrine is the adrenal gland [31].

## THE CARDIOPROTECTIVE RESPONSE OF THE β-ADRENERGIC RECEPTOR TO ELEVATED LEVELS OF ANGIOTENSIN II

From the aforementioned text, it is obvious that Ang II-mediated myocardial damage is the result of an Ang II-stimulated local release of catecholamines. Therefore, the fact that this damage is an acute process may be related to β-adrenergic receptor downregulation.

β-adrenergic receptors are present throughout the myocardium and on smooth muscle cells in coronary arteries and arterioles [32]. The largest percentage of these receptors is found on the smooth muscle of small arterioles, with a smaller percentage on coronary arteries [33]. It has been suggested that β-adrenergic receptor desensitization may protect the myocardium from catecholamine-induced damage and thus explain the restriction of catecholamine-induced myocyte necrosis primarily to the first three days of infusion [34]. There is evidence that the β-adrenergic

receptor agonist, isoproterenol, causes β-adrenergic receptor downregulation and that this downregulation occurs rapidly and is then maintained until the isoproterenol is cleared [34].

In order to determine whether β-adrenergic receptor downregulation could be cardioprotective after the first few days of chronic Ang II infusion, we [35] recently determined β-adrenergic receptor densities in crude membrane preparations of rat left ventricles from the following groups: (1) control, (2) Ang II infused for 3 days (AngII3day), (3) Ang II infused for 3 days, followed by removal of the Ang II for 5 days (AngII3on/5off), and (4) Ang II infused for 8 days (AngII8day). β-adrenergic receptor density after 3 days of Ang II infusion was significantly reduced by 38% from that in untreated controls (i.e., 112 ± 30 fmol/mg protein for control versus 70 ± 20 fmol/mg protein for AngII3day). When the osmotic minipump was removed after 3 days of Ang II infusion and the rats were sacrificed 5 days later, there was a significant increase in β-adrenergic receptor density when compared to Ang II-infused hearts for 3 days (70 ± 20 fmol/mg protein for AngII3day versus 133 ± 29 fmol/mg protein for AngII3on/5off). The density of β-adrenergic receptors in the AngII3on/5off group was not statistically different from that in the control group (112 ± 30 fmol/mg protein). This would explain the previously mentioned findings of Kabour et al. [7] where de novo necrosis occurred during a second, 2-day Ang II infusion which was started 5 days after an initial 2-day Ang II infusion. Finally, when Ang II was infused for 8 consecutive days, β-adrenergic receptor density was significantly less than that in the control (55% decrease, $p < 0.05$) and AngII3day (29% decrease, $p < 0.05$) groups, indicating a progressive down-regulation of the β-adrenergic receptors.

## CLINICAL SIGNIFICANCE OF ANGIOTENSIN II-INDUCED MYOCARDIAL DAMAGE

Hearts from patients with heart failure secondary to renovascular hypertension have extensive myocardial damage [1] similar to that observed after experimental elevations in Ang II or catecholamines. It is unknown whether this damage is due to elevated plasma levels of Ang II, norepinephrine, epinephrine, or perhaps all three. Since these agents may cause myocardial damage in heart failure, the short-term compensatory benefits may be at the cost of damage to myocytes and coronary arteries.

Patterns of myocyte necrosis similar to those elicited by elevated Ang II have been observed in the nonischemic myocardium from patients after myocardial infarction (MI). In 1971, Page et al. reported myocyte necrosis in the noninfarcted myocardium post-MI in autopsy samples from patients [36]. This remote myocyte necrosis (i.e., necrosis in the nonischemic myocardium) was described as foci of necrosis occurring in both the left and right ventricles. They remarked that the pattern of damage was similar to that seen after infusion of exogenous catecholamines, but did not investigate the status of endogenous catecholamine production in these hearts. Corday et al. [37] reported remote myocyte necrosis post-MI in an animal model and speculated that catecholamines could be responsible. More re-

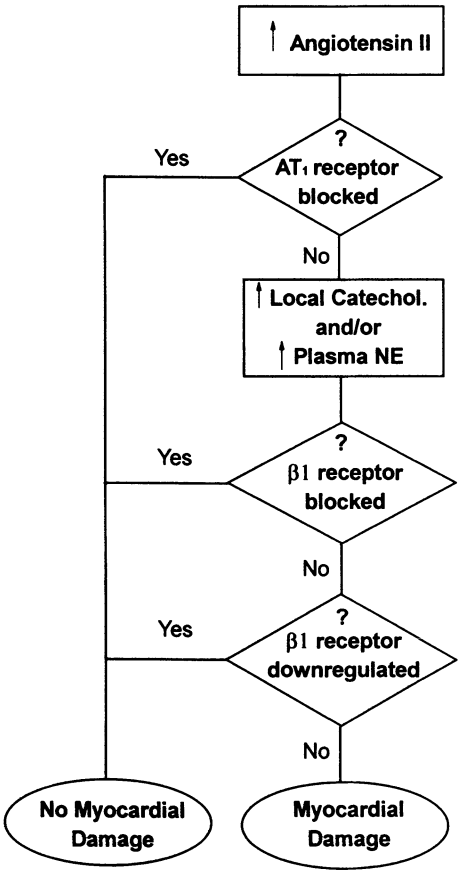


**Figure 8.** Flow chart summarizing how coronary and myocyte damage may occur due to chronic angiotensin II infusion. If angiotensin II levels are elevated but the $AT_1$ receptor is blocked, no myocardial damage is observed. If the $AT_1$ receptor is not blocked, angiotensin II induces a local release of norepinephrine. If the β-adrenergic receptors and, more specifically, the β1-adrenergic receptors are blocked, no myocardial damage is observed. If the β1-adrenergic receptors are not blocked but the receptors are downregulated, no myocardial damage is observed. If the β1 receptors are not downregulated, then myocardial damage is observed.

cently Beltrami et al. [38] found that, in patients with ischemic cardiomyopathy, cell loss from the MI together with the remote myocyte necrosis resulted in a 28% loss in the total number of myocyte nuclei in the left ventricle (LV). The average volume of the MI in these patients was 9%. Thus, the remote necrosis involved 19% of the total cell loss. The right ventricle (RV) had a 30% decrease in cell number which was attributed entirely to remote myocyte necrosis. Therefore, remote myocyte necrosis can involve a large percentage of the myocardium. Obviously, this magnitude of myocardial damage could significantly impair cardiac function and

may play a key role in the transition from compensated to decompensated heart failure following MI.

Our laboratory has also observed areas of remote necrosis following MI in rats [39]. The remote necrosis was found to be present in both the LV and the RV. In this study, remote myocyte necrosis could be prevented with the administration of an ACE inhibitor initiated one day prior to the creation of the MI. This finding implies that Ang II is responsible for this remote necrosis. ACE inhibition has been shown to significantly decrease mortality in rats [40] and in humans [41] post-MI. Accordingly, the ability of ACE inhibition to prevent remote necrosis may explain, at least in part, the decreased mortality associated with ACE inhibition post-MI.

## CONCLUSIONS

In figure 8, a schematic representation of the mechanism of Ang II-mediated myocardial damage is presented. Chronic, pathophysiological elevations of Ang II cause myocyte necrosis and coronary vascular damage. These adverse effects are mediated by the Ang II type 1 ($AT_1$) receptor and are therefore preventable with $AT_1$ receptor blockade. However, the intracellular signaling cascade stimulated by elevations in Ang II results in an $AT_1$ receptor-mediated catecholamine release, and the myocardial damage can also be attenuated by β1-adrenergic receptor blockade. The restriction of myocardial damage to the first 3 days of Ang II infusion is due to subsequent downregulation of the β1-adrenergic receptor population. Discontinuation of the Ang II infusion results in a return to normal β1-adrenergic receptor density, which makes the myocardium susceptible once again to subsequent elevations of Ang II. This Ang II-related myocardial damage could play an important role in the pathogenesis of heart failure post-MI or in other cardiac disease states.

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# COMPARISON OF DIRECT ANGIOTENSIN II RECEPTOR BLOCKADE WITH CONVERTING ENZYME INHIBITION IN THE RAT MODEL OF HEART FAILURE

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***Summary.*** The purpose of our investigations was to compare the effects of ACE inhibition with captopril to direct $AT_1$ receptor blockade with losartan in the rat postinfarction model of congestive heart failure. In the first group of studies, hemodynamic and neurohormonal effects of treatment for 2 weeks with captopril (2 g/liter in drinking water) or losartan (40 mg/kg by gavage) were compared, starting 3 weeks after infarction. Losartan and captopril reduced LV end-diastolic pressure and LV end-diastolic volume index and increased venous compliance. In both treatment groups, these changes were significant compared to placebo treatment ($p < 0.05$). Serum angiotensin II and aldosterone levels were lower after treatment with captopril, whereas only aldosterone tended to be lower after treatment with losartan. A second study compared the survival of heart failure rats treated with captopril (2 g/liter drinking water, $n = 46$) or losartan (2 g/liter drinking water, $n = 51$). Treatment was started immediately after coronary artery ligation and continued for 1 year. Uncensored median survival in captopril-treated rats that survived at least 48 h was 201.5 days versus 236.0 days for losartan-treated rats ($p = 0.066$). Median survival censored for rats with lung infections was 201.5 days in captopril-treated rats versus 243.0 days for losartan-treated rats ($p = 0.028$). Conscious hemodynamic measurements and remodeling data obtained at one year in the surviving rats ($n = 5$ for captopril, $n = 9$ for losartan) revealed no differences except for an increase in heart rate and a decrease in developed LV pressure in losartan-treated rats. We conclude that in this experimental model of heart failure, there was no difference between hemodynamic effects and survival after $AT_1$ receptor blockade with losartan and with ACE inhibition with captopril.

Activation of the renin-angiotensin-aldosterone system in heart failure contributes very importantly to the pathophysiology of this symptom complex [1–3]. Inhibition

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of angiotensin (Ang) II formation with angiotensin-converting enzyme (ACE) inhibitors has been shown to improve hemodynamic performance, exercise tolerance, symptoms, and mortality in both animal models [4,5] and in patients with heart failure [6–12]. The use of ACE inhibitors is now generally accepted therapy for patients with chronic congestive heart failure and for those with systolic dysfunction after myocardial infarction [13,14]. ACE inhibitors also appear to decrease the incidence of recurrent myocardial infarction, unstable angina, and sudden death among those with reduced left ventricular (LV) function after myocardial infarction [11–13]. Although these beneficial effects are well documented, the mechanism of action of these agents is not entirely clear. It is thought that ACE inhibitors exert their cardiac effects primarily by decreasing LV afterload through inhibition of the conversion of Ang I to Ang II [15]. The mechanism of action of ACE inhibitors may be more complex than simple afterload reduction, however, because experimental [16] and clinical studies [11,17] have shown that a relatively pure afterload-reducing agent, such as hydralazine, is less beneficial than ACE inhibitors. Furthermore, serum renin, and by inference Ang I and II, is not elevated during the chronic compensated phase of congestive heart failure [18], which makes it unlikely that blocking the activity of ACE can be entirely responsible for the hemodynamic benefits. Additional actions may include the effects of ACE inhibitors on the kinin and prostaglandin systems [15,19–21].

Losartan is an imidazole derivative that exerts its effects by competitive antagonism of Ang II for one class of its receptors, referred to as $AT_1$ receptors. These are GTP-coupled proteins with seven membrane spanning domains [22,23]. There is evidence that they may be coupled to all three major G protein coupled signaling pathways: (1) activation of phospholipase C, resulting in generation of 1,4,5-inositol triphosphate and diacylgylcerol and subsequent release of intracellular $Ca^{2+}$; (2) activation of dihydropyridine-sensitive $Ca^{2+}$ channels; and (3) inhibition of adenylate cyclase activity [24–26]. $AT_1$ receptor subtypes are found in various organs, including liver, brain, adrenal, heart, and vascular muscle, where they are thought to mediate, among other effects, the characteristic actions of Ang II on blood pressure regulation and aldosterone secretion.

These observations suggest that direct $AT_1$ receptor blockade and ACE inhibition should share many cardiovascular actions, possibly including beneficial effects in congestive heart failure. When the hemodynamic effects of losartan and ACE inhibition with captopril were compared in an experimental heart failure model, both agents decreased LV end-diastolic pressure, LV end-diastolic volume, and increased venous compliance without changing heart rate [27]. In addition, enalapril, another ACE inhibitor, and losartan equally attenuated the development of myocardial fibrosis in the noninfarcted rat LV [28]. Because both ACE inhibitors and losartan improved hemodynamic parameters after infarction and because ACE inhibitors have been shown to improve survival, a study was designed to compare the effects of captopril and losartan in the rat postinfarction model of heart failure [29]. The purpose of this report is to summarize our studies comparing the effects on hemodynamic and the renin-angiotensin-aldosterone system of ACE inhibition

**Table 1.** Ventricular weight, body weight, and myocardial infarction size in control postinfarction rats and postinfarction rats treated for 2 wks with either captopril or losartan

| | Control | Captopril | Losartan |
|---|---|---|---|
| *Body Weight (kg) × $10^{-1}$* | 3.76 ± 0.07 | 3.54 ± 0.08 | 3.37 ± 0.06* |
| *LV Weight (g) × $10^{-1}$* | 6.798 ± 0.305 | 6.722 ± 0.305 | 6.632 ± 0.122 |
| *RV Weight (g) × $10^{-1}$* | 3.229 ± 0.267 | 2.621 ± 0.221 | 2.640 ± 0.152 |
| *LV Weight/Body Weight (g/kg)* | 1.82 ± 0.07 | 1.90 ± 0.05 | 1.97 ± 0.05 |
| *Infarct Size (% LV)* | 44.9 ± 2.7 | 48.6 ± 3.0 | 46.3 ± 2.4 |
| *N* | 9 | 9 | 10 |

Note: Values are means ± SEM. LV, left ventricular; N, sample size. *P < 0.05 by Dunnett's test for multiple comparisons against a single control.

versus direct $AT_1$ receptor blockade and to review recent evidence that the effects of captopril and losartan on survival in the rat postinfarction model may be similar.

## HEMODYNAMIC OF LOSARTAN AND CAPTOPRIL

In the initial hemodynamic studies, male Sprague-Dawley rats (175 to 225 g) underwent coronary artery ligation using techniques similar to those described earlier [16,27]. After three weeks, rats were anesthetized and nine-lead electrocardiograms with six limb leads and three chest leads were performed. Using criteria described previously [5], rats with evidence of large myocardial infarctions were selected for study. Three groups of rats were used for the hemodynamic study protocol: one group was untreated, a second group was treated with captopril (2 g/l drinking water), and a third group was treated with losartan (40 mg/kg/day by gastric gavage). In both treatment groups, the drug was administered for two weeks. The dose of captopril chosen was the same as that used by Pfeiffer et al. [5] in their study on the effects of ACE inhibition in the rat postinfarction heart failure model. The dose of losartan selected for use was shown in preliminary studies to be sufficient to shift the log pressure-response curve to an infusion of Ang II rightward by approximately $10^2$ [27].

Body weight, heart weight, and infarct size in the untreated, captopril-treated, and losartan-treated postinfarction rats are shown in table 1. In all three groups, the average infarct size was large, approximately 45% to 49%. Treatment with either captopril or losartan did not change any of these parameters, with the exception that losartan decreased body weights. The hemodynamic changes in the untreated heart failure animals and those treated with captopril and losartan are shown in table 2. There were no differences in heart rate among the untreated and treated heart failure rats. While there were lower average right atrial and mean aortic pressures in both treatment groups, these decreases were not statistically significant. LV end-diastolic pressure and LV end-diastolic volume index were decreased significantly ($P < 0.05$ and $P < 0.01$ for captopril and losartan, respectively) in both treatment groups as compared to untreated heart failure animals. Mean circulatory filling pressure, an important index of preload [28], was decreased with losartan, and

**Table 2.** Heart rate, mean aortic pressure, right atrial pressure, LV end-diastolic pressure, mean circulatory filling pressure, venous compliance, total blood volume index, and LV end-diastolic volume index in control postinfarction rats and postinfarction rats treated for 2 wks with either captopril or losartan

| | Control | Captopril | Losartan |
|---|---|---|---|
| *Heart Rate (bpm)* | 333 ± 9 | 341 ± 8 | 330 ± 9 |
| *Mean aortic pressure (mm Hg)* | 107 ± 3 | 94 ± 5 | 97 ± 6 |
| *Right atrial pressure (mm Hg)* | 2.3 ± 0.1 | 2.1 ± 0.4* | 1.9 ± 0.2 |
| *LV end-diastolic pressure (mm Hg)* | 26.7 ± 1.5 | 15.8 ± 2.2* | 14.2 ± 3.0† |
| *Mean circulatory filling pressure (mm Hg)* | 9.6 ± 0.3 | 8.5 ± 0.4 | 7.0 ± 0.3† |
| *Venous compliance (mL/mm Hg/kg)* | 2.27 ± 0.06 | 3.02 ± 0.21† | 2.80 ± 0.18* |
| *Total blood volume index (mL/kg)* | 65.8 ± 1.1 | 64.9 ± 3.9 | 59.4 ± 3.0 |
| *End-diastolic volume index (mL/kg)* | 2.71 ± 0.10 | 2.18 ± 0.15* | 2.03 ± 0.17* |
| *N* | 9 | 9 | 10 |

Note: Values are means ± SEM. N, sample size. *$P < 0.05$; †$P < 0.01$ by Dunnett's test for multiple comparisons against a single control.

**Table 3.** Plasma renin activity, angiotensin II, and aldosterone concentrations in postinfarction control rats and postinfarction rats treated for 2wks with either captopril or losartan

| | Heart Failure | Captopril | Losartan |
|---|---|---|---|
| *Renin Activity (ng/ml/hr)* | 17.9 ± 3.5 | 11.5 ± 1.6 | 12.9 ± 2.0 |
| *Angiotensin II (pg/ml)* | 93 ± 43 | 18.7 ± 2.7* | 72.9 ± 15.8 |
| *Aldosterone (pg/ml)* | 561 ± 65 | 298 ± 2.7† | 345 ± 51 |
| *N* | 7 | 6 | 9 |

Note: Values are means ± SEM. N, sample size. *$P = 0.006$ vs. losartan-treated group, †$P = 0.02$ versus untreated heart failure group by one-way analysis of variance.

venous compliance was increased with captopril ($P < 0.05$). While total blood volume index was decreased in both groups, this change was not significant ($P > 0.05$).

### EFFECTS ON RENIN-ANGIOTENSIN-ALDOSTERONE SYSTEM

In the animals studied hemodynamically, measurements of circulating levels of renin, Ang II, and aldosterone also were made. Average plasma renin activity was decreased in both treatment groups (table 3), but the changes were not significant ($P > 0.05$). Angiotensin II levels in the group treated with losartan (72.9 ± 15.8 pg/ml) were nearly the same as in the control group (92 ± 43 pg/ml), whereas the values were lower, on average, after treatment with captopril (18.7 ± 2.7 pg/ml) ($p < 0.006$). Aldosterone levels in the group treated with captopril (298 ± 2.7 pg/ml) were decreased significantly ($p < 0.02$) as compared to the untreated heart failure group (561 ± 65 pg/ml). The aldosterone levels were also lower in the group treated with losartan (345 ± 51 pg/ml), but in this case, the difference did not achieve statistical significance. Generally, the trends in this data are consistent with

what might be anticipated from the pharmacological actions of these drugs, but it would be necessary to study larger numbers of animals before drawing any firm conclusions.

The results of this initial study suggested that losartan is as effective in the treatment of heart failure as captopril. The beneficial hemodynamic effects of $AT_1$ receptor blockade were similar to those of converting enzyme inhibition: LV end-diastolic pressure and LV end-diastolic volume decreased, and venous compliance increased. Since captopril has been shown to improve long-term survival in the rat model, a study was designed to directly compare the effects of captopril and losartan on survival.

## COMPARISON OF THE EFFECTS OF CAPTOPRIL AND LOSARTAN ON SURVIVAL

A one-year treatment trial comparing the effects of captopril with those of losartan was designed with mortality as the primary end-point [29]. The study was planned with the intent to detect a 20% difference between treatments with a two-sided significance level of 0.05. A placebo arm was considered but would have required too large a sample size. Inclusive of operative mortality, about 35% of rats undergoing coronary ligation in our laboratory have moderate to large myocardial infarctions. Had we chosen to include a placebo arm, to achieve the desired level of significance and power would have required us to operate on 944 rats. Because the beneficial effects of ACE inhibition is already well-defined in the rat postinfarction model, we concluded that there was no compelling reason to warrant a placebo heart failure group.

Male Sprague-Dawley rats (175 to 275 g) underwent coronary artery ligation. Afterwards, animals were returned to their cages and randomly assigned to treatment with losartan (2 g/liter drinking water) or captopril (2 g/liter drinking water). Ten to 14 days after operation, rats were anesthetized with methoxyflurane, and a nine-lead electrocardiogram was recorded to estimate infarct size. Only rats with ECG evidence of moderate to large myocardial infarctions on the basis of standard criteria [5] were continued in the study. Cages were inspected for dead animals daily. A postmortem examination was performed, and the lungs were inspected for gross signs of consolidation. The lungs and heart were placed in formalin for subsequent necropsy studies. Group stratification was confirmed at postmortem examination by quantitative histopathological studies. As in the previous work, rats were considered to have a moderate to large infarction when $\geq$20% of the LV surface area was occupied by fibrous scar tissue.

A total of 237 animals were entered into the study; 119 were randomized to captopril treatment and 118 to losartan treatment. In the captopril treatment group, 41 rats died before the ECG was recorded, 1 rat died during the ECG anesthetic period, and 31 rats had small infarctions ($<$10%) by ECG criteria. In the losartan treatment group, 45 rats died before the ECG was recorded, 1 died immediately after the ECG, and 21 had small infarctions. All rats that died before the ECG was recorded died within 48 h after coronary ligation (36% initial mortality). Thus, 46

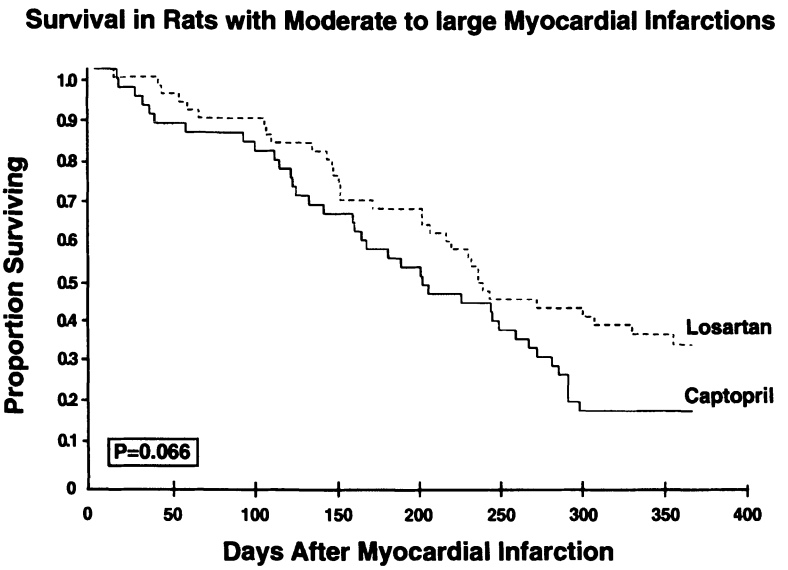


**Figure 1.** Kaplan-Meier survival curves in rats with moderate to large infarction treated with captopril or losartan (2 g/liter drinking water for each). One-year median survival in captopril-treated rats was 201.5 days versus 236.0 days in losartan-treated rats (log rank p = 0.066).

rats with moderate or large myocardial infarctions were treated with captopril, and 51 rats with moderate to large myocardial infarctions were treated with losartan.

There were no significant differences in body weight or age at the time of myocardial infarction ($P < 0.05$). Infarct size in rats treated with captopril that died during the trial was 37 ± 4% (range 18 to 49%) versus 38 ± 4% (range 19 to 56%) in rats treated with losartan. In both treatment groups, there was one rat with an infarct size <20% and one rat with an infarct size >50%.

### SURVIVAL DATA

After 97 animals had been randomized into the study, statistical analysis indicated that inclusion of a larger number would be unlikely to significantly change the outcome. Accordingly, no further rats were entered into the trial, and all surviving animals were followed for one year. The survival curves for rats with moderate to large infarctions that survived until the ECG are shown in fig. 1. The mean and median survival data is summarized in table 4. Although there is no statistically significant difference in median survival between the two treatment groups, 201.5 days for captopril versus 236.0 days for losartan (log rank p = 0.066), the survival curve for the losartan-treated rats is above that for the captopril treatment group starting within two weeks of coronary artery ligation. When the analysis included all rats that died within 48 h of the infarction, median survival in the captopril-treated group was 34.0 days versus 54.0 days for losartan treatment (p = 0.132).

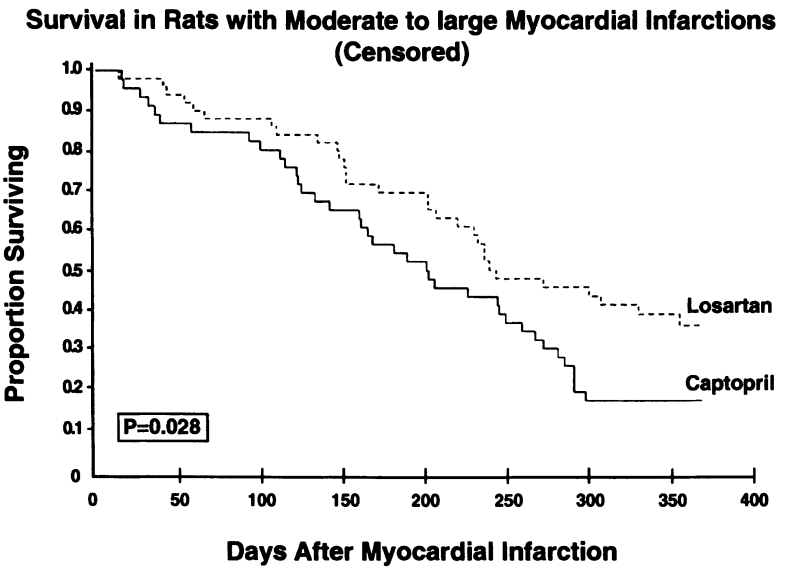


**Figure 2.** Kaplan-Meier survival curves in rats with moderate to large infarction treated with captopril or lorsartan as described in figure 1. after the data were censored for rats dying with lung infections. One-year survival was significantly greater for the losartan-treated rats (243.0 days versus 201.5 days, p = 0.028).

**Table 4.** Survival estimates for postinfarction rats treated with either captopril or losartan

| | | Captopril | Losartan |
|---|---|---|---|
| Median Survival (Days) | *TOTAL* | 46 | 51 |
| | *Censored (Lost on Follow-up)* | 191.4 ± 14.1 (201.5) | 238.3 ± 15.6 (236.0) |
| | *Censored (All Causes)* | 191.4 ± 14.1 (201.5) | 246.4 ± 15.8* (243.0) |

Note: Values are means ± SEM. Median values are shown in parenthesis. *P < 0.03 by Log-Rank Test.

Among the rats with moderate to large infarctions that survived until the ECG was recorded, three rats in the losartan group had class 3 lung consolidation consistent with pneumonia [see ref. 5 for definition of lung histopathological classes]. There were no cases of lung consolidation consistent with pneumonia in the captopril treated group. Because lung consolidation was not evenly distributed between the two groups, data from these three animals were not censored for the primary analysis. As shown in table 4, when these three animals were considered censored, the median survival for captopril-treated rats was significantly greater than survival for the losartan-treated rats (201.5 days versus 243.0 days, p = 0.028). A plot of the censored data (fig. 2) shows even more clearly that the survival curve for the losartan-treated group is above that for the captopril-treated group.

**Table 5.** Heart rate, LV pressures, body weight, heart weight, and heart weight/body weight ratio in infarcted rats treated with either captopril or losartan that survived to 1 year

| | Captopril | Losartan |
|---|---|---|
| *Heart Rate (bpm)* | 266 ± 15 | 293 ± 19* |
| *LV EDP (mm Hg)* | 14 ± 7 | 11 ± 9 |
| *LVSP (mm Hg)* | 109 ± 19 | 99 ± 26 |
| *Body Weight (g)* | 495 ± 17 | 519 ± 73 |
| *LV Weight (mg)* | 1073 ± 98 | 946 ± 213 |
| *LV/Body Weight* | 2.18 ± 0.26 | 1.84 ± 0.39 |

Note: Values are mean ± SD. LV EDP, LV end-diastolic pressure mm Hg; LV SP, LV systolic pressure mm Hg. N = 5 for rats treated with captopril and 9 for rats treated with losartan. *P = 0.018 compared to captopril by Student's *t* test for unpaired values.

**Table 6.** Indices of LV systolic and diastolic function in postinfarction rats treated with either captopril or losartan that survived to 1 year

| | Captopril | Losartan |
|---|---|---|
| *Tau (ms)* | 20.2 ± 3.4 | 19.4 ± 4.1 |
| *dP/dt (mm Hg/s)* | 5271 ± 886 | 4836 ± 1276 |
| *CII (ml/kg/min)* | 315 ± 57 | 276 ± 58 |
| *SVI (ml/kg/min)* | 1.17 ± 0.20 | 0.97 ± 0.25 |
| *PDP (mm Hg)* | 180 ± 16 | 153 ± 21* |
| $K_c$ | 2.35 ± 0.71 | 2.20 ± 0.98 |

Note: Values are means ± SD. CI, cardiac index; $K_c$, chamber stiffness constant; PDP, peak developed pressure; SVI, stroke volume index; Tau, time constant of LV relaxation. N = 5 for rats treated with captopril and 9 for rats treated with losartan. *P = 0.024 compared to captopril by Student's *t* test for unpaired values.

## HEART WEIGHT, BODY WEIGHT, HEMODYNAMICS, AND LV REMODELING PARAMETERS IN SURVIVING RATS

Body weight, heart weight, and heart weight/body weight ratio, hemodynamic data, and LV remodeling parameters in the captopril- and losartan-treated groups obtained at one year in the surviving rats are shown in table 5. There were no significant differences in these parameters between the two treatment groups. When hemodynamic variables in captopril-treated and losartan-treated groups were compared (table 6), the only differences were an increase in heart rate (293 ± 19 vs. 266 ± 15 beats/min, p = 0.018) and a decrease in peak developed pressure (153 ± 21 vs. 180 ± 16 mm Hg, p = 0.024) in the losartan-treated rats. None of the indices of LV remodeling in rats surviving one year were different in the two groups (table 7).

This study confirmed that mortality is increased in rats with heart failure after moderate to large myocardial infarctions and showed that there is no major differ-

**Table 7.** Indices of LV remodeling in postinfarction rats treated with either captopril or losartan that survived to 1 year

| | Captopril | Losartan |
|---|---|---|
| *$V/V_w$* | 0.79 ± 0.21 | 1.09 ± 0.40 |
| *Length (mm)* | 17.5 ± 0.9 | 18.7 ± 1.5 |
| *LV Diameter (mm)* | 9.49 ± 0.78 | 10.01 ± 1.66 |
| *Thickness (mm)* | 2.22 ± 0.24 | 2.03 ± 0.21 |
| *LV Thickness/Diameter* | 0.23 ± 0.04 | 0.21 ± 0.04 |
| *% MI Infarct Size* | 31 ± 4 | 38 ± 7 |

Note: Values are means ± SD. LV, left ventricular; $V/V_w$, LV chamber volume/LV wall volume. N = 5 for captopril and 9 for losartan.

ence in survival with specific $AT_1$ receptor blockade with losartan versus ACE inhibition with captopril. Also, there were no differences in indices of myocardial function and left ventricular geometry obtained at one year in surviving rats. Although other studies have examined the hemodynamic differences between specific $AT_1$ receptor blockade and ACE inhibition in heart failure, to our knowledge, this is the first study to examine survival.

There are few published animal studies of survival after myocardial infarction. Our study was based on the original captopril survival study by Pfeffer et al. [5], with the major difference that treatment was initiated on the day of the infarction; in the earlier study, treatment was started on day 14. In that study, captopril improved survival in rats with large infarctions. The median survival in the moderate and large infarct groups treated with captopril was 329 and 181 days, respectively. If the 14 days before initiation of therapy is subtracted from the survival reported in our study, the median survival of 201.5 days for captopril-treated rats with moderate to large infarctions in our study is comparable. In our trial, after elimination of the rats that died before the ECG, 46 rats were treated with captopril and 51 with losartan compared with 35 rats with moderate infarction and 37 with a large infarction assigned to captopril in the earlier study. Thus, despite differences in study design and numbers, the survival data with captopril in both of these studies are similar.

An interesting parallel can be drawn between the results of our studies and the recently completed ELITE trial [30]. The latter study evaluated the long-term safety of losartan treatment compared with captopril treatment in older patients (age >65 years) with heart failure. In total, 722 patients with symptomatic heart failure (NYHA Class II-IV), who had not received therapy with an ACE inhibitor previously, were randomized to treatment with captopril or losartan for 48 weeks. Preliminary results showed that there was no difference between losartan and captopril in the primary endpoint (persistent increase in serum creatinine) of the study. Interestingly, the incidence of death and/or hospitalization for heart failure, the secondary endpoint, was 9.4% in the losartan-treated patients compared to

13.2% in captopril-treated patients ($p = 0.075$); this difference was entirely due to a 46% decrease in total mortality in the losartan-treated patients ($p < 0.05$).

These recent clinical results seem entirely consistent with the trend toward increased survival observed in postinfarction rats treated with losartan as compared to those receiving captopril. Moreover, the similarity in the overall results again confirms that the rat postinfarction model is useful for testing the efficacy of drugs designed for treatment of heart failure.

## ACKNOWLEDGMENTS

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# EFFECTS OF CAPTOPRIL ON MYOCARDIAL OXIDATIVE STRESS CHANGES IN POST-MI RATS

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***Summary.*** Changes in oxidative stress as indicated by the redox ratio as well as lipid peroxidation were characterized in rat hearts at different time points, subsequent to myocardial infarction (MI). In the severe heart failure stage at 16 weeks of post-MI, oxidative stress was significantly increased. Treatment with afterload reducing drugs, captopril or prazosin, started at 4 weeks post-MI and continued up to 16 weeks, resulted in a significant modulation of the oxidative stress changes with an improved hemodynamic function. It is suggested that the improved prognosis in MI patients with afterload reduction reported earlier may involve an improvement of the antioxidant reserve coupled with a reduction in the oxidative stress in the infarcted heart.

## INTRODUCTION

Oxygen free radicals have been suggested to play a role in the pathogenesis of cardiac dysfunction in different pathological conditions [1–5]. Under normal physiological conditions, there is a delicate balance between oxygen free radical production and antioxidants. However, during disease states or pathological conditions, the balance may shift in favor of a relative increase in free radicals, resulting in an increased oxidative stress. Generally, the redox (oxidation-reduction) state, i.e., the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG), is considered to be inversely proportional to the oxidative stress and is used as an index for the latter. Thus, an increase in the redox state indicates reduced oxidative stress, and a decrease in the redox state indicates increased oxidative stress [4]. Myocardial antioxidants have been reported to change under different physiological and pathophysiological

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conditions [5–7]. Hypertrophy of the heart, because of chronic pressure overload, has been shown to be associated with increased antioxidant activities in both rats [8] and guinea pigs [3], whereas heart failure has been reported to be associated with a depressed antioxidant reserve [3,7,9,10].

Recent studies from our laboratory, using the coronary artery ligation model of myocardial infarction (MI) and congestive heart failure (CHF) in rats, have shown that sustained cardiac function in nonfailing hearts is accompanied by the maintenance and/or a trend towards an increase in antioxidants and a decrease in oxidative stress, while the converse is true during severe heart failure [9,10]. In another study using this animal model, we examined the effects of afterload reduction on changes in antioxidant enzyme activities in relation to the hemodynamic function. It was reported that afterload reduction using captopril or prazosin resulted in improved hemodynamic function in post-MI rats [10]. This improved cardiac function was accompanied by an increase in different antioxidant enzyme activities. However, there have been no studies to date examining the effects of captopril (an ACE inhibitor) on oxidative stress changes, as measured by the redox ratio in CHF subsequent to (MI).

The present study was designed to examine the effects of captopril on myocardial reduced glutathione (GSH) content, myocardial oxidized glutathione (GSSG) content, and GSH/GSSG ratio to assess the degree of oxidative stress at 16 weeks postsurgery duration. The changes in lipid peroxidation as measured by thiobarbituric acid-reactive substances (TBARS) were also examined. Prazosin, an $\alpha_1$-blocker and another afterload reducing drug, was used for comparison.

## MATERIALS AND METHODS

### Animal model and study groups

Male Sprague-Dawley rats (150 ± 10 g) were maintained on standard rat chow and water ad libitum unless mentioned otherwise. Myocardial infarction was produced via occlusion of the left anterior descending coronary artery [11,12]. In this procedure, the animals were anesthetized with 2% isoflurane, and the skin was then incised along the left sternal border. The third and fourth ribs were cut proximal to the sternum with the subsequent insertion of retractors, and the heart was exteriorized through the intercostal space. The left coronary artery was ligated about 1–2 mm from its origin with a 6-0 silk thread. Following ligation, the heart was gently repositioned in the chest. Excess air was withdrawn into a syringe, and the chest was closed. The rats were maintained on a positive pressure ventilation, delivering 2% isoflurane mixed with oxygen, throughout the surgery. Control animals were treated in a similar fashion with the exception that the suture around the coronary artery was not tied, and the thread was only passed through the muscle.

There were five experimental groups, and each group had its own age matched sham control as follows: (1) 1-week post-MI and 1-week control; (2) 4-week post-MI and 4-week control; (3) 16-week post-MI and 16-week control; (4) 16-week captopril-treated post-MI and 16-week control; and (5) 16-week prazosin-treated

post-MI and 16-week control. In groups 4 and 5, captopril (2 g/l in drinking water) and prazosin (0.2 mg/kg s.c. daily) treatments, respectively, were started 4 weeks after the surgery and were continued up to 16 weeks. Animals were monitored daily for their general behavior as well as their food and water intake.

### Glutathione assay

Concentration of total glutathione (GSSG + GSH) was measured in the myocardium by the glutathione reductase/5, 5′-dithiobis-(2-nitrobenzoic acid) (DTNB) recycling assay [13]. The rate of DTNB formation is followed at 412 nm and is proportional to the sum of GSH and GSSG present. Myocardial tissue was homogenized in 5% sulfosalicylic acid. The tissue homogenate was centrifuged for 10 min at 10,000 g. Supernatant was stored at 4°C until assayed. GSSG alone was measured by treating the sulfosalicylic acid supernatant with 2-vinylpyridine and triethanolamine. The solution was vigorously mixed, and final pH of the solution was checked to be between 6 and 7. After 60 min, the derivatized samples were assayed as described above in the DTNB-GSSG reductase recycling assay. GSH values were calculated as the difference between total (GSSG + GSH) and GSSG concentrations.

### Thiobarbituric acid-reactive substances (TBARS)

Lipid peroxide content in hearts was determined by measuring the TBARS by the method described previously [14]. Hearts were homogenized in (10% wt/vol) 0.2 Mol/l Tris-0.16 Mol/l KCl buffer, pH 7.4, and incubated at 37°C for 1 hour. After one hour, a 2 ml aliquot was collected from the incubation mixture and poured into a Corning culture tube. This was followed by the addition of 2.0 ml of 40% trichloroacetic acid and 1.0 ml of 0.2% thiobarbituric acid (TBA). 100 μL of 2% butylated hydroxy-toluene was added to the TBA reagent mixture in order to minimize peroxidation during the assay procedure. The mixture was then boiled for 15 min and then allowed to cool on ice for 5 min. Next, 2 ml of 70% trichloroacetic acid was added, and tubes were allowed to stand for 20 min. After 20 min, the sample was centrifuged at 800 g for 20 min. The developed color was read at 532 nm on a spectrophotometer. Commercially available malondialdehyde was used as a standard.

### Statistical analysis

Data are expressed as the mean ± SEM. For a statistical analysis of the data, group means were compared by one-way ANOVA followed by Bonferroni's test. Values of $P < 0.05$ were considered significant.

## RESULTS

### General characteristics, mortality, and body weight

All sham-control and coronary-ligated animals were monitored daily for their general behavior and body weight. Nothing unusual was noted in any of the sham control, post-MI, or drug treated groups with respect to their general appearance

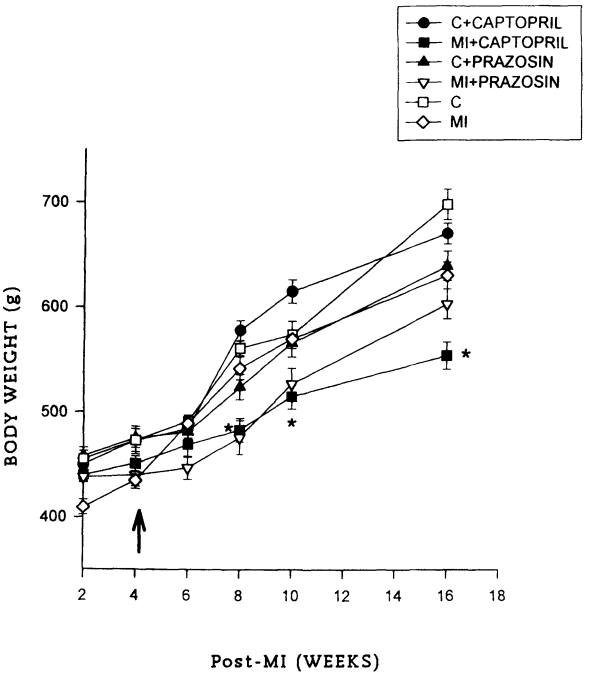


**Figure 1.** Body weight of sham control and post-MI rats at different postsurgery durations with and without treatments with captopril and prazosin. Drug treatment was started at 4-weeks postsurgery duration as described in the Material and Methods section. Data are mean ± SE of 10–12 rats. C, Sham Control; MI, Infarcted Group; post-MI, post-myocardial infarction. Arrow indicates the time at which the drug treatment was started. *Significantly different ($P < 0.05$) from its sham control animals treated with captopril.

and behavior. Mortality in the coronary-ligated animals during or immediately after the surgery was about 20%. Another 12% of the animals died within 24 hrs following the surgery. Body weight gain of animals in all post-MI groups was slightly lower than their respective sham control groups (figure 1). Body weight gain in the captopril-treated 16-week post-MI group was significantly less than the respective untreated post-MI group. Body weight gain in the prazosin-treated group was not different from the untreated post-MI groups.

### Redox state

Myocardial GSH and GSSG contents as well as GSH/GSSG ratio were examined in 1-, 4-, and 16-week sham and post-MI groups without treatment and at 16 weeks in groups treated with captopril or prazosin. These data are shown in table 1 and figure 2, respectively. GSH content in experimental hearts showed no significant change at 1- and 4- weeks post-MI compared to respective sham control groups. GSH content was decreased by about 34% in the 16-week post-MI group relative

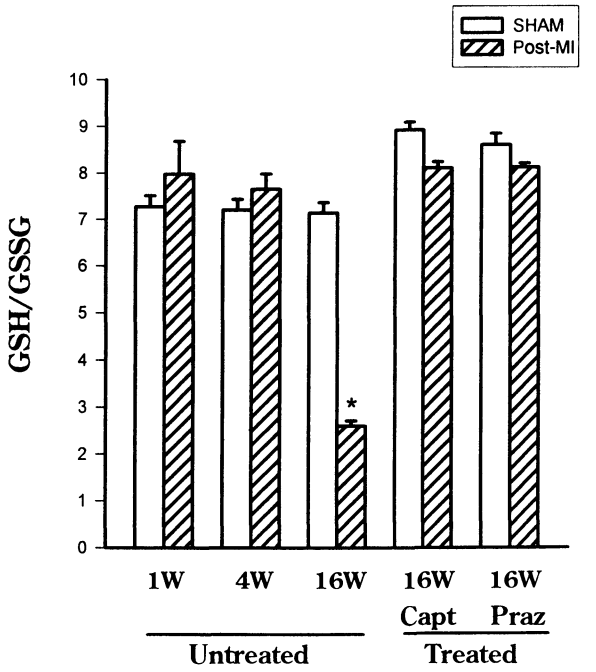


**Figure 2.** GSH/GSSG ratio in sham and infarcted post-MI hearts at 1, 4, and 16 weeks of postsurgery duration without treatment and at 16 weeks postsurgery duration with captopril (Capt) or prazosin (Praz) treatment. Drug treatment was started at 4 weeks postsurgery duration as described in the Material and Methods section. Data are mean ± SE of 6–7 experiments. *Significantly different ($P < 0.05$) from respective sham as well as 1- and 4-week experimental groups by ANOVA followed by Bonferroni test.

**Table 1.** Myocardial reduced (GSH) and oxidized (GSSG) glutathione levels in sham control and post-MI rats at 1, 4, and 16 weeks without treatment and at 16 weeks with captopril or prazosin treatments

| | GSH (μmol/L) | | GSSG (μmol/L) | |
|---|---|---|---|---|
| post-MI Duration (weeks) | Sham | post-MI | Sham | post-MI |
| *Without Treatment* | | | | |
| 1 | 63.8 ± 1.8 | 60.3 ± 1.8 | 8.5 ± 0.3 | 7.6 ± 0.5 |
| 4 | 62.6 ± 1.3 | 59.8 ± 1.9 | 8.8 ± 1.7 | 8.1 ± 0.4 |
| 16 | 66.8 ± 1.9 | 44.1 ± 1.4* | 9.2 ± 0.2 | 18.2 ± 2.1* |
| *With Treatment* | | | | |
| Captopril | 73.2 ± 1.9 | 72.1 ± 3.7† | 8.2 ± 0.3 | 8.8 ± 0.4† |
| Prazosin | 73.5 ± 2.9 | 71.3 ± 1.4† | 8.3 ± 0.2 | 8.7 ± 0.2† |

Note: Values are mean ± SE of 6–7 hearts. *) Significantly different ($P < 0.05$) from respective sham control as well as 1- and 4-weeks post-myocardial infarction (post-MI) groups. †) Significantly different from the 16-week untreated post-MI group.

to respective sham control, and this decrease was statistically significant ($P < 0.05$). GSSG content remained unchanged at 1- and 4-weeks post-MI; however, it was significantly increased at 16-weeks post-MI compared to its respective sham control group (table 1). The redox ratio (GSH/GSSG) was marginally higher in the 1- and 4-week post-MI groups compared to sham controls; however, the change was not statistically significant. This ratio in the 16-week post-MI group was significantly lower than its respective sham control group (figure 2).

The GSH content in the 16-week post-MI group was significantly improved in both the captopril and prazosin-treated groups compared to the 16-week post-MI untreated group. The GSSG content was significantly decreased in both the captopril and prazosin treated-16-week post-MI groups compared to their respective untreated 16-week post-MI group (table 1). Captopril and prazosin treatments also significantly improved the redox ratio in the 16-week post-MI group compared to the untreated 16-week post-MI group (figure 2).

### Lipid peroxidation

Lipid peroxidation, which is another indicator of oxidative stress as well as injury, was assessed in 1-, 4-, and 16-week sham and post-MI groups as well as in the 16-week group treated with captopril or prazosin by evaluating myocardial TBARS, and these data are shown in figure 3. TBARS in the 1-week post-MI group remained unchanged compared to its respective control. Although TBARS were increased in the 4- and 16-week post-MI groups as compared to their sham controls, the increase was statistically significant only in the 16-week post-MI group.

Captopril or prazosin had no effect on the level of TBARS in sham controls. However, in the captopril-treated group, TBARS were significantly lower as compared to its respective untreated post-MI group. TBARS in the prazosin-treated post-MI group were also lower than the untreated post-MI group (figure 3).

## DISCUSSION

Beneficial effects of vasodilators in the management of hypertension and congestive heart failure have been very well established. Many investigators have demonstrated that captopril improves hemodynamic function as well as influences ventricular remodeling in congestive heart failure subsequent to MI in rats [15] and dogs [16]. Captopril has also been reported to improve survival in the rat model [17]. The present study demonstrates for the first time that treatment with captopril results in a significant decrease in oxidative stress, as evidenced by an increase in the redox state ratio.

In our previous study, using this animal model, we characterized changes in antioxidants in relation to the hemodynamic function at different stages of heart failure. Based on the hemodynamic function and other clinical data, the animals at 1, 4, and 16 weeks of MI duration were classified to be in nonfailure, mild failure, and severe failure stages, respectively. The maintenance of antioxidant enzyme

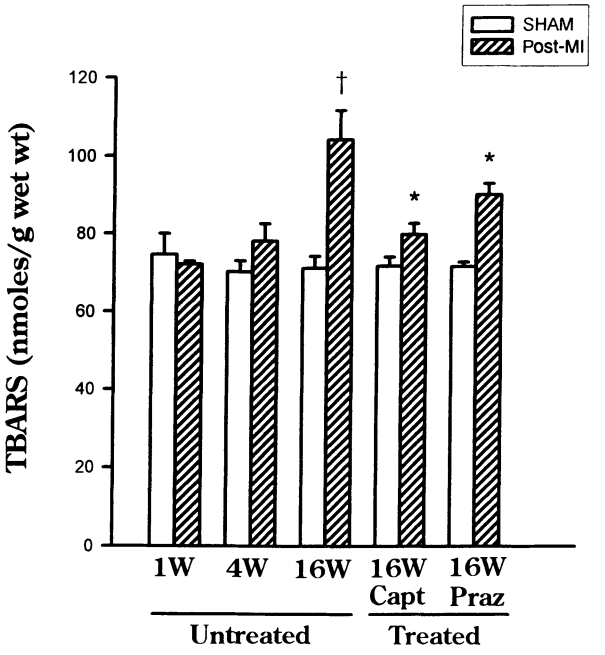


**Figure 3.** Lipid peroxidation as indicated by thiobarbituric acid reacting substances (TBARS) in sham and infarcted post-MI hearts at 1-, 4-, and 16-weeks postsurgery durations without treatment and at 16-weeks with captopril (Capt) or prazosin (Praz) treatment. Drug treatment was started at 4 weeks postsurgery duration as described in the Material and Methods section. Data are mean ± SE of 6–8 experiments. †Significantly different ($P < 0.05$) from respective sham as well as 1- and 4-week experimental groups by ANOVA. *Significantly different from the 16-week untreated post-MI group (ANOVA). (Taken with permission from the Journal of the American College of Cardiology, *20 (4)*, March 15, 1997.)

activity at 1 week and the decrease in activity at 4 and 16 weeks of MI duration suggested that an antioxidant deficit plays a role in the pathogenesis of heart failure subsequent to MI [9,10]. Afterload reduction by captopril or prazosin was accompanied by an improved hemodynamic function and better maintenance of myocardial endogenous antioxidant reserve at the severe failure stage [10]. In this study, improved redox ratio by these two drugs in the post-MI groups complements the previous findings of an improved antioxidant status in these hearts.

An increase in lipid peroxidation at the severe failure stage in this study provides further evidence of free radical involvement in heart failure. Measurement of TBARS as an indicator of lipid peroxidation has been criticized because of its nonspecificity [18]. However, when used in conjunction with the study of redox state as well as the antioxidant enzymes, it provides meaningful information. Increase in lipid peroxidation has also been reported in heart failure conditions in humans [19–22]. A strong correlation between lipid peroxidation and depressed cardiac

function has also been reported in other pathological conditions [2,23]. In the present study, we also report that treatment of animals with captopril results in decreased lipid peroxidation in the hearts at 16 weeks of MI duration. A decrease in breath pentane levels in CHF patients treated with captopril has also been reported [24]. Prazosin, another afterload reducing drug, also decreased lipid peroxidation at the severe failure stage. In addition to being a vasodilator, captopril also possesses antioxidant properties [25]. The exact mechanism by which captopril decreases oxidative stress is not known.

In most mammalian cells, glutathione is present in high concentrations as GSH (millimolar range) with minor fractions as GSSG. This tripeptide plays a crucial role in modulating cell damage resulting from reactive oxygen species as well as hydrogen peroxide [26]. In the heart, glutathione exists predominantly in the form of GSH. It acts as a cosubstrate of glutathione peroxidase for the detoxification of $H_2O_2$ and other organic peroxides [26]. Since superoxide dismutase and catalase are present in relatively low concentrations in the heart, the antioxidant role of glutathione is even more important in protecting the heart against oxidative stress injury [26–29]. In the present study, myocardial GSH content was significantly depressed at the severe failure stage and was accompanied by a significant increase in the GSSG content. Changes in tissue concentration of GSH have been reported in a variety of experimental models. In a chronic pressure overload model of heart failure, myocardial GSH content was significantly increased at the hypertrophy stage and significantly reduced at the failure stage [30]. Increase in oxidative stress was also evidenced by a decrease in the redox ratio [30]. Decrease in GSH levels in the hearts of adriamycin-treated animals [31] and diabetic rats [32] have also been reported. In our study, the redox ratio was significantly decreased in the 16-week post-MI group compared to the 1- and 4-week post-MI groups as well as their respective controls. After captopril or prazosin treatment, both the myocardial GSH content and the redox ratio were improved. The role of increased oxidative stress in the pathogenesis of heart failure from a chronic pressure overload has been established by using antioxidant treatment [30]. Since vasodilation is a common effect of both captopril and prazosin, these beneficial effects on the oxidative stress changes may be secondary to the afterload reduction.

In conclusion, there is an increase in lipid peroxidation and a decrease in the redox state during the severe failure stage. Modulation of these changes by captopril and prazosin treatments, as well as improved hemodynamic function, indicates that heart failure subsequent to MI is associated with an increase in oxidative stress.

**ACKNOWLEDGMENTS**

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# ROLE OF ANGIOTENSIN IN ANGIOGENESIS AND CARDIAC FIBROSIS IN HEART FAILURE

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***Summary.*** Cardiac hypertrophy is associated with the accumulation of extracellular matrix (ECM), cardiac fibrosis, and abnormal diastolic stiffness. Intra-coronary fibrosis in the hypertrophied myocardium can lead to occlusion of a major coronary artery with resulting ischemia. Angiotensin (Ang) II is associated with cardiac hypertrophy and fibrosis as well as angiogenesis, but the mechanisms are unknown. Tissue levels of angiotensin are elevated in the ischemic and nonischemic regions of the failing heart, and there is evidence that Ang II contributes to myocardial cellular hypertrophy and cardiac fibrosis. Also, Ang II may contribute to angiogenesis in the infarcted heart, thereby increasing collateral circulation. Angiotensin-converting enzyme inhibitor (ACEI) treatment opposes hypertrophy and ischemic heart failure. The mechanisms responsible for this cardioreparative effect of ACEI are largely unknown, but may due to inhibition of Ang II formation as well as other mechanisms. In the infarcted and noninfarcted ischemic heart, interstitial matrix metalloproteinases (MMPs) are activated. Recent experiments suggest that ACEI improves cardiac function and reduces myocardial ischemic damage, in part, by inhibiting MMP activity.

## ROLE OF ANGIOTENSIN II IN CARDIAC HYPERTROPHY

Increased levels of the octapeptide angiotensin (Ang) II have been associated with the pathological states of the heart [1–3]. However, the role of Ang II in the pathophysiology of cardiomyopathy has not been fully elucidated. Several in vivo

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studies suggest that Ang II may be a critical factor in mediating cardiac hypertrophy [4]. In vitro studies have also shown that mechanical stretch releases Ang II from cardiac myocytes and that Ang II mediates, in part, the stretch-induced hypertrophic response [5]. Locally produced Ang II may therefore act as an endogenous growth factor for myocardial cells [6].

The mechanisms by which Ang II mediates cardiac hypertrophy are not fully understood. However, Ang II is believed to stimulate early genes in cardiac myocytes and nonmyocyte cells, leading to hypertrophy and mitogenesis [7]. The induction of immediate early genes alters posttranslational processing of the preexisting gene transcription factors through cellular second messenger systems; these systems then influence the expression of late genes, particularly those which regulate the extracellular matrix (ECM) components.

The receptor systems involved in Ang II activity are $AT_1$ and $AT_2$ receptors. The $AT_1$ receptor is primarily involved in the regulation of structural ECM component genes [8]. The precise role of the $AT_2$ receptor is still not well understood, but some studies suggest that the $AT_2$ receptor may be involved in cellular proliferation and cell division [9]. Collectively, multiple studies have suggested a physiological role for Ang II in cardiovascular remodeling.

To compensate for the myocardial damage after ischemic injury, the entire heart undergoes hypertrophy [10,11]. However, the cardiac hypertrophy also can lead to increased wall stress, interstitial fibrosis, diastolic dysfunction, and increased risk for myocardial infarction (MI). Coronary vessels may become stiffer because of perivascular fibrosis, and this may increase the risk for coronary occlusion [12–15], resulting in the development of transmural MI and heart failure [16].

During the compensatory hypertrophic response, the myocytes and fibroblasts function normally. As an adaptive response to normalize wall stress and compensate for an increased load, cardiac muscle cells proliferate and increase in size, and there are changes in extracellular matrix that result in a remodeling of the entire ventricular chamber [17]. Remodeling, by its very nature, implies synthesis and degradation of ECM including interstitial fibrillar collagen. Accumulation of collagen leads to fibrosis. An abnormal accumulation of collagen is present within the interstitium and adventitia of intramyocardial coronary arterioles under hypertrophic conditions [18]. This perivascular and interstitial fibrosis may be responsible for abnormal myocardial stiffness in hypertrophied hearts [19–24]. We have previously demonstrated that Ang II induced collagen formation and reduced MMP in cardiac fibroblasts [25].

We have also demonstrated that chronic infusion of Ang II increases vascular permeability and consequently increases interstitial volume and edema [26]. This may also cause accumulation of ECM and cardiac fibrosis.

Experimentally induced renal artery stenosis (two-kidney, one clip [2K1C] Goldblatt hypertension) yields an increase in arterial pressure that is associated with an increase in Ang II, and these two changes may act synergistically to induce the cardiac and renal structural changes associated with renal stenosis [27–29].

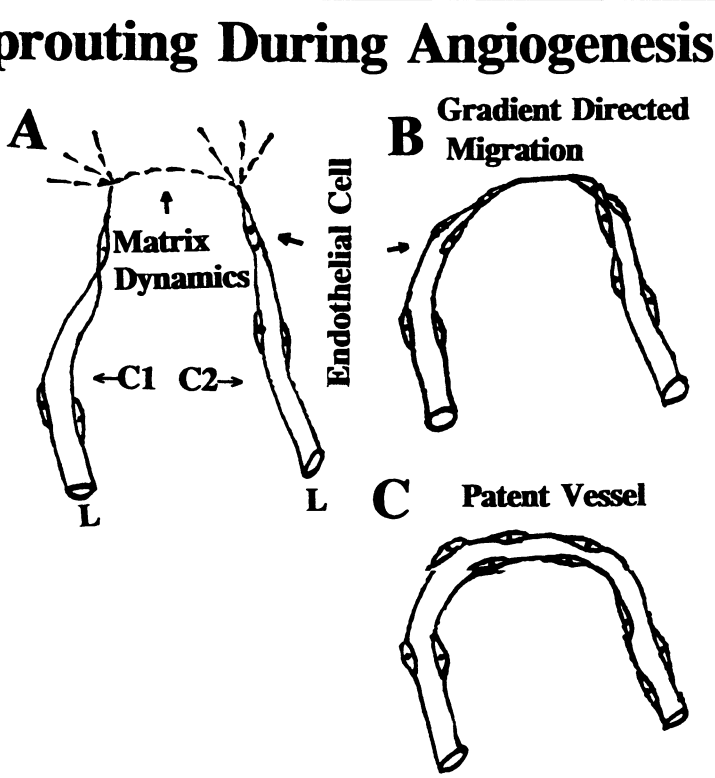


**Figure 1.** Hypothetical role of matrical pathways in the sprouting of capillaries (C1, C2) of blood vessels. *A*, migratory endothelial cells at tips of adjacent sprouts create a connecting pathway of aligned ECM (matrical pathway) as a consequence of a traction-mediated two-center effect; *B*, migratory endothelial cells approach one another via the gradient-directed pathway (ECM chemosensory); and *C*, The vascular loop is completed as endothelial cells meet, adhere, and interact to form a patent lumen (L).

## ANGIOTENSIN II AND ANGIOGENESIS

The vasculogenesis (formation of new vasculature or enlargement of the preexisting vessels) and angiogenesis (development of capillaries) are the structural processes by which new vessels are generated by sprouting from existing blood vessels (figure 1) [30,31]. Angiogenesis is not only necessary for normal embryonic development and postnatal growth but also occurs in adult life in a variety of physiological and pathological conditions. Transient, regulated growth of new capillaries occurs during formation of the corpus luteum, wound healing, bone fracture repair, and collateral development following myocardial ischemia [32,33]. In response to gradual occlusion of a major coronary artery, new collateral vessels develop to provide an alternate pathway for blood supply to the ischemic myocardium [34].

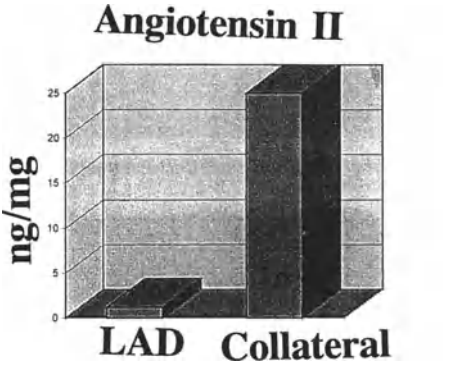


**Figure 2.** Representative experiment showing the level of angiotensin II in the coronary collateral vessels as compared to normal left anterior descending artery (LAD) [35]: Angiotensin was measured as described [6].

We evaluated specific mechanisms involved in vasculogensis and angiogenesis utilizing a canine model of coronary artery occlusion, which consisted of gradual closure of the left circumflex (LCx) artery by an ameroid constrictor [35]. The canine heart responds to chronic coronary occlusion by development of numerous collateral arteries easily identified on the epicardial surface as tortuous vessels extending from the unoccluded to the occluded (collateral-dependent myocardial region) arteries [34,35]. Elevated levels of Ang II were observed in the coronary collateral vessels as compared to left anterior descending (LAD) artery (figure 2). Also, in the ischemic heart, Ang II levels are elevated [36].

Other investigators have also demonstrated that increased activity of the renin-angiotensin system is correlated with the development of collateral formation after renal ischemia [37]. Furthermore, in a study of a chick embryo model of angiogenesis, infusion of Ang II for 14 days after gestation resulted in an increased number of new blood vessels in the chorioallantoic membrane as compared to the chorioallantoic membrane of control saline-infused chick embryos [38]. This study clearly demonstrated that Ang II plays a significant role in angiogenesis in this model. These authors also found that neither blockade of $AT_1$ nor the $AT_2$ receptors prevented this Ang II-induced angiogenesis. However, a prostaglandin synthesis inhibitor reduced Ang II-mediated angiogenesis [38]. Prostaglandin is synthesized by cycloxygenase-dependent pathway and oxidative cell-membrane metabolites. Collectively, these studies suggested that an antioxidant-sensitive pathway may be involved in the Ang II-induced angiogenesis [37,38], particularly at the site of ischemic injury, since reactive oxygen intermediates are involved in the intracellular signal transduction of Ang II-mediated cellular metabolic function [39].

Cellular proliferation and division are the prerequisite steps in Ang II-induced angiogenesis. Proliferative cells produce ECM when the tissue is restructured.

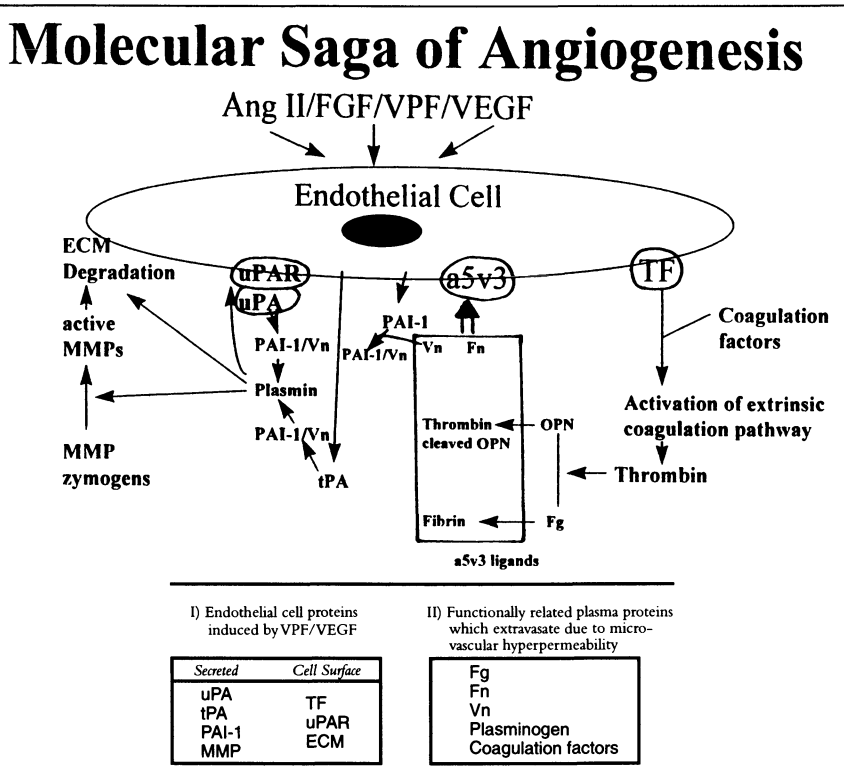


**Figure 3.** Functional relationship between the ECM proteins induced in endothelial cells by angiotensin or VEGF and proteins that extravasate from blood plasma as a consequence of microvascular hyperpermeability suggest the hypothesis that multiple and complex interactions between these two protein populations are fundamental to the mechanism by which angiotensin and other cytokines promotes angiogenesis. Ang II, angiotensin II; Fg, fibrinogen; Fn, fibronectin; MMP, matrix metalloproteinase; uPA, urokinase plasminogen activator; Vn, vitronectin; PAI, plasminogen activator inhibitor.

Iruela-Arispe et al. [40] demonstrated that collagen, ECM, and growth factors are actively synthesized by endothelial cells undergoing angiogenesis in vitro. Also, factors that increase vascular hyperpermeability induce angiogenesis (figure 3). Ang II induces vascular hyperpermeability in vivo, and this effect may contribute to angiogenesis and ECM formation [26].

Increased ECM may also enhance the storage of angiogenic growth factors such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and vascular permeability factor (VPF) [81]. All these factors function in conjunction with ECM to enhance angiogenesis in the tissue [41–44]. A bolus injection of an angiogenic factor, VEGF, has been shown to improve collateral blood supply to the myocardium at ischemic risk [45]. This may be indirectly related to the production of Ang II by VEGF in cardiovascular cells. Potentially, altered ECM produced in

the intima of collateral arteries is the result of a coordinated expression of the angiogenic factor(s) involved in development of these vessels and Ang II, which may promote production of growth factors [46]. It would be of great interest to evaluate the role of Ang II in vivo models of angiogenesis and vasculogenesis.

## ACTIVATION OF MATRIX METALLOPROTEINASES IN THE INFARCTED AND NONINFARCTED ENDOCARDIUM OF THE FAILING HEART

Occlusion of a major coronary artery impairs nutrient supplies to a specific region of the heart and can result in cardiac myocyte death [47]. The anatomic impact of a large infarct is evident by myocardial wall thinning and ventricle dilatation [48]. Focal ischemia leads to inflammatory cell proteolytic and oxidative responses, endothelial damage, and myocyte necrosis and apoptosis [49–51].

Hemodynamic adaptations occur in an infarcted heart that increase left ventricular filling pressure [52]. Although chamber dilation may be compensatory for the maintenance of stroke volume [53], chronic elevation of diastolic pressure and increased wall stress may induce structural changes that are detrimental in the long-term and may further impair myocardial energetics and cardiac performance. Despite the structural and functional alterations, the ejection fraction often decreases in direct proportion to the amount of damaged myocardium [54], leading to speculation as to the adaptive nature of these physiological events [55].

During the compensatory response, the noninfarcted and infarcted portions of the ventricle undergo remodeling, and increased ventricular stress causes myocardial cell hypertrophy and proliferation. At the site of infarction, the connective tissue matrix continues to be disrupted, and the wall becomes thinned, whereas the noninfarcted area undergoes hypertrophy. The physiological mechanisms of this dual remodeling (i.e., hypertrophic remodeling response in the noninfarcted area and myocardial wall thinning and dilatation in the infarcted area) in the same ischemic heart are not known.

Cardiac work requires energy [56] and the proper structural geometry of the heart muscle [57–60]. A great deal of information is available on the role of energetics in myocardial function [56,61,62]. However, the structural basis of cardiac function and dysfunction remains to be elucidated. Normally, cardiac myocytes are aligned in a proper three-dimensional arrangement by the ECM, in particular, the interstitial type I fibrillar collagen, connected with its receptor integrins and with its intracellular myofilamental cytoskeletal proteins [63]. Remodeling is associated with synthesis and disruption of ECM components [10]. Along with others [50], we [64] have demonstrated disruption of ECM, particularly interstitial collagen, at the site of infarction and at the site of the thin wall. This disruption in ECM components contributes to myocyte misalignment and heart failure.

Extracellular MMPs are involved in the disruptive process of ECM components. We have demonstrated that in the normal heart, the majority of MMPs are present in the latent, inactive form [65]. However, in the infarcted and noninfarcted heart,

MMPs are activated [66]. In the normal heart, we observed constitutive expression of a basal level of a 66kD MMP-2, gelatinase A. However, in the noninfarcted heart, the expression of gelatinase A was significantly increased [64,66]. At the site of infarction, gelatinase A and gelatinase B, MMP of 92kD, as well as interstitial collagenase, MMP-1, were induced [51,64,66].

We previously observed that in end-stage heart failure, the majority of MMPs originate from myo-fibroblast-like cells [51]. The mechanism of this differential expression of MMPs at the site of infarction and noninfarction in heart failure are not fully understood.

In dilated failing human hearts, secondary to previous MI or dilated cardiomyopathy (DCM), MMP activity is increased [66], especially within the endocardium of the infarcted and noninfarcted portions of either ventricle with MI and in both ventricles in DCM. This suggests that an activation of collagenase throughout the myocardium may contribute to ECM remodeling and to heart failure.

To examine whether the MMP activation was due to gene and/or posttranslational modification, we analyzed tissue from 10 hearts explanted because of coronary heart disease (CHD) and normal left atrial tissue from 5 donor hearts [64]. Our data suggests that gelatinase B (92kDa) is induced in heart failure. Moreover, the results suggest that tissue plasminogen activator (tPA) converts plasminogen to plasmin which, in turn, activates MMPs and inactivates the tissue inhibitor of metalloproteinase (TIMP) post-translationally following ischemic cardiomyopathy and heart failure [64].

## TREATMENT OF HEART FAILURE WITH ANGIOTENSIN-CONVERTING ENZYME INHIBITORS

Every year approximately 400,000 new cases of heart failure occur, resulting in 900,000 hospitalizations. More than 2 million American are affected with heart failure. The five-year mortality of heart failure is still about 50% [67,68].

Treatment with ACEI has been shown to improve survival of patients affected with congestive heart failure [69–71]. When patients were treated with ACEI after an acute MI without symptoms of heart failure, left ventricular (LV) enlargement was delayed and a significant reduction in mortality resulted [72]. ACEI may protect against myocardial failure partly by lowering blood pressure [73,74]. Himeno et al. [75] demonstrated that quinapril lowered both blood pressure and fibronectin expression. Richer et al. [76] found that quinapril treatment reduced the heart weight to body weight ratio. These studies collectively suggested that ACEI may modulate myocardial remodeling and altered ECM expression [75–77].

The mechanisms by which ACEIs ameliorate deterioration of LV structure and function remain to be determined. One possible explanation is that ACEI blunts structural ventricular remodeling partly through hemodynamic improvement (reduction of afterload) as well as by blocking local Ang II formation and subsequent activation of other paracrine growth factor systems. Stimulation of the renin-

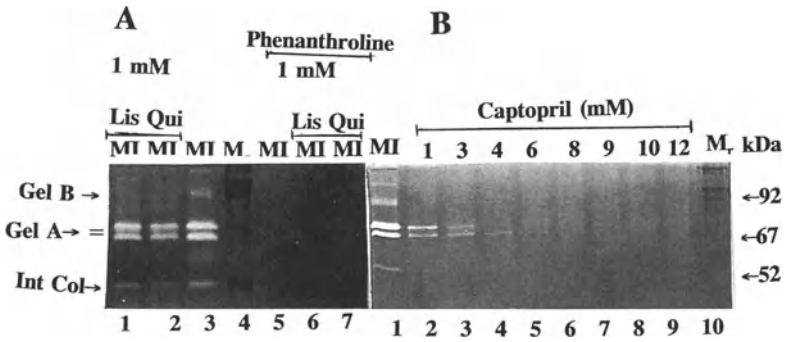


**Figure 4.** Zymographic analysis of human myocardial infarcted (MI) heart extract: Fifty micrograms of MI extract was incubated with 1 mM of lisinopril and quinapril (*A*) and various concentrations of captopril (*B*). The SDS-PAGE-zymography was performed as described [82]. *A*, lane 1, MI extracted, incubated with 1 mM lisinopril prior to loading on to the gel; lane 2, MI extracted, incubated with quinapril; lane 3, MI extract alone; lane 4, molecular weight standard; lanes 5–7 same as 1–3 except samples in lanes 5–7 were incubated with phenathroline. *B*, lane 1, MI extract; lanes 2–12, MI extract incubated with 1, 3, 4, 6, 8, 9, 10, 12 mM concentration of captopril, respectively. Lane 10, molecular weight standard.

angiotensin system following an initiating event (i.e., MI) may lead to structural remodeling of the heart with progressive LV enlargement and impairment of the contractile function [53,54]. A net balance between collagen synthesis and degradation is required for cardiac remodeling. MMPs may be responsible for collagen degradation and myocardial wall thinning [64]. In vitro MMPs are inhibited by converting enzyme inhibitors (figures 4 and 5). Gelatinase A is constitutively expressed during remodeling process [78]. However, MMP-1 and gelatinase B are inducible enzymes and are elevated following MI [64,66,79,80].

Figures 4 and 5 demonstrate that captopril, lisinopril, and quinapril at 30 mg/kg concentration (1 mM) significantly inhibited MMP-1 and gelatinase B. However, gelatinase A was partially inhibited. These results suggested that under pathological conditions, ACEI reduces MMP activity and, therefore, inhibits ECM disruption, which may be important in helping to restore cardiac structure and function and in preventing further progression of heart failure.

## CONCLUSION

Final resolution of the mechanism by which ACE inhibitors ameliorate cardiac failure will require additional experimentation. In vivo comparative studies with other drugs that decrease afterload and mechanical stress on the heart and investigation of multiple doses of converting enzyme inhibitors that cause substantial MMP inhibition will be necessary to understand the mechanism by which ACE inhibition helps to prevent adverse ECM remodeling and heart failure. These studies are in progress. If we can understand the differences in the dual remodeling process that takes place in noninfarcted and infarcted region of the heart, it might then be

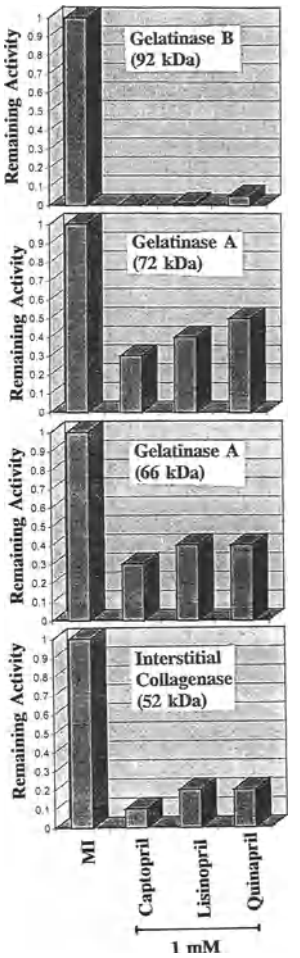


**Figure 5.** Histographic presentation of the scanned data of zymographic analysis of MI extract incubated with 1 mM (30 mg/kg) of captopril, lisinopril, and quinapril, respectively.

possible to develop therapeutic interventions to reverse or halt the process of cardiac wall thinning, dilatation, and, ultimately, heart failure.

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# ROLE OF ANGIOTENSIN II IN MYOCARDIAL ISCHEMIA/ REPERFUSION INJURY

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***Summary.*** Treatment with angiotensin-converting enzyme inhibitors has now become front-line therapy in the chronic phase following myocardial infarction. In addition to its role in the long-term consequences of myocardial infarction, recent evidence suggests that the cardiac renin-angiotensin system is also activated during acute myocardial infarction. This phenomenon might contribute to tissue injury induced by ischemia-reperfusion. In fact, Ang II has been shown to exert several actions that could be detrimental in this setting. Ang II is a potent vasoconstrictor and might thus exacerbate ischemia. Activation of Ang II receptors is also coupled to stimulation of protein kinase C, which can induce tissue injury in cardiac myocytes by altering cytoplasmatic calcium levels and kinases activity. In addition, evidence has accumulated that Ang II might also promote neutrophil activation and adhesion, major mechanisms of tissue injury during postischemic reperfusion. Several studies have focused on the role of the renin-angiotensin system in different models of acute ischemia, such as myocardial stunning and prolonged ischemia, with or without reperfusion. Results obtained are conflicting, but taken together, the available evidence strongly suggests that activation of the renin-angiotensin system may contribute to the pathophysiological consequences of myocardial ischemia/reperfusion. This issue warrants further investigation, aimed at better understanding the mechanisms of this phenomenon and at identifying the most appropriate and clinically useful therapeutical approaches.

It has long been appreciated that activation of the renin-angiotensin system plays an important role in hypertension and heart failure [1,2]. More recently it has been proposed that this system might also have important pathophysiological conse-

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quences in ischemic heart disease. Treatment with angiotensin-converting enzyme (ACE) inhibitors in the chronic phase following myocardial infarction has been shown to reduce left ventricular remodeling and infarct expansion and improve survival in experimental [3] and clinical studies [4–6]. In addition to its role in the long-term consequences of myocardial infarction, evidence has accumulated indicating that the cardiac renin-angiotensin system may also become activated during acute myocardial ischemia [7,8].

It is now appreciated that the heart is capable of synthetizing angiotensinogen and transforming it into angiotensin (Ang) I and then into Ang II [9–11]. Renin and angiotensinogen mRNA are expressed in rat hearts [12], and myocardial Ang II production has been observed in nephrectomized rats, in which plasma Ang II is not detectable [13]. Direct demonstration of myocardial production of Ang II comes from the observation that when Ang I is infused in isolated rat hearts, Ang II is released in the coronary sinus [9,12]. The presence of ACE in the myocardium has been demonstrated by radioactive-binding of enalapril [14]; however, other pathways of Ang II production exist within the heart. One is represented by a chymotrypsin-like protease (chymase) contained in mastcells [15]; another source is cathepsin G present in neutrophils [16]. Importantly, the activity of these enzymes is not affected by ACE inhibitors [7,11,17]. Thus, ACE inhibitors might have little influence on local Ang II formation in the heart. Both experimental [7,8] and clinical [18] studies have shown that acute myocardial ischemia is accompanied by activation of the renin-angiotensin system, and formation of Ang II might have several deleterious consequences on ischemic tissue. Thus, it has been hypothesized that local formation of Ang II might contribute to tissue injury associated with acute myocardial infarction [19,20].

## POTENTIAL MECHANISM OF ANGIOTENSIN II TOXICITY IN ISCHEMIC HEARTS

Ang II may exert several actions that could be detrimental in the setting of acute myocardial ischemia (figure 1). It is a potent vasoconstricting agent [8,21]; therefore, it could conceivably exacerbate ischemia during coronary artery occlusion and increase myocardial oxygen demand because of the increase in afterload. Angiotensin II also facilitates the activity of the sympathetic nervous system [22]. The consequent increase in norepinephrine release may result in arrhythmias and increase oxygen demand [9,22]. In addition, activation of Ang II receptors of cardiac myocytes and other cell types is coupled to stimulation of phospholipase C [23–25], with resulting increased formation of diacylglycerol and inositol triphosphate. Stimulation of this pathway might lead both to toxic effects secondary to increased cytoplasmic calcium concentrations and to changes in the activity of protein kinases [26]. Finally, very recent studies suggest that Ang II, like other agents that stimulate protein kinase [27], might also promote neutrophil recruitment and activation, with consequent release of proinflammatory substances (e.g., PAF, oxygen radicals, lytic enzymes) in the ischemic territory. This hypothesis stems from the observation that Ang II and III stimulate chemotaxis of neutrophils [28,29] and that it can enhance

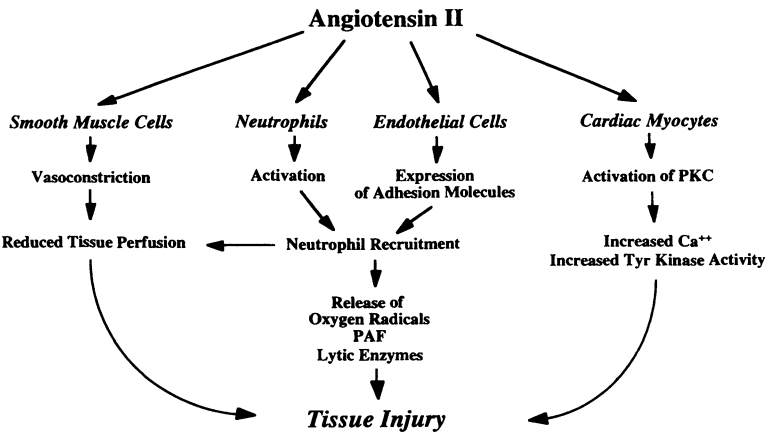


**Figure 1.** Diagram of possible mechanisms by which increased Angiotensin II levels may potentiate tissue injury in the setting of acute myocardial ischemia.

the expression on cardiac endothelial cells of E-selectin, a protein that promotes adhesion of neutrophils [30]. These various effects might have important consequences. On the one hand, neutrophil adhesion and activation is now recognized as a major mechanism of tissue injury during acute myocardial ischemia and reperfusion [31–36]. On the other hand, the effects of vascular plugging by adhering neutrophils combined with the effects of coronary vasoconstriction may also impair restoration of tissue perfusion upon recanalization [31,32,35,37], thus blunting the benefits of timely thrombolytic therapy in patients with acute myocardial infarction.

## EFFECTS OF ACE INHIBITION ON MYOCARDIAL ISCHEMIC INJURY

These various observations strongly suggest that interventions aimed at reducing Ang II formation may exert a protective effect on acutely ischemic hearts, and several studies have addressed this issue. Earlier studies were conducted in the setting of permanent coronary artery occlusion (table 1). A protective effect of captopril treatment was first reported by Erl et al. [38], who administered captopril to open-chest anestethized dogs with a fixed coronary artery occlusion and showed a reduction in infarct size after 6 hours. Other studies subsequently confirmed this finding in anestethized cats and rats, with the use of captopril or other ACE inhibitors, such as enalapril and ramiprilat (table 1). However, these beneficial effects could not be reproduced when experiments were performed under more physiological conditions, i.e., in conscious dogs subjected to 24 hours of coronary artery occlusion (table 1). One explanation for this discrepancy is that the use of anesthesia might have influenced treatment outcome, since barbiturate anesthesia per se stimulates renin release and potentiates the effects of ACE inhibitors [39,40]. Thus, while ACE inhibition might have been effective in preventing Ang II formation in

**Table 1.** Effect of ACE inhibitors during permanent coronary occlusion

| Author | Experimental model | Duration of CO | Drug | Protection |
|---|---|---|---|---|
| Ertl et al. | dog, anestethized | 6h | captopril | yes |
| Liang et al. | dog, conscious | 24h | captopril | no |
| Lefer et al. | cat, anestethized | 5h | enalapril | yes |
| Daniell et al. | dog, conscious | 24h | captopril | no |
| Hock et al. | rat, anestethized | 48h | enalapril | yes |
| Martorana et al. | dog, anestethized | 6h | ramipril | yes |

Note: CO = coronary occlusion.

**Table 2.** Effect of ACE inhibitors on myocardial stunning

| Author | Drug | Protection |
|---|---|---|
| Przyklenk et al. | enalapril | yes |
| Westlin et al. | captopril | yes |
| Westlin et al. | enalapril | no |
| Przyklenk et al. | zofenopril | yes |
| Przyklenk et al. | enalapril | yes |
| Bittar et al. | captopril | yes |

Note: All studies were conducted on dogs subjected to 15 minutes of coronary occlusion followed by 3 hours of reflow.

anesthetized animals, most Ang II generation in conscious animals might have occurred via non-ACE mechanisms. Therefore, ACE inhibitors might have been unable to block Ang production, despite adequacy of dosage and of administration scheme. In addition, in positive studies, infarct size was typically measured after only 6 hours of ischemia. Therefore, it is possible that the drug could have simply delayed the onset of necrosis, without inducing permanent myocardial salvage, as it has previously been shown for other interventions aimed at reducing infarct size [41,42].

## EFFECTS OF ACE INHIBITION ON MYOCARDIAL STUNNING

Other studies have examined the effects of ACE inhibition in the setting of coronary artery occlusion followed by reperfusion. It is known that brief ischemic episodes, insufficient to induce myocardial necrosis, may nevertheless result in myocardial stunning [43]. Several studies have been conducted in the classical model of myocardial stunning, i.e., dogs with 15 min coronary occlusion, and almost all of them have shown a protective effect of various ACE inhibitors against contractile dysfunction (table 2). This effect has been attributed to alterations in systemic hemodynamic parameters and to increased myocardial blood flow. An additional beneficial role in this situation is probably played by the SH- group present in the molecule of many of these drugs. Presence of SH- groups confers antioxidant properties, and generation of oxygen radicals plays a major role in the pathogenesis of myocardial stunning, as shown by the fact that administration of antioxidants is

**Table 3.** Effect of ACE inhibitors on ischemia/reperfusion

| Author | Species | Exp. protocol | Drug | Protection |
|---|---|---|---|---|
| Hock et al. | rat | 10m CO + 48h reflow | enalapril | yes |
| de Graeff et al. | pig | 1h CO + 2h reflow | captopril | yes |
| de Graeff et al. | pig | 1h CO + 14 days reflow | captopril | yes |
| Brown et al. | dog | 3h CO + 3h reflow | enalapril | no |
| Tio et al. | pig | 45m CO + 14 days reflow | zofenopril | no |
| Noda et al. | dog | 90m CO + 3h reflow | captopril | yes |
| Tobe et al. | pig | 45m CO + 14 days reflow | perindopril | no |
| Hartman et al. | rabbit | 30m CO + 2h reflow | ramipril | yes |
| de Lorgeril et al. | dog | 2h CO + 6h reflow | captopril | yes* |

Note: CO = coronary artery occlusion. *only in a subgroup with high collateral flow.

beneficial in preventing contractile dysfunction [44]. In accordance with this hypothesis, different investigators have consistently shown protection by captopril and zofenopril [45,46], whereas enalapril (which does not contain an SH- group) was either ineffective [46] or its protective effects were largely reversed by indomethacin, thus suggesting they were due to a prostaglandin-mediated mechanism, and not to antioxidant properties [45].

## EFFECTS OF ACE INHIBITION ON MYOCARDIAL ISCHEMIA/REPERFUSION INJURY

With the advent of thrombolytic drugs, emphasis on clinical management of patients with acute myocardial infarction has shifted to the condition of prolonged coronary artery occlusion followed by reperfusion. Thus, experimental studies have also focused on the use of ACE inhibitors in models of postischemic reperfusion after periods of coronary occlusion prolonged enough to induce myocyte necrosis (table 3). It has been shown that, irrespective of treatment, mortality is higher in animals with high baseline renin levels, thus confirming the important role of the renin-angiotensin system in the deleterious events that accompany acute myocardial ischemia [47].

Several studies have shown that ACE inhibition may exert positive results in this setting. These beneficial results have been attributed to various effects of these drugs, including increase in collateral blood flow to the ischemic region, blunting of sympathetic nervous system activation, inhibition of bradykinin breakdown and scavenging of oxygen radicals [20]. As already pointed out, inhibition of ACE activity can also reduce bradykinin breakdown, since the proteolytic activity of ACE extends to catabolize bradykinin. Increased bradykinin levels would result in increased myocardial glucose uptake and increased formation of nitric oxide and prostacyclin, which in turn would induce vasodilation and inhibition of platelet aggregation. These various effects might conceivably protect the heart during acute ischemia/reperfusion. Thus, administration of ACE inhibitors might be protective via a dual mechanism of reduced Ang II level and increased concentration of bradykinin. Interestingly, it has been reported that the reduction in experimental

infarct size observed with ramiprilat was independent of ACE inhibition, and it was instead secondary to an increase in myocardial levels of bradykinin [48,49].

In spite of several positive studies, other investigators were not able to document reduction in infarct size in reperfused hearts through use of ACE inhibitors [3,50,51] (table 3). Several factors might have contributed to these discrepancies. As in the permanent occlusion studies, barbiturate anesthesia per se might have stimulated renin release and potentiated the effects of ACE inhibitors in some studies [39,40]. Anti-oxidant properties of certain ACE inhibitors containing SH- groups might also have contributed to the protective effects seen with some compounds (e.g., captopril), but not with other drugs, since it is known that postischemic reperfusion is accompanied by generation of a large amount of oxygen radicals that can induce tissue injury [52].

Another explanation for the apparent divergent results of various studies relates the benefit of ACE inhibitors to the degree of myocardial ischemia during the period of occlusion. In this respect, de Lorgeril and coworkers noted that captopril was protective in a subgroup of dogs with very low collateral flow during occlusion (i.e., when ischemia was severe), while it apparently worsened myocardial injury in animals with high collateral flow [50]. This finding raises the issue that enhanced bradykinin activity by ACE inhibition, with its attendant effect on vasodilation and on nitric oxide production, may not necessarily be regarded as beneficial under severe ischemia, because of either induction of "coronary steal" during ischemia [50] or toxic effect of increased nitric oxide production during reflow [53]. However, another, perhaps, more compelling argument to explain these controversial findings is that although elevated concentrations of Ang II may be detrimental, these increased levels are responding poorly to inhibition of ACE.

## RATIONALE FOR TESTING ANGIOTENSIN II RECEPTOR ANTAGONISTS ON ISCHEMIA/REPERFUSION INJURY

As already mentioned, alternative enzymatic pathways of Ang II production within the heart may be represented by chymase and cathepsin G of mastcells and neutrophils [15,16], the activity of which is not affected by ACE inhibitors [7,11,17]. It should be pointed out that Ang II formation through alternate pathways might be particularly relevant in reperfused hearts, when activated neutrophils accumulate in large amounts within the previously ischemic tissue and release the content of their granules [54]. This might explain the often disappointing results obtained with ACE inhibitors in reperfused hearts, since these drugs might have little influence on cardiac Ang II formation in this setting. Recently, the availability of Ang II receptor antagonists has provided scientists with a powerful tool to directly investigate the role of the renin-angiotensin system in many pathophysiological conditions, independently of the route of formation. Since they act at the receptor level, these antagonists could also provide a more efficient tool to prevent Ang II effects. In addition, blocking the $AT_1$ receptor should also result in increased stimulation of the $AT_2$ subtype [55], which in turn may induce the synthesis and release of local kinin [55,56].

In reperfused tissues, neutrophils may be linked to Ang II in a more complex fashion, in addition to their being a potential source of Ang II. Neutrophil recruitment and activation in postischemic hearts are accompanied by release of several mediators, such as oxygen free radicals, platelet-activating factor, leukotrienes, and proteolytic enzymes, which can all contribute to tissue damage, either directly or secondary to "inflammatory" amplification of injury (figure 1) [54]. Since experimental observations suggest that Ang II can promote neutrophil chemotaxis and adhesion [28–30], it can be hypothesized that in reperfused hearts, neutrophils might contribute to local Ang II production, which in turn would induce further recruitment and activation of neutrophils. This would result in amplification of Ang II production and neutrophil-mediated injury. Administration of Ang II receptor antagonist, but not ACE inhibitors, might effectively hinder this mechanism.

In this respect, recent studies have investigated the effects of losartan, an angiotensin II $AT_1$ receptor antagonist, on myocardial ischemic injury. In rats with permanent coronary artery occlusion, losartan administration did not influence infarct size, but it significantly improved cardiac function and angiogenesis and attenuated myocardial hypertrophy [57]. In isolated rat hearts, losartan treatment also improved functional recovery after ischemia and reperfusion [58].

In light of the observations linking Ang II and neutrophil activation [27–30], we have evaluated the effects of losartan in an experimental model aimed at investigating the role of neutrophils in myocardial ischemia/reperfusion injury. In this model, developed and validated in our laboratory [17,35], isolated rat hearts were subjected to 20 minutes of ischemia; at the onset of reflow, hearts were perfused for 5 min with neutrophils and plasma and then with standard perfusion buffer for an additional 40 min. Under these experimental conditions, losartan significantly improved recovery of contractile function and reduced myocardial neutrophil infiltration, at concentrations similar to those achieved clinically [59]. If confirmed in subsequent in vivo studies, these findings would indicate that effective inhibition of Ang II stimulation, through $AT_1$ receptor blockade, might reduce myocardial inflammatory injury by counteracting some deleterious effects resulting from neutrophil activation.

## CONCLUSION

Taken together, the evidence available strongly suggests that activation of the renin-angiotensin system may contribute to the pathophysiological consequences of myocardial ischemia/reperfusion. This issue warrants further investigation, aimed at better understanding the mechanisms of this phenomenon and at identifying the most appropriate and clinically useful therapeutical approach.

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# EFFECTS OF ANGIOTENSIN II RECEPTOR ANTAGONIST ON CARDIAC REMODELING IN CARDIOMYOPATHIC HAMSTER HEARTS

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***Summary.*** Recent reports have shown that angiotensin-converting enzyme inhibitors have a role in cardiac remodeling and have beneficial effects on congestive heart failure. Several studies have described alterations in sarcoplasmic reticulum gene expression in the failing heart. These results indicate that calcium homeostasis in myocytes may be disturbed in congestive heart failure. The present study examines the effects of long-term treatments with the angiotensin-converting enzyme inhibitor Enalapril and Ang II subtype1 receptor antagonist TCV-116 on the morphological changes in the extracellular matrix, progressive left ventricular dysfunction in cardiomyopathic hamsters. Between age 5–20 weeks, 24 BIO53.58 hamsters (model of dilated cardiomyopathy) received 10 mg/kg per day orally either of TCV-116 or no treatment. Between age 5–30 weeks, 24 BIO53.58 hamsters (model of dilated cardiomyopathy) received 20 mg/kg per day orally either of Enalapril or no treatment. During the study period, cardiac function was assessed by echocardiography in a noninvasive manner. At 20 or 30 weeks of age, each heart was fixed with 10% formalin, embedded in paraffin, and serial sections were stained with Gomori's aldehyde fuchsin using the Masson-Goldner method. High framerate ultrasonoscopic echocardiograms revealed that the left ventricular percent fractional shortening (%FS) tended to improve in the Enalapril group (22 $\pm$ 4% vs 20 $\pm$ 3%) and TCV-116 group (24 $\pm$ 4% vs 21 $\pm$ 4%). The fibrous tissue volume significantly decreased in Enalapril group (25.2 $\pm$ 0.5 $mm^3$, $P < 0.05$) compared with the untreated group (27.6 $\pm$ 2.3 $mm^3$). TCV-116 did not significantly decrease the fibrous tissue volume. Enalapril can prevent cardiac remodeling, but TCV-116 is not as effective as Enalapril. Enalapril may nevertheless, suppress fibrosis.

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## INTRODUCTION

The accumulation of fibrillar collagen in the cardiac interstitium is one of the major morphological features of left ventricular hypertrophy accompanied by genetic hypertension, acquired hypertension, and myocardial infarction. This morphological change is called structural remodeling, and may account for the abnormal ventricular function that eventually leads to congestive heart failure. Several lines of evidence suggest that both circulating and tissue renin-angiotensin systems may be involved in the remodeling of the myocardium. Receptors for Ang II, aldosterone, endothelins, and bradykinin have been identified in the heart. The heart also has the capacity to generate Ang II, bradykinin, and endothelin. Locally generated hormones therefore could alter tissue structure in an autocrine and/or paracrine manner. Hormones such as Ang II, endothelin, and aldosterone, serve as stimulators to enhance collagen formation and reduce collagenolytic activity. Hormones such as bradykinin, prostaglandins, and glucocorticoids, serve as inhibitors to counterbalance the influence of these stimulators. Treatment with only a low dose of angoiotensin-converting enzyme inhibitor (ACEI), without subsequent lowering of blood pressure, causes a decrease in left ventricular hypertrophy [1]. Treatment with the ACEI lisinopril, has been shown to reverse interstitial collagen accumulation in spontaneously hypertensive rats with established left ventricular hypertrophy [2]. These results suggest that Ang II acts as a growth factor for myocytes and other cells in the heart. Ang II may affect ventricular remodeling by acting as a growth factor, as previously suggested, thus promoting myocyte hypertrophy [3]. The increased DNA synthesis generally found in interstitial cells following myocardial infarction was found to be inhibited by an ACEI via a mechanism independent of its effect on afterload change [4]. Other research suggests that Ang II may directly affect the collagen and connective tissue architecture [5]. Cardiac fibroblasts are predominantly responsible for the synthesis of fibrillar type I and III collagen [6]. Excessive production of collagen fibers may impair systolic and diastolic function. Ang II is known to stimulate collagen synthesis and bradykinin inhibits its influence. Several clinical [7,8] and experimental [2,9] studies have demonstrated that ACEI suppresses cardiac fibrosis.

The purpose of the present study is as follows: 1) to evaluate the effects of long-term treatments with the ACEI Enalapril, and Ang II receptor antagonist TCV-116 on the morphological changes in the extracellular matrix and progressive left ventricular dysfunction in an animal model of dilated cardiomyopathy, and 2) to investigate whether there is any difference between the effects of Enalapril and TCV-116.

## MATERIALS AND METHOD

### Experimental animals

The BIO53.58 strain of cardiomyopathic golden Syrian hamsters develops abnormalities of the cardiac and skeletal muscles that are inherited as an autosomal recessive trait [10]. Between 4–20 weeks of age, BIO53.58 hamsters gradually

develop cardiac dilation that is accompanied by diffuse cell death. This strain also has a significantly shorter life span, and demonstrates reduced cardiac function at an earlier age than the hypertrophic cardiomyopathic hamster (BIO14.6 hamster) [10,11].

In contrast to the BIO14.6 hamster, BIO53.58 hamsters do not develop myolysis or hypertrophy before dilation [12]. Therefore, the BIO53.58 hamster provides a good model of cardiac dilation and congestive heart failure. Experiments were carried out using 48 male, dilated cardiomyopathic hamsters (BIO53.58), aged 5 weeks (BIO Breeders, Fitchburg, MA, USA). Male F1b hamsters (n = 48), a non-cardiomyopathic F1 hybrid of BIO1.5 and BIO87.2 hamsters, were used as controls. BIO53.58 hamsters were randomly assigned to one of three groups, receiving either Enalapril (20 mg/kg per day po, BANYU Pharmaceutical Co., Ltd.), TCV-116 (10 mg/kg per day po, TAKEDA Pharmaceutical Co., Ltd.), or no treatment. The study period was 15 weeks.

### Echocardiography

During the study period, we performed transthoracic echocardiography on each hamster under uletan anesthesia (0.5 mg/g body mass intraperitoneal injection) with an ultrasound system (Hitachi EUB565A), using a 7.5 MHz sector scanner. We recorded M-Mode echocardiograms at chorda level, measured the left ventricular diastolic dimension and left ventricular systolic dimension using the conventional "leading edge" method [13], and calculated percent fractional shortening (%FS) as the percent differance between the left ventricular diastolic (LVDd) and systolic dimensions (LVDs); $\%FS = 100 \times (LVDd\text{-}LVDs)/LVDd$.

### Histological analysis

*Tissue preparations*

Hamsters from each group were also used for histological analysis. The ventricles and atria were excised from each heart and the blood was carefully washed out with saline. Hearts were fixed with 10% formaldehyde and embedded in paraffin after dehydration through a graded alcohol series, and were sectioned transversely using a microtome (Leitz Wetzlar: 33776) from the atria to the apex in 8 μm serial sections. The sections were stained with Gomori's aldehyde fuchsin using the Masson-Goldner method and periodic acid shiff (PAS)-hematoxylin for light microscopy.

*Volumetry*

Cardiac tissue volumes were determined by a point counting method [14,15]. The sections were picked up at 400 μm intervals from serial sections, and enlarged to 44× with a light microscope projector on a sheet of paper which had regular triangle lattice of points spaced 20 mm from the nearest neighbors. At such a magnification, each point is 0.45 mm apart in the sections and represents a hexagonal area of $0.45\,mm \times 0.45\,mm \times \sqrt{3}/2\,mm^2$. The numbers of points lying in the

myocytes, nonmyocytes, calcified, or fibrotic area projected on the paper were counted. The volume (V) was obtained from the sum of the points, representative area (a) for one point and the sectional interval being:

$$V(mm^3) = \Sigma(\text{point} \times a \times 0.4mm)$$

The volume of ventricles (VV) was largely divided into the volume of myocytes (MV) and the other space (NMV):

$$VV(mm^3) = MV(mm^3) + NMV(mm^3)$$

In the present study, because the volume of nonmyocytes was negligibly small compared with the volume of fibrous tissue and calcified lesions, NMV was considered as the volume of fibrous tissue and calcified lesions. VV, MV, NMY, and NMV to VV ratio (= NMV/VV × 100) were calculated.

*Histometry*

The section which had the largest diameter of the left ventricle was selected from the serial sections to measure myocyte breadth and to count nuclear density.

*Myocyte breadth*

Short diameters of myocytes were measured using an eyepiece micrometer with an 1 μm scale at a magnification of 1000×. Lines which ran transversely on the left ventricular wall were selected from the anterior, lateral, posterior, and septal wall. The myocyte diameters were measured along each line from epicardium to endocardium. The mean value was used as a representative for each specimen.

*Nuclear density*

Numbers of the myocyte nuclei were counted in 16 randomly selected fields from the left ventricular myocytes space through an eyepiece with a 250 μm square micrometer at a magnification of 400× under the light microscope. The nuclei which seemed to be degenerated were not counted. Numbers of nuclei calculated per square millimeters were used as density of viable myocytes.

Hamsters from each group were also used for histological analysis. The ventricles and atria were excised from each heart and the blood was carefully washed out with saline. Hearts were fixed with 10% formaldehyde and embedded in paraffin after dehydration through a graded alcohol series, and were sectioned transversely using a microtome (Leitz Wetzlar: 33776) from the atria to the apex in 8 μm serial sections. The sections were stained with Gomori's aldehyde fuchsin using the Masson-Goldner method. At intervals of 200 μm, sections were observed using a projector (Neo Vision: 102S) with ×33.3 magnification. The area of myocyte, fibrous tissue and vessels were calculated by the point-counting method and the volume of myocyte occupying region, nonmyocyte region, and total myocardium (summation of myocyte occupying region and nonmyocyte region) of the ventricles were estimated.

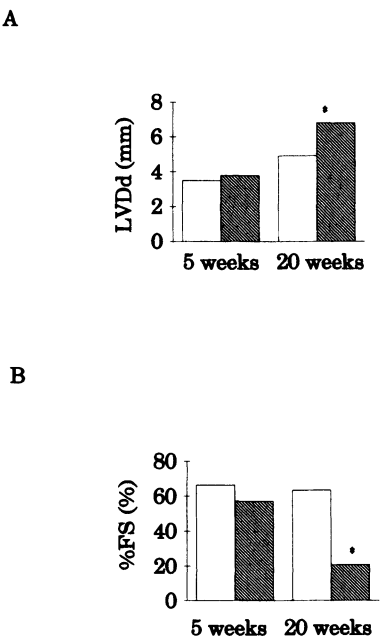


**Figure 1.** *A*) Changes in left ventricular diastolic dimension (LVDd). Left ventricular diastolic dimension in BIO53.58 (hatched bar) was measured by M-mode high frame rate ultrasonoscopic echocardiography. Values are expressed as mean, $*P < 0.05$ compared with age-matched F1b hamsters (open bar); *B*) Changes in percent fractional shortening (%FS). Percent fractional shortening was calculated by M-mode high frame rate ultrasonoscopic echocardiography. Values are expressed as mean $*P < 0.05$ compared with age-matched F1b hamsters.

## STATISTICAL ANALYSIS

Values are given as mean ± SD. Comparisons between 2 groups were performed with unpaired Student's t-test or Mann-Whitney U-test when t-test is inappropriate. $P < 0.05$ was considered the limit of significance [16].

### Effects of TCV-116 or Enalapril treatment on cardia function

As shown in figure 1A, the left ventricular diastolic dimension was significantly ($P < 0.05$) enlarged in cardiomyopathic hamsters (6.8 ± 0.4 mm) at 20 weeks of age as compared with age-matched F1b hamsters (4.9 ± 0.4 mm). The percent fractional shortening of the left ventricles of BIO53.58 hamsters was significantly decreased at 5 weeks of age (57.3 ± 7.9%vs. 66.5 ± 5.9%; $P < 0.05$) and decreased further at 20 weeks of age (20.8 ± 4.0%vs. 63.3 ± 5.8%; $P < 0.01$) as compared with age-matched F1b hamsters (figure 1B).

The left ventricular diastolic dimension decreased significantly in the Enalapril group (6.1 ± 0.4 mm, $P < 0.05$) but not in TCV-116 compared with the no treatment group (6.8 ± 0.4 mm) (figure 2A).

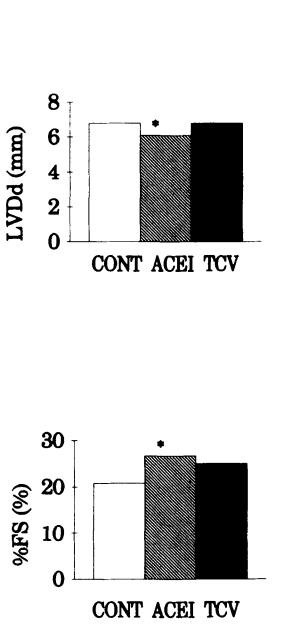


**Figure 2.** *A*) Changes in left ventricular diastolic dimension (LVDd) of the no treatment (CONT, open bar), Enalapril (ACEI, hatched bar), and TCV 116 (TCV, solid bar) treatment groups of cardiomyopathic hamsters BIO53.58. Values are expressed as mean, $*P < 0.05$ compared with the no treatment group; *B*) Changes in percent fractional shortening (%FS) of the no treatment (CONT, open bar), Enalapril (ACEI, hatched bar), and TCV 116 (TCV, solid bar) treatment groups of cardiomyopathic hamsters BIO53.58. Values are expressed as mean, $*P < 0.05$ compared with the no treatment group.

Left ventricular percent fractional shortening increased in the Enalapril group (26.7 ± 4.6%, $P < 0.05$) and tended to increase in TCV-116 compared with the no treatment group (20.8 ± 4.0%) (figure 2B).

### Effects of TCV-116 or Enalapril treatment on cardiac structure

The area of necrosis, fibrosis, and calcification were decreased in both the Enalapril and TCV-116 groups. The left ventricular wall remained thick in both the Enalapril and TCV-116 groups compared with the thinning ventricular wall of the no treatment group.

Total ventricular volume tended to increase in the Enalapril group. Fibrous tissue volume tended to decrease in the Enalapril group, but not significantly. At 25 weeks of treatment, it decreased significantly in the Enalapril group (25.17 ± 0.48 $mm^3$, $P < 0.05$) compared with the no treatment group (27.64 ± 2.34 $mm^3$) (figure 3).

Histological findings indicated that the Enalapril group was improved compared with the no treatment group. The area of fibrosis was decreased. Total ventricle volumes were calculated by semi-serial section of right and left ventricles. As

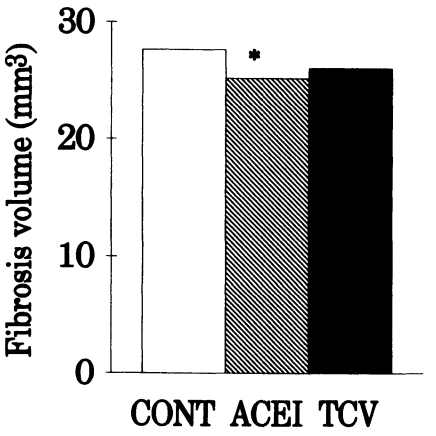


**Figure 3.** Fibrous tissue volume of cardiomyopathic hamsters (BIO53.58) of no treatment (CONT, pen bar), Enalapril (ACEI), and TCV 116 (VC, solid bar) group. Values are expressed as mean. *P $< 0.05$ compared with no treatment group.

mentioned above, ventricular weight of the Enalapril group decreased. Figure 8 indicates the relative tissue volume normalized by the mean value of the untreated group. Total ventricle volume tended to decrease in the Enalapril group. Ventricular myocyte volume (total ventricle volume-nonmyocyte volume) also tended to decrease in the Enalapril group. Total ventricle and ventricular myocyte volume tended to increase in the TCV-116 group. Nonmyocyte volume significantly decreased in the Enalapril group ($25.2 \pm 0.5\,mm^3$, $P < 0.05$) compared with the no treatment group ($27.6 \pm 2.3\,mm^3$). Nonmyocyte volume tended to decrease in the TCV-116 group, it was not significant. Myocyte size was smaller and myocytes were more concentrated in both of Enalapril and TCV-116 groups than in the no treatment group.

## DISCUSSION

Both Enalapril and TCV-116 tended to improve cardiac function but did not prevent cardiac dilation.

The total amount of collagen increase in BIO53.58 hamsters correlated with the pathological progression of fibrosis. The increase of collagen gives ventricles stiffness and impairs the cardiac diastolic function. Furthermore, it is known that Ang II stimulates collagen synthesis in cultured cardiac fibroblasts [17]. The 15-week treatment with ACEI may not have been enough time to change collagen metabolism, especially the degradation of collagen, even if Ang II-induced collagen synthesis was inhibited. In our preliminary experiments, Enalapril treatment for 25 weeks significantly suppressed the fibrosis of ventricles in cardiomyopathic hamsters compared with the no treatment group. A recent clinical study reported

that ACEI caused regression of cardiac hypertrophy. In addition, the remarkable effectiveness of ACEI in preventing heart failure and mounting evidence for additional cardioprotective effects of drugs related to the renin-angiotensin system have promoted an intense interest in the cardiac tissue renin-angiotensin system and its role in both normal and diseased hearts. Therefore, the tissue renin-angiotensin system may be implicated in cardiac hypertrophy and other cardiac disorders in man.

Fifteen week treatment with Enalapril improved cardiac function and prevented cardiac dilation, but prevention of fibrosis was not significant. The 15-week treatment with ACEI may not have been enough time to change collagen metabolism, especially the degradation of collagen, even if Ang II-induced collagen synthesis was inhibited. In our preliminary experiments, Enalapril treatment for 25 weeks significantly suppressed the fibrosis of ventricles in cardiomyopathic hamsters compared with the no treatment group. The total amount of collagen increase in BIO53.58 hamsters correlated with the pathological progression of fibrosis. The increase of collagen gives ventricles stiffness and impairs the cardiac diastolic function. Furthermore, it is known that Ang II stimulates collagen synthesis in cultured cardiac fibroblasts [17]. A recent clinical study reported that ACEI caused regression of cardiac hypertrophy. In addition, the remarkable effectiveness of ACEI in preventing heart failure and mounting evidence for additional cardioprotective effects of drugs related to the renin-angiotensin system have promoted an intense interest in the cardiac tissue renin-angiotensin system and its role in both normal and diseased hearts. Therefore, the tissue renin-angiotensin system may be implicated in cardiac hypertrophy and other cardiac disorders in man.

In the present study, Enalapril tended to inhibit myocyte cell death and cell growth, and significantly improved cardiac fibrosis in cardiomyopathic hamsters. Our data demonstrated the mild increase in the volume of myocytes space and significant decrease in fibrosis in the Enalapril group. Enalapril significantly decreased ventricular weight compared with the no treatment group. It seems that the decrease in ventricular weight is largely due to the reduction of fibrosis. In our preliminal experiments, calcium antagonist such as manidipin-HCl and amlodipine significantly inhibited cell death by ameliorating calcium overload of myocytes. Major effects of Enalapril are thought to suppress the fibrosis by inhibiting the production of Ang II. A recent clinical study demonstrated that ACEI improve cardiac function and suppress cardiac dilation in chronic heart failure [18]. Even in acute myocardial infarction, early treatment with ACEIs improve cardiac remodeling. Our results support the clinical study, and have proved that ACEI suppresses the development of fibrosis in heart failure.

## CONCLUSIONS

Enalapril significantly suppressed fibrosis and improved cardiac function in the cardiomyopathic hamster. TCV-116 was not so effective on prevention of fibrosis as Enalapril. It seemed that the longer period of treatment with Enalapril is the most

effective on prevention of fibrosis and not only repression of Ang II but also potentiation of bradykinin is important for prevention of the cardiac remodeling.

These data suggest that the major effect of Enalapril is the suppression of fibrosis.

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