The Local Cardiac Renin Angiotensin-Aldosterone System

EDITED BY
EDWARD D. FROHLICH
RICHARD N. RE

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THE LOCAL CARDIAC RENIN ANGIOTENSIN-ALDOSTERONE SYSTEM

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THE LOCAL CARDIAC RENIN ANGIOTENSIN-ALDOSTERONE SYSTEM

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PREFACE

How exciting it is to see a field so well established as the renin-angiotensin system continue to grow and mature. Originally, following the original identification of renin by Tigerstedt and Bergman over 100 years ago, workers in this area spent years attempting to establish its role in experimental and renal hypertension. The early work by Goldblatt, in 1934, demonstrated that the placement of a clip around a renal artery was clearly related to the subsequent development of hypertension. However, it wasn't until the simultaneous finding by two different geographically separated teams, Page, et al, in the United States and Braun-Menendez, et al, in Argentina that the peptide angiotensin was identified. Thus, the rate-limiting enzyme renin was released from the kidney and catalyzed a biochemical cascade which was eventually shown to produce the elevated arterial pressure. Subsequently, many workers contributed to the elucidation of the concept and sequence of angiotensin II generation. Thus, the enzyme renin acted upon its protein substrate, produced in the liver, to liberate the decapeptide angiotensin I which, upon circulating through the pulmonary circulation, finally produced the potent octapeptide angiotensin. Several important subsequent findings demonstrated that angiotensin II promoted the release of the adrenal corticosteroid from that gland, thereby resulting in a larger system, the renin-angiotensin-aldosterone system. Further, this system demonstrated a classical biofeedback and the circulating octapeptide was shown to have additional biological activities in organs other than heart, vessels, kidney, adrenals, and even brain. Indeed, the story became exceedingly complex in a rapidly moving field.

One new dimension to this fascinating story appeared with the demonstration of the existence of local renin-angiotensin systems. Indeed, a number of organs were shown to be the source of each component of this renin-angiotensin system. Controversy still exists as to whether the rate-limiting enzyme renin was produced in each of those organs with putative local systems. Initially, renin was demonstrated to be produced in ovary, but at the present time the question as to whether renin is produced in heart and arteries remains unsettled. Suffice it to say, each of the other components of the renin-angiotensin system has been shown to be produced locally in heart and vasculature. Moreover, there is evidence that even the adrenal steroid aldosterone is produced locally in the heart.

Complicating things even further is the role of the renin-angiotensin-aldosterone system in heart and arterioles. Evidence continues to grow that not only is angiotensin II generated in the cardiac myocyte, but the peptide has important independent biological cardiac actions as well through its autocrine-paracrine regulation of other hormonal and growth factors. As a result, there are direct effects of angiotensin on the extracellular matrix and perivascularly - to promote fibrosis and inflammatory responses. Furthermore, these local actions also affect the cardiac myocyte and are responsible for hypertrophic, apoptotic, and other responses.

Because of the rapid growth of this area, we organized a series of workshops at our institution with the purpose of bringing together the active workers concerned with the local effects of the cardiac renin-angiotensin-aldosterone system. The first of these workshops was in 2002 at the Ochsner Clinic Foundation. It was a resounding success and the proceedings were promptly published in the Journal of Molecular and Cellular Cardiology. Subsequently, the participants of that meeting urged us to organize a second meeting which was held in November 2004 at our institution. The proceedings of that meeting are the substance of this monograph published with the assistance of our colleague from Paris, Bernard Swynghedauw.

Clearly the success of our meetings must be attributed to the investigators from around the world who provided the impetus for us to meet and to discuss at length this rapidly changing field. We also have to express our grateful appreciation to our pharmaceutical partners who share with us a deep-seated interest in the local cardiovascular renin-angiotensin-aldosterone system; to these colleagues from AstraZeneca and Novartis we extend our warm and hearty thanks. And, finally, we want to express our enthusiastic and very warm and heartfelt appreciation to Lillian Buffa, Caramia Fairchild, and Joan Patterson. These creative and hard-working women not only support our clinical and administrative responsibilities, but also worked diligently to see to it that all of the submitted papers were properly rendered camera-ready for the rapid publication of these second proceedings. None of the following material would have appeared in these proceedings without their support.

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Chapter 1
HYPERTENSIVE HEART DISEASE: TIME FOR NEW PARADIGMS

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At the Ochsner Clinic Foundation's first workshop on the cardiac renin-angiotensin-aldosterone system about two years ago, I emphasized that left ventricular hypertrophy (LVH) has been a useful clinical marker of increased cardiovascular risk for over 40 years (1,2), yet our concept of LVH and its risk was far from complete. Over the preceding decades, this clinical marker of risk was established with increasingly more refined diagnostic methods (first chest roentgenology, then electrocardiography and then echocardiography). Each technique established earlier clinical recognition of LVH. However, the fundamental mechanisms underlying that risk were not developed although the underlying biological events of its development had been unraveling. They continue to be identified further today. Thus, at the time of that first workshop, we demonstrated that the biological risk factors associated with LVH could be ascribed to ventricular ischemia, fibrosis and probably, apoptosis (1-3). They remain the underlying pathophysiological alterations associated with LVH development, experimentally and clinically. Among other factors, inflammation is currently being explored; and, it is highly conceivable that other factors will also be identified and linked with several other co-morbid diseases that are associated with LVH in hypertension (e.g., atherosclerosis, diabetes mellitus, exogenous obesity). Yet, even these pathophysiological and clinical epiphenomena associated with LVH do not provide the full picture.

Our discussions of two years ago have provided a necessary impetus to establish a more comprehensive understanding of the fundamental biological risk mechanisms underlying LVH (4). We are

now at the beginning of an exciting and more comprehensive understanding of this important cardiovascular concern. One of the important biological mechanisms involves the local renin-angiotensin systems in heart, vessels, brain, kidney and other organs. The existence of the local cardiac system no longer is in doubt; and aldosterone has also been added to the story, but the existence of cardiogenic renin remains to be established with clearer certainty. Even though locally produced cardiac aldosterone seems to be a reality, how it is linked to LVH risk and disease remains to be elucidated.

Thus, clinical practicality of a local cardiac renin-angiotensin-aldosterone system (RAAS) is established. It can explain each of the pathophysiological alterations underlying myocytic hypertrophy, ischemia, fibrosis, apoptosis, and inflammatory responses. Although pressure and volume overload still remain important pathogenetic physiological events that participate in inducing myocardial hypertrophy (3), it is apparent that the foregoing epiphenomena provide important explanations for the adverse pathological events that can account for the inherent risk associated with LVH. Indeed, these pathological bio-endpoints seem to be mediated at the very least, in part, through locally produced angiotensin II and its "by-products" through autocrine, paracrine and, even, intracrine events. However, It would be premature to suggest that these RAAS events exclude interplay with other important biological factors.

In addition to the necessity for identifying the role of a local cardiac RAAS, it is necessary to develop additional intellectual and useful clinical paradigms. The biologist must continue to expand this new concept; and the clinician must be able to recognize its existence and participation with useful and unambiguous clinical markers. Efforts must continue to develop new and more specific biomarkers of the underlying mechanisms of risk associated with LVH. Techniques are already available to demonstrate and quantify ischemia, but measurement of coronary flow reserve is highly specialized and costly. Myocardial biopsy remains a very restricted investigative method. However, it now seems possible to relate clinically the extent of myocardial fibrosis to the measurement of its circulating collagen fragments which are directly related to the extent of extracellular fibrosis in hypertensive patients with LVH (5,6). This recent finding must be confirmed and developed further. Similarly, other methods must be developed to detect and quantify the extent of apoptosis clinically if we are to confirm the hypothesis that the extent of ventricular myocytic apoptosis could explain the high frequency of cardiac failure in patients with hypertension and LVH (7). And, further, more specific tissue

biomarkers are necessary to understand the role of inflammatory changes in hypertensive heart disease. Quantitative measurement of circulating C-reactive protein levels are neither specific for heart, vessels nor kidney; and they are as inappropriate for the 21st century as the erythrocyte sedimentation rate was for active disease in the latter decades of the 20th century.

Finally, with respect to the local cardiac RAAS, current thinking must be focused on innovative concepts to explain more clearly the pathogenesis of the underlying mechanisms of cardiovascular risk in hypertensive heart disease. For example, the consequences of salt-loading on the systemic endocrine RAAS are well-known to all physicians and investigators. Thus, as a result of salt-loading there is suppression of renin release from the juxtaglomerular apparatus of the kidney; and in some patients, arterial pressure increases. But, in this workshop, salt-loading also stimulates the local cardiac RAAS; and this cardiac effect seems to be independent of pressure elevation and volume expansion (8,9). In another presentation in this workshop, the duality of a local RAAS within the kidney also seems possible (10). How these findings will play out is, of course, the subject of future workshops.

Thus, I have emphasized that it is not sufficient today to consider LVH as a risk factor, per se. The clinical development and application of more accurate methods to demonstrate the participation of the underlying risk mechanisms of LVH remains exceedingly important if we are to understand clinical outcomes more comprehensively. It is therefore essential to obtain a clearer understanding of the associated epiphenomena of LVH and their participation in related co-morbid conditions if we are to identify risk much earlier in the disease and with greater sophistication. To this end, efforts are underway to identify highly specific biomarkers of these underlying pathophysiological factors. And, further, we must expand our thinking about the intra-organ RAAS and, perhaps, other intra-organ dual systems that may have opposing effects. On one hand there may be suppression of the endocrine RAAS while, on the other hand, stimulation of the local cardiac system produces adverse perivascular and interstitial fibrosis as well as specific alterations in function. We must elucidate the interactions of this local cardiac RAAS system with other intra-organ (i.e., cardiac) systems including the catecholamines, endothelin, the natriuretic peptides, oxytocin, growth factors, and, other yet to be identified factors. Although it was suggested four decades ago that the heart was an endocrine organ this suggested was concerned with the role of catecholamines (11). This concept now has far greater potential!

These fundamental considerations should not be restricted solely

to pathophysiological and biological considerations. There is greater need today for specific, yet clinically practical, considerations including cost-effective methods to demonstrate earlier risk from hypertensive heart disease. Is it really useful to spend tremendous amounts of funds to determine with present day clinical techniques (e.g., echocardiography) whether certain therapies may be more effective to diminish left ventricular mass or wall thicknesses? We know that all pharmacotherapies are effective to this end. But reduced cardiac mass and wall thickness is not synonymous with the reduced risk associated with LVH. Is it necessary to learn whether one agent reduces cardiac mass better than another when we are unable to demonstrate risk reversal with more precise and tissue specific biomarkers. Thus, the amount of funds spent today on multicenter trials that compare different drugs may very well be *passé*. After all, we do not know whether all agents even within one class are identical qualitatively or quantitatively in their mutual effects?

To these ends, this second workshop focuses on various biological aspects of the RAAS. Studies that are discussed concern the cardiac renin receptor, intracellular renin isoforms, transgenic studies designed to elucidate more clearly the RAAS, angiotensin receptors in heart and kidney, and the real existence of a reciprocal RAAS within kidney and other target organs of hypertensive disease. In addition, the intracellular role of the cardiac RAAS and other peptides in heart and in other hemotopoietic system remains to be defined more clearly. In this regard, we also consider the role of chymase and bradykinin. And, further, we also explore the role of the RAAS not only in hypertension but in atherosclerosis.

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Chapter 2
CARDIAC (PRO)RENIN RECEPTORS: FUNCTIONAL PROPERTIES AND POTENTIAL SIGNIFICANCE

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ABSTRACT

In many tissues, local angiotensin generation depends on uptake of circulating renin and/or prorenin. Such uptake involves both diffusion into the interstitial space and binding to (pro)renin receptors. This review describes the current status of cardiac (pro)renin receptors, and focusses on their potential significance. (Pro)renin receptors bind both renin and prorenin, and prorenin undergoes a conformational change ('non-proteolytic' activation) following binding, thereby allowing cell surface angiotensin generation with both renin and prorenin. Renin and prorenin binding also induced direct, angiotensin-independent effects (e.g., ERK1/2 activation), suggesting that renin and prorenin may act as agonists. Finally, under certain conditions binding resulted in internalization of renin and prorenin. Internalized renin and prorenin were either degraded or, in the case of unglycosylated prorenin, contributed to intracellular angiotensin generation. Taken together, (pro)renin receptors could provide an important new drug target, allowing selective interference with angiotensin production at tissues sites and/or direct (pro)renin-induced effects.

INTRODUCTION

The renin-angiotensin system (RAS) is classically described as a circulating regulatory system that plays a key role in controlling blood pressure, fluid balance and salt balance in mammals. Renin, an aspartyl protease, is

considered to have a unique function and an exclusive substrate: it cleaves angiotensinogen to generate angiotensin (Ang) I. Subsequently, the decapeptide Ang I is converted into the octapeptide Ang II, either by angiotensin-converting enzyme (ACE), a zinc metallopeptidase, or by chymase, a serine protease [1].

In addition to the circulating RAS, so-called "tissue RAS" or "local Ang II-generating systems" have been proposed, based on the observation that blockers of the RAS (ACE inhibitors and Ang II type 1 (AT_1) receptor antagonists) exert beneficial effects beyond their blood-pressure lowering effects (for review, see [2]). Tissue RAS appear to have a critical role in organ damage under pathological conditions such as hypertension and diabetes [3,4]. To allow local Ang II generation, either renin, angiotensinogen and ACE must be synthesized locally, or one or more of these components needs to be taken up from the circulation, e.g. through diffusion into the interstitial space [5] or via binding to receptors.

Particularly in the heart, most, if not all, renin is derived from the circulation [6-8]. Moreover, cardiac Ang I generation depends exclusively on renin, and without renin, neither Ang I nor Ang II can be detected in cardiac tissue [6,9,10].

Thus, it is important to delineate exactly how renin (and/or its inactive precursor, prorenin [11]) enters the heart. In the case of prorenin, its local activation mechanism should also be unraveled: through cleavage of the prosegment ('proteolytic' activation) or due to temporal unfolding of the prosegment ('non-proteolytic' activation). These issues became even more important when it was discovered that renin has direct cellular effects, independently of Ang II [12].

Currently, two (pro)renin receptors have been identified [13-16], and the existence of a third receptor has been proposed [17]. In addition, several "(pro)renin-binding proteins" ((P)RnBP) have been investigated, either in membranes prepared from rat tissues [18,19], or in intracellular compartments [20]. Of these (P)RnBPs, only the intracellular RnBP has been cloned and characterized [21]. Although it inhibits renin, it is also identical to the enzyme N-acyl-D-glucosamine 2 epimerase [22]. Mice lacking RnBp display normal blood pressure and plasma renin activity [23]. Therefore, it is unlikely that this intracellular RnBP is a determinant of renin activity and/or metabolism in vivo.

(PRO)RENIN RECEPTORS

The mannose-6-phosphate/insulin-like growth factor II receptor

The mannose-6-phosphate (M6P) receptor binds renin and prorenin with high affinity ($K_d \approx 1$ nM) in neonatal rat cardiac myocytes and fibroblasts [13,15] as well as in human endothelial cells [14,24]. This receptor is identical

to the insulin-like growth factor II (IGFII) receptor, and as such it contains binding domains for both IGFII and phosphomannosylated (M6P-containing) proteins like renin and prorenin [25]. It does not bind unglycosylated (pro)renin [15,24]. Following binding, both renin and prorenin are rapidly (within minutes) internalized, and internalized prorenin is proteolytically cleaved to renin [15,24].

(Pro)renin binding to M6P/IGFII receptors did not result in extra- or intracellular angiotensin generation [26], and (prorenin-derived) intracellular renin was found to be degraded slowly (within hours) [15,24]. Thus, M6P/IGFII receptors most likely serve as clearance receptors for both renin and prorenin, thereby determining the extracellular levels of (pro)renin. Alternatively, since binding of M6P-containing proteins to M6P/IGFII receptors results in activation of second messenger pathways in a G-protein-dependent manner [27,28], it is possible that renin and prorenin act as agonists for this receptor.

A receptor for unglycosylated (pro)rennin on adult rat cardiomyocytes

Rats transgenic for the mouse *ren-2^d* renin gene (coding for unglycosylated prorenin) are known to have extremely severe hypertension and cardiac damage [29,30]. Using this model, with an inducible expression of the *ren-2^d* renin gene restricted to the liver, Peters et al. [17] have found that increased synthesis of *ren-2^d* renin was associated not only with high circulating levels of *ren-2^d* prorenin but also with high cardiac levels of *ren-2^d* (pro)renin. Subsequent studies in isolated adult rat cardiomyocytes revealed that these cells internalized prorenin (both endogenous rat prorenin and mouse *ren-2^d* prorenin), and not (or very weakly) rat or mouse renin. Interestingly, only the internalization of mouse *ren-2^d* prorenin resulted in angiotensin generation, possibly because internalization induced a conformational change in mouse prorenin ('non-proteolytic' activation), thereby increasing its enzymatic activity from 0.7% to 3.3% [17]. The authors contributed the absence of angiotensin generation following internalization of rat prorenin to the difference in glycosylation between rat and mouse prorenin. Such a difference may determine the use of different pathways of internalization and/or different degrees of intracelllar activation of both proteins. These results revive the controversy on the existence of an intracrine RAS, and the mitogenic effect of intracellular Ang II [31-34].

A functional receptor specific for renin and prorenin

A functional receptor for renin was first identified on human mesangial

cells in culture [12]. Renin binding to this receptor increased ^{3}H-thymidine incorporation and plasminogen activator inhibitor-1 (PAI-1) synthesis. The receptor was subsequently cloned from an adult human kidney expression library (GenBank accession number AF 291814) [16]. It is a 350-amino-acid protein with a single transmembrane domain displaying no homology with any known membrane protein. The receptor was found to bind prorenin equally well (i.e., renin's active site is not involved in the binding process), and in contrast to the above described receptors, cell surface-bound renin and prorenin were neither internalized nor degraded.

Importantly, binding of renin to this receptor induced a fourfold increase of the catalytic efficiency of angiotensinogen conversion to Ang I, and receptor-bound prorenin became fully enzymatically active. These data are in complete agreement with the high efficiency of angiotensin generation in the cell surface, allowing Ang II to bind immediately to AT_1 receptors following its synthesis, without leaking into the extracellular space [26].

Furthermore, in the presence of the AT_1 receptor antagonist losartan, (pro)renin binding resulted in rapid activation of the MAP kinases ERK1 (p44)/ERK2 (p42), thereby demonstrating for the first time Ang II-independent effects of renin and prorenin.

Immunohistochemistry and *in situ* hybridization studies have localized the receptor in vascular smooth muscle cells in human heart and kidney, in glomerular mesangial cells and in distal and collecting tubular cells in the kidney ([16] and J.-M. Gasc and G. Nguyen, personal communication).

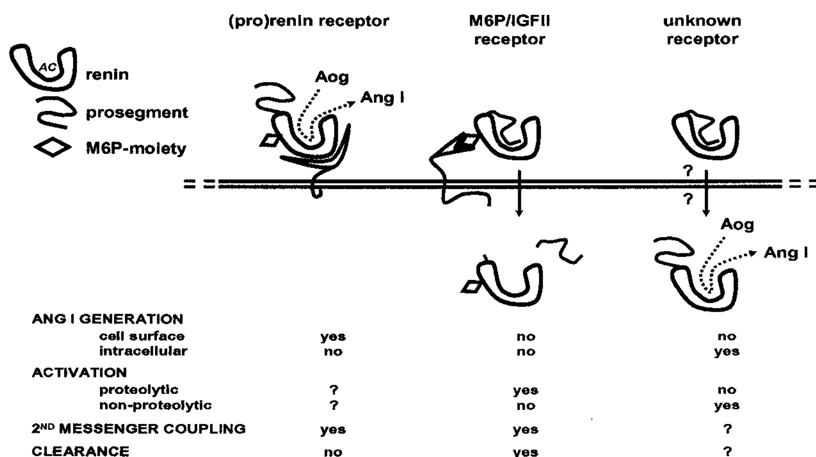
POTENTIAL SIGNIFICANCE OF (PRO)RENIN RECEPTORS

The physiological implications of (pro)renin receptors are important (Figure 1). They may be essential players at the level of the tissue RAS by:

- *Concentrating (and extracellularly activating) renin and prorenin on the cell surface.* This facilitates Ang II generation in the immediate proximity of AT_1 receptors, thus leading to efficient receptor activation with little or no loss of Ang II into the extracellular space. For instance, the localization of (pro)renin receptors on vascular smooth muscle cells of coronary and renal cortical arteries [16] indicates that the receptor may contribute to the control of vascular tone in heart and kidney. In cardiomyocytes, the receptor may underlie the efficient AT_1 receptor activation by Ang II generated on the cell surface following the addition of prorenin and angiotensinogen [26]. Finally, a recent study employing a decoy peptide (corresponding to the 'handle' region for non-proteolytic activation of prorenin) was found to inhibit diabetic nephropathy in streptozocotin-treated rats by lowering

renal angiotensin levels [35]. This finding strongly supports the idea that non-proteolytical activation of prorenin (e.g., through receptor binding) contributes to angiotensin generation at tissue sites.

- *Internalizing (and intracellularly activating) renin and prorenin.* Internalization may lead to intracellular degradation [15,24] or, under defined conditions (mouse *ren-2*^{d} prorenin in adult rat cardiomyocytes), to intracellular angiotensin generation. This phenomenon could underlie the severe, blood pressure-independent cardiac damage in transgenic rats expressing the mouse *ren-2*^{d} renin gene [36,37]. It may also determine the degree of extracellular angiotensin generation, through its influence on extracellular (pro)renin levels.
- *Allowing renin and prorenin to act as agonists.* Both the (pro)renin receptor and the M6P/IGFII receptor couple to second messenger systems [16,27,28], and (pro)renin binding to the (pro)renin receptor results in ERK1/2 activation, increased ^{3}H-thymidine incorporation and PAI-1 release [12,16]. This could be of particular importance under conditions where high renin and/or prorenin levels exist. For instance, direct renin-induced effects may underlie the MAP kinase-dependent renal damage in homozygous TGR(mRen2)27 rats [38].
- *Providing a functional role for prorenin.* In circulating blood plasma, prorenin represents 70-90% of total renin in normal subjects [39]. Prorenin levels rise even further under certain physiological (pregnancy) and pathological (diabetes) conditions [39-43], partly due to its synthesis at extrarenal sites, like ovary, testis and eye [43-45]. For many years, the consequences of this prorenin rise were unknown, as prorenin was considered to have no physiological role. Studies in transgenic animals [11,36,37] now suggest that prorenin may be more than the inactive precursor of renin, either because it contributes to angiotensin generation at tissue sites [11,35] or because it acts as an agonist in an angiotensin-independent manner [16]. Vitreous fluid in diabetic subjects with proliferative retinopathy contains high levels of prorenin [46], and interruption of the RAS can prevent retinal neovascularization [47]. The possibility that these high prorenin levels play a pathological role needs to be considered.



	(pro)renin receptor	M6P/IGFII receptor	unknown receptor
ANG I GENERATION			
cell surface	yes	no	no
intracellular	no	no	yes
ACTIVATION			
proteolytic	?	yes	no
non-proteolytic	?	no	yes
2ND MESSENGER COUPLING	yes	yes	?
CLEARANCE	no	yes	?

Figure 1. Current status of (pro)renin receptors, prorenin internalization and prorenin-induced effects. The (pro)renin receptor cloned by Nguyen et al. [16] facilitates cell surface angiotensin (Ang) generation from angiotensinogen (Aog), mannose 6-phosphate/insulin-like growth factor II (M6P/IGFII) receptor-induced internalisation of M6P-containing prorenin results in prorenin clearance [15,24], and an unknown mechanism allows non-glycosylated (i.e., non-M6P-containing) prorenin to internalize and to subsequently generate Ang I intracellularly [17]. AC, active center.

Overall, (pro)renin receptors could provide an important new drug target, allowing selective interference with angiotensin production and/or direct (pro)renin-induced effects at tissues sites. Their existence may have a high impact because the RAS and the renin receptor are not restricted to the cardiovascular area. Activation of tissue RAS is also involved in several physiological and pathological processes such as growth and development [48,49], inflammation [50], thrombosis [51], obesity [52], and learning and memory [53].

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Chapter 3
ON THE RELATIONSHIP BETWEEN THE RENIN RECEPTOR AND THE VACUOLAR PROTON-ATPASE MEMBRANE SECTOR-ASSOCIATED PROTEIN (M8-9)

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Angiotensin (Ang) II plays an important role in the regulation of blood pressure, volume homeostasis, salt balance (by triggering salt and water retention) and vasoconstriction. The formation of Ang II results from the initial cleavage of angiotensinogen by renin. It has been established that Ang II is produced in tissues such as the vasculature, the brain and the heart. Renin, which is mainly produced in the kidney (as prorenin), is also found in tissues where it may come from *de novo* synthesis or uptake. Although controversial, von Lutterotti *et al.* concluded that renin synthesis does not occur in the heart. Evidence of renin uptake by the heart is accumulating, though the mechanisms of this uptake remain unclear. Recently, a specific renin and prorenin receptor was identified in human tissues. Binding of this receptor to renin and prorenin resulted in increased cleavage of angiotensinogen and stimulation of a MAP kinase signaling pathway.

We report, here, over 95% amino acid sequence identity between the human renin receptor and a previously identified vacuolar proton-ATPase membrane sector-associated protein (M8-9). Protein homology was found in a variety of species including mouse, chicken and *C.elegans*. In addition, we have shown that, at the cDNA level, the mouse and the human nucleotide sequences are highly similar. Transcripts were found (using RT-PCR) in all adult tissues examined and in the kidney from E9.5dpc suggesting that the molecule is widely, if not

ubiquitously, expressed in the mouse. Our findings suggest that the renin receptor may also function as part of a vacuolar ATPase protein complex and that they are the product(s) of a single gene.

INTRODUCTION

The renin-angiotensin system (RAS) is an enzymatic cascade involved in the regulation of systemic blood pressure (BP) and maintenance of electrolyte homeostasis. The initial cleavage of angiotensinogen by renin in the circulation and the subsequent cleavage of angiotensin (Ang) I by the angiotensin-converting enzyme (ACE; on the luminal surface of the vascular endothelium) produces Ang II, the effector molecule of the RAS cascade. Ang II plays an important role in the regulation of BP, volume homeostasis, salt balance and tissue remodeling. The effects of Ang II are exerted via its receptors, AT_1 and AT_2, causing increased sodium and water retention in the distal renal tubule, increased aldosterone release from the adrenal gland and vasoconstriction.

It is now well established that the RAS is not just an endocrine (ie: circulating) system since Ang II can be produced locally in a number of tissues such as the vascular beds, the brain or the heart [1, 2, 3]. In addition, anephric patients have a plasma level of renin that does not reach zero and retain the ability to produce Ang II [4,5,6] suggesting that Ang II can be produced independently of renal renin production. Similar findings were obtained in animals which had undergone bilateral nephrectomy [7]. Secondly, recent reports have demonstrated that all the components of the RAS are present in some tissues suggesting the existence of a local paracrine or autocrine RAS. ACE mRNA was found in the heart, by autoradiography [8] as was angiotensinogen [9]. However, it is unclear whether cardiac renin comes from uptake or *de novo* synthesis. Renin is primarily synthesized as a pre-pro-hormone in the juxtaglomerular cells of the kidney. After removal of the signal peptide, prorenin can either be secreted from the Golgi by the constitutive pathway via clear secretory vesicles or packaged in immature granules where it is processed into mature renin. Renin is stored in dense core granules and released when needed by regulated exocytosis. Other sites including the reproductive organs, the adrenal and the pituitary [10] have been shown to produce prorenin but it is unclear whether prorenin produced is converted to renin in the circulation or taken up as prorenin for conversion.

Although controversial, von Lutterotti *et al.* (1994) concluded that renin synthesis does not occur in the heart under normal physiological conditions [11]. There is also evidence of prorenin uptake by the heart although the mechanisms remain unclear. Müller *et al.* (1998) showed that hearts isolated from transgenic rats overexpressing the human angiotensinogen gene and perfused with human renin, generated Ang II [12]. This continued after renin infusion was stopped, suggesting that renin was taken up and sequestered in cardiac tissues, and the effect was abolished by the administration of a specific human renin inhibitor. De Mello and Danser (2000) concluded that Ang II synthesis in the heart depends on renin and angiotensinogen being taken up by cardiac tissues [13]. Additional reports of prorenin uptake come from transgenic rat studies.

Several molecules have been identified as renin-binding molecules. The mannose-6-P receptors (M6Pr) are involved in the internalisation of prorenin in endothelial cells [14] and cardiac cells [15] leading to rapid conversion of prorenin to renin, proteolytically. The process is not unique to prorenin since M6Pr also bind to thyroglobulin. The renin-binding protein (RnBp) binds tightly and specifically to renin (as observed in kidney homogenates) and masks its protease activity. It was, however, shown to be identical to an epimerase [16] and is thought to be involved in modulation of the release of active renin from renin-producing cells [17]. In addition, RnBp null mouse mutants displayed no effects on renin (and other RAS components) or on BP [18].

Nguyen *et al.* (2002) [19] recently described the cloning of a human renin receptor that was found to be expressed in multiple tissues and was localised to the mesangium of glomeruli, the coronary artery and the vascular bed of the placenta. Transfection of the cDNA into human foetal mesangial cells (HMC) resulted in expression of a membrane-associated protein that specifically bound to renin and prorenin but not other aspartyl proteases. Binding resulted in a 5-fold increase in angiotensinogen cleavage (compared with renin or prorenin in solution), the rapid phosphorylation of the receptor and the activation of ERK1 and ERK2 (MAP kinases).

We report here sequence comparison of the human renin receptor with a vacuolar proton-ATPase membrane sector-associated protein (M8-9 [20]) in a variety of species including mouse, chicken and *C.elegans*. Our findings suggest that the renin receptor is conserved between these species. The protein identified as the renin receptor may also function as part of a vacuolar ATPase protein complex and is the product of a single gene. In addition, we studied the mouse homologue and found that at the cDNA and protein levels, mouse and human

sequences are highly similar. Transcripts were found (using RT-PCR) in all adult tissues examined and in the kidney from E9.5dpc indicating that the molecule is ubiquitously expressed in the mouse.

MATERIALS & METHODS

Cell lines: AS4.1 (ATCC no: CRL2193, a mouse kidney tumour cell line) and E14TG2a (ATCC no: CRL 1821, a mouse embryonic stem cell line), were obtained from the American Tissue Culture Collection and HMC (a human mesangial cell line) were a kind gift from Dr B. Banas (University of Regensburg, Germany).

AS 4.1 and HMC cells were grown in monolayers in DMEM/F12 supplemented with 10% foetal calf serum (FCS) and 2mM L-glutamine. E14TG2a were cultured in monolayer on gelatin coated-cell culture flasks in GMEM supplemented with 2mM L-Glutamine, 10% FCS, 100μM sodium pyruvate, 1% non-essential amino-acids, 100μM 2-mercapto-ethanol and 10^3 units of leukaemia inhibitory factor. All cells were passaged every three days with trypsin/EDTA.

Sequence Identity Analysis: A sequence identity search was conducted using BLAST (Basic Local Alignment Search Tool; http://www.ncbi.nlm.nih.gov/BLAST/) which is a set of similarity search programs designed to explore all of the available sequence databases with the human renin receptor cDNA clone, N14F [19] (GenBank: AF291814). The resulting mouse sequences were located in the mouse genome sequence using ENSEMBL (http://www.ensembl.org/Mus_musculus/). Vector NTI 5.3.0 was used to translate cDNAs into amino-acid sequence and ClustalW was used for sequence alignments [21]. Hydrophobicity prediction plots were obtained using TMHMM [22].

Tissue Collection: Mice were given free access to water and standard commercial mice chow containing 0.27% sodium and 0.395% chloride (CRM, Special Dietary Services, Witham, UK). The mice were purchased from Charles River (Margate, UK).

The breeding, maintenance and study of animals were performed according to Home Office regulations. The animals were paired up and the females examined for vaginal plugs the following morning. The time of the plug detection was termed E0.5. On the selected timepoint, the embryos were dissected out of the uterus rapidly under a dissecting

microscope. One placental cone was saved and treated as described below for adult tissues. Using fine forceps, the heart and lungs, liver and gut were removed to expose the dorsal part of the embryos' abdomen. Gender determination was done visually for embryos older than E15.5. The kidneys were then removed, placed in RNA later (Ambion, Texas, USA) and kept at 4°C until needed.

A similar technique was used to collect tissue from the mother such as heart, lungs, submandibular gland, liver, kidneys, adrenal glands, ovary, mesenteric fat, muscle and brain in that order. The adult tissues were also placed in RNA later and kept at 4°C until processed for RNA extraction.

RNA Preparations: A range of 60mg to 100mg of tissue was used with the exception of embryonic kidneys, adrenal glands and ovaries where the amount of tissue was in the range of 5 to 10 mg. Homogenisation with a bead mill (Mixer Mill MM 300, Retsch, UK) was optimised for each tissue type.

Trizol™ (Invitrogen, UK) was used as described by the manufacturer. The RNA samples were DNase-treated using an RNase-free DNase kit (Ambion, Texas, USA) then stored at –80°C until required for further analysis. RNA was extracted from cells using an RNA preparation kit (Midi kit, Qiagen, UK). The cells were rinsed with PBS before being collected and lysed. The RNA was extracted as per manufacturer's instructions and stored at –80°C until required for further analysis.

Reverse Transcription: Two micrograms of RNA were used in a reaction containing random octamers (10mM) which was incubated at 65°C for 5 minutes. Forty units of RNase Inhibitor (Sigma, UK), 10mM DTT and 25 units of Superscript II Reverse Transcriptase (Invitrogen, UK) were added. The samples were incubated under the following conditions: 25°C for 10 minutes, 42°C for 50 minutes, 70°C for 15 minutes. Negative controls were generated by replacing the enzyme by 0.5μl of first strand buffer.

Mouse Renin Receptor Polymerase Chain Reaction: Polymerase chain reaction (PCR) was performed on the reverse transcribed samples using the following primers (MGW Biotech, Germany):

1. GGA<u>agatct</u>GGCACCATGGCTGTGCT (forward primer; underlined sequence is a restriction site for BglII).
2.<u>gaattc</u>CTCAACTTGTCAACACTATAAATCACTCTAAAA

(reverse primer; underlined sequence is a restriction site for EcoRI).

3.gaattcTTCATGTGCAAATGGACCAATATC (reverse primer; underlined sequence is a restriction site for EcoRI).

Primers 1 and 2 were used to amplify the coding region only with an expected product size of 1.1Kb while primers 1 and 3 were used to amplify the entire cDNA with an expected product size of 2Kb.

The PCR conditions were as follows: 30s at 94°C, 30s at 60°C for primers JJM 515 and 516 or 61°C for primers JJM 515 and 517 and 2 min at 72°C. Two and a half units of a Taq polymerase (HotStartaq, Qiagen, UK) was used with 10pMol of each primer, 1.5mM$MgCl_2$ and 10mM dNTP. The samples were amplified for 30 cycles and analyzed by agarose gel electrophoresis.

PCR Product Purification and TA cloning: The PCR product was excised from a 0.8% agarose gel under ethidium bromide UV illumination, and purified using a gel purification kit (QiaQuick, Qiagen, UK). The product was then cloned using a TA cloning kit (pGEM-t easy kit, Promega, UK). Plasmid DNA was prepared (Plasmid Maxi prep, Qiagen, UK) for sequencing (ABI Prism 377 DNA sequencer) using BigDye 2 sequencing kit.

Northern Blotting: 10μg of RNA was mixed with formaldehyde, formamide and bromophenol blue, incubated at 65°C for 15mins and loaded onto a formaldehyde/1% agarose. The samples separated on the agarose gel at a voltage of 3-4V/cm until bromophenol blue had migrated 8cm. The blotting of the resulting gel was performed as described previously [23].

RESULTS

BLAST results: Three mouse cDNA sequences were found by BLAST search with the human renin receptor cDNA clone, N14F (GenBank: AF291814). One of them was from a mouse mammary tumor EST library FVB/N strain (GenBank: BC014706). The other two clones were from an 8-day mouse embryo whole body library and from a mouse neonatal head library, both of C57/Bl6 strain (GenBank: AK017482 and AK029405, respectively). The cDNA were all approximately 2Kb in length and had a STOP codon located within exon 9 producing a long 3' untranslated region of approximately 800bp (Figure 1).

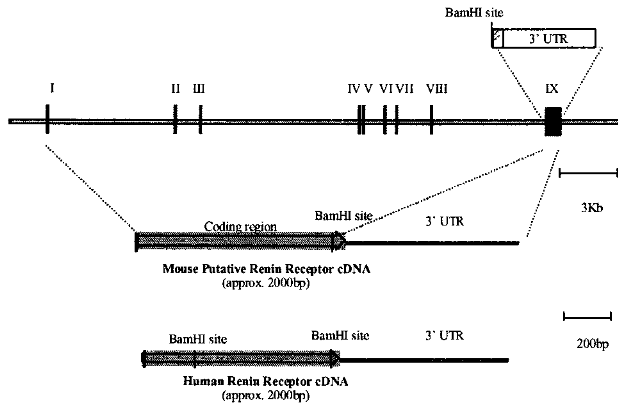
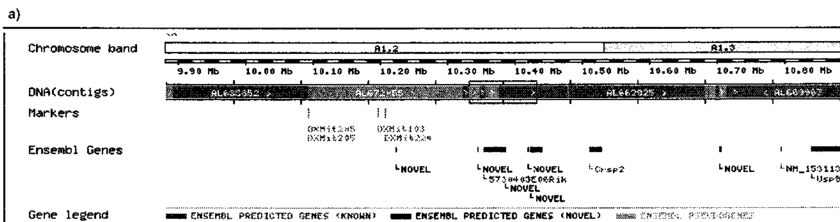


Figure 1: Top: Genomic structure of the putative mouse renin receptor. Roman numerals represent the exons. The striped area indicates the coding region of exon 9 while the plain area indicates the 3' untranslated region of exon 9. Bottom: Mouse putative renin receptor and human renin receptor cDNAs. The arrow denotes the coding region of the cDNA while the grey double bar denotes the 3' untranslated region.

The mouse cDNAs mapped to chromosome X at locus A1.2 (Figure 2a). The genomic sequence from ENSEMBL shows that the mouse renin receptor gene is 25Kb in length and has 9 exons and 8 introns (Figure 1).

Sequence alignments: DNA sequences of the 3 mouse renin receptor cDNA clones found by BLAST were aligned with the human renin receptor clone (Figure 2b). The data clearly show that the cDNA BC014706 was highly similar to the human sequence.

a)



b)

Mouse cDNA	Whole cDNA	Coding Region	3' UTR
BC014706	82.9%	88.8%	75%
AK017482	81.6%	88.2%	73.2%
AK029405	83.8%	88.1%	80.2%

Figure 2: a) Contig from Chromosome X of the mouse genome containing clone BC014706, b) Nucleotide sequence alignment matching of the three mouse cDNA clones, BC014706, AK017482 and AK029405 against the human renin receptor nucleotide sequence. Results are expressed in percentages of nucleotides matching the human renin receptor within the whole cDNA, the coding region only and the 3' untranslated region.

At the amino acid level, human and mouse proteins displayed over 80% sequence identity. Only 22 amino acids differed between the two sequences which contain 350 amino acids (Figure 3a). The mouse renin receptor protein has two hydrophobic regions located at amino acid position 219-235 and 336-350, and very few differences in sequences were observed between mouse strains.

a)

```
Mouse   MAVLVVLLFFLVAGALGNEFSILRSPGSVVFRNGNWPIPGDRIPDVAALSMGFSVKEDLS 60
Human   MAVFVVLLA-LVAGVLGNEFSILKSPGSVVFRNGNWPIPGERIPDVAALSMGFSVKEDLS 59
        ***:****  ****.********:*****************:*******************

Mouse   WPGLAVGNLFHRPRATIMVMVKGVLKLALPAGSVISYPLENAVPFSLLDSVANSIHSLFSE 120
Human   WPGLAVGNLFHRPRATVMVMVKGVNKLALPPGSVISYPLENAVPFSLDSVANSIHSLFSE 119
        ****************:*******:*****.*****************************

Mouse   ETPVVLQLAPSEERVYMVGKANSVFEDLSVTLRQLRNRLFQENSLLNSLPLNSLSRNNEV 180
Human   ETPVVLQLAPSEERVYMVGKANSVFEDLSVTLRQLRNRLFQENSVLSSLPLNSLSRNNEV 179
        ********************************************:*.*************

Mouse   DLLFLSELQVLHDISSLLSRHKHLAKDHSPDLYSLELAGLDELGKRYGEDSEQFRDASKI 240
Human   DLLFLSELQVLHDISSLLSRHKHLAKDHSPDLYSLELAGLDEIGKRYGEDSEQFRDASKI 239
        ******************************************:*****************

Mouse   LVDALQKFADDMYSLYGGNAVVELVTVKSFDTSLVRKSRTILEAKQE-NTQSPYNLAYKY 299
Human   LVDALQKFADDMYSLYGGNAVVELVTVKSFDTSLIRKTRTILEAKQAKNPASPYNLAYKY 299
        **********************************:**:********  *. *********

Mouse   NLEYSVVFNLVLWIMIGLALAVIITSYNIWNMDPGYDSIIYRMTNQKIRID 350
Human   NFEYSVVFNMVLWIMIALALAVIITSYNIWNMDPGYDSIIYRMTNQKIRMD 350
        *:*******:******.*********************************:*
```

b)

```
Bovine M8-9        ETKQVKDPSTTYNLAYKYNFEYPVVFNLVL 30
Human (fragment)   EAKQAKNPASPYNLAYKYNFEYSVVFNMVL 30
                   *:**.*:*::.***********.****:**
```

c)

```
Bovine M8-9        ETKQVKDPSTTYNLAYKYNFEYPVVFNLVL 30
Mouse (fragment)   EAKQ-ENTQSPYNLAYKYNLEYSVVFNLVL 29
                   *:** ::..:.*********:**.*******
```

Figure 3: CLUSTAL alignment of a) amino acid sequences between human renin receptor and mouse renin receptor; of b) amino acid sequences between human renin receptor and bovine M8-9 vacuolar ATPase sub-unit, and of c) amino acid sequences between mouse renin receptor and bovine M8-9 vacuolar ATPase sub-unit. Numbers in brackets indicate the amino acid number. * refers to residues in alignment that are identical; : and . refer to conservative and semi-conservative substitutions respectively.

When carrying out the BLAST search, a truncated vacuolar ATPase protein called M8-9 [20] from adrenal chromaffin cells (co-purifying with V-ATPases) was found to have homology with the human renin receptor and also the mouse cDNA. Amino acid alignment between the bovine M8-9 V-ATPase sub-unit (30 amino acids) [20] and the human renin receptor translation showed a high degree of sequence homology (Figure 3b). Eight out of the 30 amino acids differed between the two sequences, 3 of which were conservative substitutions. Similar analyses revealed that 10 amino acids differed between the murine and the bovine sequences (Figure 3c), 3 of which were conservative substitutions. Two additional human M8-9 cDNAs were found to have sequence identity with the bovine M8-9 and the human renin receptor. The first one (GenBank NM_005765) is approximately 2100bp long [24] whereas the other (Swissprot 075787) is 300bp long. The alignment of their translations with the human renin receptor protein sequence and the bovine M8-9 is shown in Figure 4.

```
renin_receptor   MAVFVVLIALVAGVLGNEFSILKSPGSVVFRNGNWPIPGERIPDVAALSMGFSVKEDLSW 60
M8-9             MAVFVVLIALVAGVLGNEFSILKSPGSVVFRNGNWPIPGERIPDVAALSMGFSVKEDLSW 60
                 ************************************************************

renin_receptor   PGLAVGNLFHRPRATVMVMVKGVNKLALPPGSVISYPLENAVPFSLDSVANSIHSLFSEE 120
M8-9             PGLAVGNLFHRPRATVMVMVKGVNKLALPPGSVISYPLENAVPFSLDSVANSIHSLFSEE 120
                 ************************************************************

renin_receptor   TPVVLQLAPSEERVYMVGKANSVFEDLSVTLRQLRNRLFQENSVLSSLPLNSLSRNNEVD 180
M8-9             TPVVLQLAPSEERVYMVGKANSVFEDLSVTLRQLRNRLFQENSVLSSLPLNSLSRNNEVD 180
                 ************************************************************

renin_receptor   LLFLSELQVLHDISSLLSRHKHLAKDHSPDLYSLELAGLDEIGKRYGEDSEQFRDASKIL 240
M8-9             LLFLSELQVLHDISSLLSRHKHLAKDHSPDLYSLELAGLDEIGKRYGEDSEQFRDASKIL 240
                 ************************************************************

renin_receptor   VDALQKFADDMYSLYGGNAVVELVTVKSFDTSLIRKTRTILEAKQAKNPASPYNLAYKYN 300
M8-9             VDALQKFADDMYSLYGGNAVVELVTVKSFDTSLIRKTRTILEAKQAKNPASPYNLAYKYN 300
                 ************************************************************
VAIN                       MYSLYGGNAVVELVTVKSFDTSLIRKTRTILEAKQAKNPASPYNLAYKYN 50

renin_receptor   FEYSVVFNMVLWIMIALALAVIITSYNIWNMDPGYDSIIYRMTNQKIRMD 350
M8-9             FEYSVVFNMVLWIMIALALAVIITSYNIWNMDPGYDSIIYRMTNQKIRMD 350
                 **************************************************
VAIN             FEYSVVFNMVLWIMIALALAVIITSYNIWNMDPGYDSIIYRMTNQKIRMD 100
```

Figure 4: CLUSTAL alignment of human amino acid sequences: renin receptor (from GenBank accession AF291814) and M8-9 (from GenBank accession NM_005765). In addition, the shorter sequence described as M8-9 (VAIN, SwissProt accession O75787) was aligned manually to show the extent of its identity with the other sequences.

Conservation of the renin receptor in several species was studied using the homologous sequences available in the ENSEMBL database. A multi-species protein sequence comparison (Figure 5a) revealed homologues to the human receptor in a variety of species including rat, mouse (as already mentioned), chicken, frog, zebrafish, mosquito or drosophila suggesting that the renin receptor gene is highly conserved between a large number of species.

a)

	Mouse	Human	Rat	Chicken	Frog	Zebrafish	Drosophila	Anopheles	C elegans
Mouse	100	92	96	79	68	67	27	23	20
Human		100	91	79	69	68	26	23	20
Rat			100	78	68	67	26	22	21
Chicken				100	69	67	24	23	22
Frog					100	63	26	22	21
Zebrafish						100	23	22	22
Drosophila							100	36	21
Anopheles								100	20
C elegans									100

b)

```
Human      MAVFVVLLALVAGVLGNEFSILKSPGSVVFRNGNWPIPGERIPDVAALSMGFSVKEDLSW 60
C_elegans  MKFVLLVSALVAACAASSLEIIAAPASLKFPDADATLKTTLISTLNENILGLSARQVDGF 60
           * .:::: ****.   ....:.*: :*.*: * :.: .:      *. :      :*:*.::  .:

Human      PGLAVGNLFHRPRATVMVMVKGVNKLALPPGSVISYPLENAVPFSLDSVANSIHSLFSEE 120
C_elegans  S--VSVDMPSRPRALAVVTVIGADNLNLG-----------------------GKTYSTK 94
           .  .  ::* **** .:* * *.::* *                         . :* :

Human      TPVVLQLAPSEERVYMVGKANSVFEDLSVTLRQLRNRLFQENSVLSSLPLNSLSRNNEVD 180
C_elegans  SDGVENVGFDQDMALIFGADR---ENVQVSAGGITGSMLALAAQQEAVDATVIKTKR--- 148
           :  * ::. .:: . :.*  .   *::.*:   : . ::   :  .::  . :. :.

Human      LLFLSELQVLHDISSLLSRHKHLAKDHSPDLYSLELAGLDEIGKRYGEDSEQFRDASKIL 240
C_elegans  STLVKELEAVYQLAAAIKSAG-VQMDNNADVFRVSITGLVGI-----TESAQKEEAVADV 202
            ::.**:.:::::: :.     :  *:..*:: :.::** *       :* * .:*    :

Human      VDALQKFADDMYSLYGGNAVVELVTVKSFDTSLIRKTR-----TILEAKQAKNPASPYNL 295
C_elegans  KAAIEALTASINNAYGGQAVIELLAPEAESGAAEKSTESREIPNHNIQKRDTKSKTPTQL 262
            *:: :: .: . ***:**:**::.:: . :  :.*.     .     *: .:. :* :*

Human      AYK-------YNFEYSVVFNMVLWIMIALALAVIITSYNIWNMDPGYDSIIYRMTNQKIR 348
C_elegans  ARENLQVTVPVSSDYPAIFAIFLGLVVILVVALIYIVVGMASIDPEKDSIIYRMTTTRMK 322
           * :          . :*..:* :.* ::: *.:*:*    .: .:**  ********. :::

Human      MD 350
C_elegans  KD 324
            *
```

c)

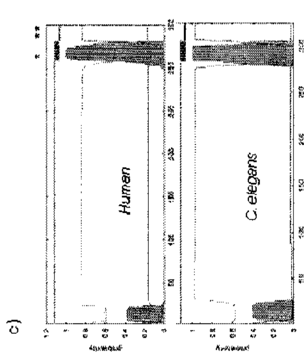


Figure 5: Conservation of amino acid sequence of renin receptor/M8-9. a) sequence identity matrix showing percentage identity of the amino acid sequence between nine species. b) CLUSTAL alignment of amino acid sequences for human renin receptor and *C.elegans* M8-9. c) Hydrophobicity plots for human renin receptor and *C.elegans* M8-9 generated by TMHMM program. * denotes transmembrane regions and ** denotes highly conserved intracellular/vacuolar domain.

The multi-species sequence alignment (Figure 5b) shows homologues in species as remotely related to humans as *C.elegans*. It is worth noting that the regions showing greater homology between the human sequence and *C.elegans* encompass the transmembrane region of the protein and the predicted intracellular/intravacuolar portion of the molecule as shown on the hydrophobic prediction plot (Figure 5c).

Analysis of the database showed that the genomic structure described above (Figure 1) is the only sequence that encodes a functional gene and therefore this gene must encode both the prorenin receptor and the protein the truncation products (M8-9) of which are associated with the V-ATPase.

Reverse Transcriptase Polymerase Chain Reaction: RT-PCR was performed as described above. Murine RNA was processed to examine the expression of the putative mouse renin receptor transcripts in a variety of tissues (Figure 6a). The PCR product encompassing only the coding region was found in both strains in all tissues examined, (namely, brain, heart, liver, lung, submandibular gland, adrenal, muscle, ovary, placenta, mesenteric fat and spleen) suggesting the molecule may be expressed ubiquitously.

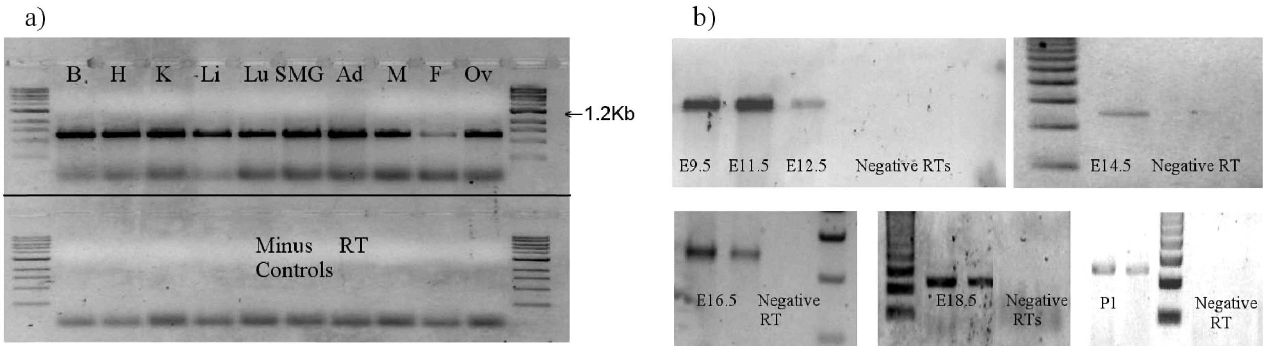


Figure 6: Reverse Transcriptase Polymerase Chain Reaction Data. a) Female adult tissue samples from mouse of FVB/N strain. The DNA ladder use is of 500bp increments. b) Embryonic kidney samples from mouse FVB/N. The second band at the bottom of the gel is unincorporated oligonucleotides. B = Brain, H = Heart, K =Kidney, Li = Liver, Lu = Lung, SMG Submandibular Gland, Sp = Spleen, Ad = Adrenal Gland, M = Muscle, F = Mesenteric fat, Ov = Ovary, E = Embryonic Day, RT = Reverse Transcriptase.

Developmental expression of the putative renin receptor was examined in mouse kidneys (Figure 6b). RT-PCR analysis was performed on RNA extracted from embryonic kidneys from E9.5 dpc.

Complimentary DNA (2kb) was generated from whole heart and kidney RNA, TA cloned and subjected to DNA sequence analysis to confirm its identity.

Expression of the putative mouse renin receptor was detected by Northern blotting (Figure 7) in several cell lines: AS 4.1 (a kidney cell line), E14TG2a (an embryonic stem cell line) and HMC (a human mesangial cell line). The latter was previously reported to be non-expressing by Northern blotting and RT-PCR.

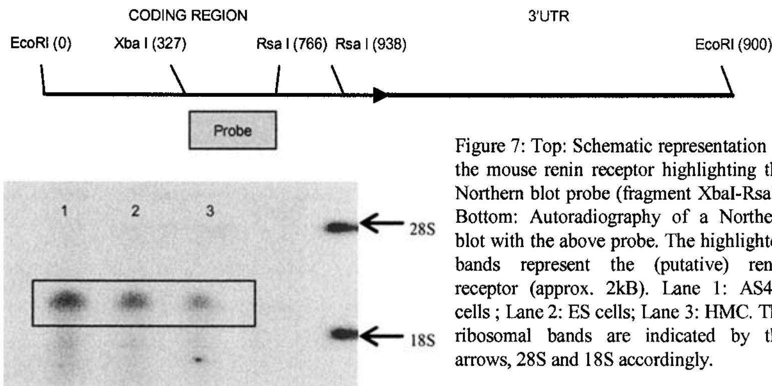


Figure 7: Top: Schematic representation of the mouse renin receptor highlighting the Northern blot probe (fragment XbaI-RsaI). Bottom: Autoradiography of a Northern blot with the above probe. The highlighted bands represent the (putative) renin receptor (approx. 2kB). Lane 1: AS4.1 cells ; Lane 2: ES cells; Lane 3: HMC. The ribosomal bands are indicated by the arrows, 28S and 18S accordingly.

DISCUSSION AND CONCLUSIONS

We report here sequence comparison between the human renin receptor and a vacuolar proton-ATPase membrane sector-associated protein (M8-9 [20]). The renin receptor was shown to be conserved in a variety of species including mouse, rat chicken, frog, zebrafish, mosquito, fruit fly and C.elegans. Our findings indicate that the renin receptor and M8-9 are encoded by the same gene, and it is possible that they serve several functions. Expression studies showed that the mouse transcript was ubiquitous to all the tissues examined and in all cell lines screened, although the cell type in which the mouse renin receptor is expressed was not determined in this study. A murine renin receptor could be a very important tool to study (pro)renin uptake in the heart and vascular tissues. In an attempt to find a suitable non-expressing mouse

cell line, we studied expression of the renin receptor in HMC, ES and AS4.1 cells. Although previously reported to be non-expressing by RT-PCR and Northern blotting, HMC were, in our studies, found to express the renin receptor limiting their use for transfection studies.

Surprisingly, BLAST analysis revealed that both the translated human cDNA (AF291814) [19] and the translated mouse cDNA (BC014706) had homology with M8-9, a truncated protein found associated with a membrane sector portion of the V-ATPase [20], but the physiological significance of this is unclear. We showed that the renin receptor is conserved between several species and multispecies alignments revealed that the human renin receptor is homologous to M8-9 sequences from a variety of species such as rat, mouse, chicken, frog, zebrafish, mosquito drosophila and *C.elegans*. In the latter, the similarity appears most notably in the C-terminus transmembrane region and the intracellular portion of the V-ATPase. This indicates that the C-terminus region has an important highly conserved function. V-ATPases are one of three types of ATPases. They contain 6-10 subunits divided into two domains: a transmembrane proton-conducting sector and an extra-membrane catalytic sector responsible for ATP hydrolysis [25,26,27]. In mammalian cells, V-ATPases play important roles in energy conservation, secondary active transport, acidification of intracellular compartments and cellular pH homeostasis [28] and play a major role in the homeostatic processes of nephron segments [29,30].

It is easy to be confused when trying to disentangle the available information relating to M8-9 and the renin receptor. It is important to remember that the name 'M8-9' refers to a group of short peptides of overlapping sequence, believed to be the truncation products of a larger, as yet unpurified, protein. In 2001 the encoded cDNA sequence and genomic structure of a gene encoding the M8-9 peptides was reported [24]. Although there is no biochemical evidence to prove that the M8-9 peptides are derived from the protein encoded by this cDNA, the fact that there are no other such sequences in the genome suggests that this must indeed be the precursor. It is not known whether the encoded protein is itself associated with the V-ATPase, or how its truncation products, which do associate with the V-ATPase, are formed. What seems clear is that this gene, termed the 'M8-9' gene, encodes sequences that co-purify with the V-ATPase. Independently, in 2002, the sequence of the human renin receptor was reported [19]. The authors commented on the homology between the peptide sequence encoded by the renin receptor cDNA and the M8-9 peptides. However, on further comparison it is clear that the renin receptor cDNA sequence must the product of the gene referred to in the database as the 'M8-9' gene. Perhaps until we

understand more fully the relationship between the renin receptor and the V-ATPase-associated peptides this gene should be referred to as the 'M8-9/renin receptor gene. There is clearly much work to do in elucidating the role(s) of the product(s) of this gene and clarification of the function(s) of the encoded protein(s) has important implications for cell function (via the V-ATPase function) and the cardiovascular system (via its role as a renin receptor).

Finally, if the role of this gene proves to be critical for cell survival, as may be the case for a protein closely associated with a V-ATPase, then careful consideration will need to be given to the design of gene targeting experiments aimed at knocking out the function of this gene.

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Chapter 4
ROLE OF THE AT_2 RECEPTOR IN CARDIOVASCULAR FUNCTION: A BRIEF SYNOPSIS

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INTRODUCTION

The renin-angiotensin system (RAS) is a coordinated hormonal cascade the major effector of which is angiotensin II (Ang II). Ang II conducts its biological functions through two major angiotensin receptors, AT_1 and AT_2. The vast majority of the actions of Ang II have been considered to be mediated by the AT_1 receptor, and the physiological actions of Ang II mediated by the AT_2 receptor have been difficult to elucidate (1,2). The AT_1 receptor mediates cellular de-differentiation and growth, vasoconstriction, anti-natriuresis, aldosterone secretion, salt-appetite, thirst, cardiac ionotropism, sympathetic outflow and inhibition of renin biosynthesis and secretion. A likely reason that AT_2 receptor actions have been difficult to demonstrate is that the AT_1 receptor is expressed in larger quantities than the AT_2 receptor in most cardiovascular tissues. Therefore, the net effect of Ang II is usually to stimulate the AT_1 receptor in preference to the AT_2 receptor. Recently, studies have begun to clarify the role the AT_2 receptor by pharmacologically inhibiting the AT_1 receptor followed by Ang II stimulation. The results of these studies have unmasked a clear vasodilator action of the AT_2 receptor. The AT_2 receptor also inhibits growth and promotes cellular differentiation. Thus, the AT_2 receptor can be conceptualized as a counter-regulatory receptor to some of the actions of Ang II via the AT_1 receptor (Figure 1). This article provides an update of the role of the AT_2 receptor as a vasodilator mediator. In addition, we shall briefly discuss the potential role of the AT_2 receptor as an inhibitor

of renin biosynthesis and secretion, a stimulator of natriuresis and as a cardioprotector.

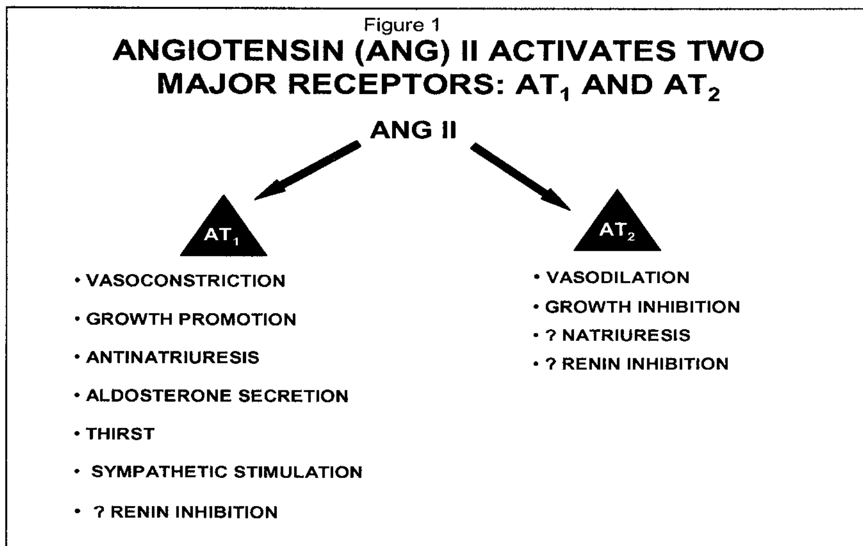

Figure 1. Presently known effects of angiotensin II type 1 and type 2 receptors stimulation.

ROLE OF THE AT_2 RECEPTOR IN VASODILATION

Studies in 1996-97 suggested that stimulation of the AT_2 receptor activates a vasodilator hormonal cascade of bradykinin (BK), nitric oxide (NO) and guanosine cyclic 3', 5'-monophosphate (cGMP) (3-5). These were followed by a large number of studies suggesting that the AT_2 receptor dilates blood vessels, counter-regulating the vasoconstrictor action of Ang II via the AT_1 receptor (6-16). Some of these studies demonstrated that AT_2 receptor stimulation could lower blood pressure, particularly if the AT_1 receptor is functionally removed by pharmacological blockade (8, 13, 16). The AT_2 receptor is up-regulated by sodium deprivation (17), and AT_1 receptor blockade during sodium depletion resulted in hypotension that could be abrogated completely by AT_2 receptor blockade (16). Furthermore, the hypotensive response to AT_1 receptor blockade in renal vascular hypertension also could be extinguished by concurrent AT_2 receptor blockade (9). In these studies, the mechanism for the counter-regulatory vasodilator actions of the AT_2 receptor was clearly linked to the BK-NO-cGMP cascade (9, 16). It was thought that AT_2 receptor function was more likely to be observed when the RAS was activated (e.g. low sodium diet, renal vascular

hypertension), and that the increase in renin secretion and Ang II formation during AT_1 receptor blockade led to stimulation of the unblocked AT_2 receptor. Thus, the beneficial effects of AT_1 receptor blockade on blood pressure were mediated by AT_2 receptor stimulation, at least during acute experimental conditions. Much more work needs to be done, however, to prove that chronic blood pressure reduction by AT_1 receptor blockers is due to this mechanism. If so, a strong theoretical case could be made for AT_2 receptor agonist development for hypertension.

Studies during the past two years have solidified the concept that the AT_2 receptor is a counter-regulatory vasodilator mediator via the BK-NO-cGMP pathway. In the rat uterine artery, AT_2 receptor blockade with PD-1123319 (PD) potentiated Ang II –induced contraction with a strong leftward shift (more sensitive) in the Ang II concentration-response curve (18). This action of PD was duplicated by the BK-B_2 receptor antagonist icatibant and by the NO synthase inhibitor N-nitro-L-arginine, and the arterial cGMP concentration was increased by Ang II. Therefore, Ang II-induced vasoconstriction in the uterine artery is inhibited by AT_2 receptor stimulation via the BK-NO-cGMP pathway (18). This information is potentially pertinent in pregnancy-induced hypertension (pre-clampsia), in which the AT_2 receptor is up-regulated (19).

In mesenteric arterial segments, Ang II in the presence of AT_1 receptor blockade induced a vasodilator response that was dependent on endothelial AT_2 receptors (20). BK B_2 receptor blockade substantially inhibited this response. In Brown-Norway-Katholick rats, which are deficient in kininogen, AT_2 receptor-mediated vasodilation was significantly impaired compared to that of wild-type controls (20). These studies indicate that AT_2 receptors in small mesenteric resistance vessels initiate vasodilation mediated by BK in a flow-dependent manner. Flow-dependent dilation appears to be dependent upon AT_2 receptors, as AT_2 receptor blockade decreased flow-induced dilation in wild-type mice but not in mice lacking tissue kallekrein (21). In wild-type mice, BK B_2 receptor antagonism decreased the dilatory response to flow, but not in animals lacking the AT_2 receptor (21). Taken together, this information suggests that resistance microvessels are the major site of AT_2 receptor-mediated vasodilation in vivo.

Recent evidence, however, also suggests a vasodilatory role for the AT_2 receptor in large capacitance vessels. When the thoracic aorta is overloaded with pressure due to aortic banding there is a large up-regulation of AT_2 receptor expression (22). In the pressure-overloaded aorta, Ang II constrictor responses were decreased, but were restored in

the presence of AT_2 receptor blockade. Removal of the endothelium eliminated the differences in Ang II –induced contraction between pressure-overloaded and control aortic rings (2). The contractile response was also restored by BK B_2 receptor blockade (23). Ang II increased aortic cGMP by 9-fold, and this increase was abolished with either AT_2 receptor blockade with PD or BK B_2 receptor blockade with icatibant (23). These studies endorse the concept of counter-regulatory AT_2 receptor-mediated dilation due to BK-NO-cGMP.

AT_2 receptor-mediated vasodilation is present in the small resistance vessels of the coronary circulation. In human coronary microarteries, Ang II-induced contraction was prevented by an AT_1 receptor antagonist and increased by AT_2 receptor antagonist PD, but vasodilator potentiation was not present when NO synthase or BK B_2 receptor antagonists were present or the endothelium had been denuded (24). When the AT_1 receptor was blocked, Ang II induced vascular relaxation that was abrogated by AT_2 receptor antagonist PD (24). Therefore, the coronary microcirculation possesses functional AT_2 receptors that induce vasodilation via the BK-NO-cGMP pathway.

The foregoing studies provide conclusive evidence that the AT_2 receptor is a vasodilator receptor counter-regulating the vasoconstrictor actions of Ang II via the AT_1 receptor. When the AT_1 receptor is blocked, the ensuing vasodilation and hypotension is, at least in part, mediated by AT_2 receptor stimulation. These observations provide a potentially exciting therapeutic target for AT_2 receptor agonists that might be developed in the future.

ROLE OF THE AT_2 RECEPTOR IN THE REGULATION OF RENIN SECRETION

Renin, the rate-limiting enzyme in the production of Ang II, is produced mainly by renal juxtaglomerular (JG) cells (25). In addition to JG cells, renin is produced in mesangial and proximal tubule cells (26, 27). Ang receptors, both AT_1 and AT_2, are present within the kidney, and Ang II is known to feedback directly to suppress renin mRNA and secretion via AT_1 receptors on JG cell membranes (28). Although AT2 receptors are present in JG cells and inhibit prorenin processing (29), no data have been available on the effects, in any, of the AT_2 receptor on renin biosynthesis and secretion.

Recently, our group demonstrated regulation of the activity of the RAS by the AT_2 receptor (30). We observed an increase in renal renin mRNA, renin protein and intrarenal Ang II levels in response to

AT_2 receptor blockade with PD. Since the renin and Ang II responses to AT_2 receptor blockade were similar to those with AT_1 receptor blockade, both AT_1 and AT_2 receptors appear to have an inhibitory effect on the activity of the RAS (30). AT_2 receptor inhibition of renin synthesis and secretion is consistent with its vasodilator and cardiovascular protective actions. Since blockers of the RAS (angiotensin converting enzyme inhibitors and receptor blockers) increase renin secretion via inhibitions of the short-loop negative feedback loop, stimulation of the AT_2 receptor by high levels of Ang II would tend to suppress renin secretion and Ang II formation, providing an additional element of protection.

ROLE OF THE AT_2 RECEPTOR IN NATRIURESIS

Although AT_2 receptors are distributed in renal blood vessels, glomeruli and tubules (17, 31), little information is available on the role of AT_2 receptors in natriuresis. The AT_2 receptor may decrease bicarbonate reabsorption in the renal proximal tubule (32). Pressure-natriuresis curves are shifted to the right (less sensitive) in mice lacking the AT_2 receptor (33). AT_2 receptor-null mice have exaggerated anti-natriuresis in response to Ang II (34). However, AT_1 receptors are up-regulated in AT_2-null mice, so the precise role of the AT_2 receptor in natriuresis remains in question. Clearly, much more work needs to be performed on the actions of Ang II on renal sodium transport via the AT_2 receptor.

ROLE OF THE AT_2 RECEPTOR IN CARDIOPROTECTION

The area of the AT_2 receptor in left ventricular (LV) remodeling and cardioprotection is an active area of investigation (34-38). Indeed, many of the beneficial effects of AT_2 receptor blockade post-myocardial infarction (MI) or of angiotensin converting enzyme inhibitors may be mediated by AT_2 receptor stimulation (39, 40).

We demonstrated in mice selectively overexpressing the AT_2 receptor in the myocardium that overexpression preserves LV size and function during post-MI remodeling. The transgenic mice preserved LV cavity size, wall thickness within the infant zone, and regional and global LV function during post-MI remodeling in comparison with wild type controls (41). Other investigators also have shown that deletion of the AT_2 receptor is injurious post-MI leading to myocardial injury, heart

failure and increased mortality (42, 43). Recently, we demonstrated that NO mediates the benefits of cardiac AT_2 receptor overexpression during post-MI remodeling (44). In transgenic mice, functional magnetic resonance imaging demonstrated that end-diastolic volume index and end-systolic volume index were significantly lower than in wild type mice at day 28 post-MI. However, treatment with NO synthase inhibitor L-NAME in transgenic mice reverted these parameters to values observed in wild type animals and eliminated the cardioprotection afforded by AT_2 receptor overexpression (44). Therefore, the NO pathway likely mediates the benefits of cardiac AT_2 receptor overexpression during post-MI remodeling.

SUMMARY

In this brief review, we have cited incontrovertible evidence that the AT_2 receptor mediates dilation in resistance microvessels. This vasodilator action is mediated by a BK-NO-cGMP autacoid cascade. In addition, we show that, similar to the AT_1 receptor, the AT_2 receptor mediates inhibition of renin biosynthesis and release. The mechanism of this action is uncertain, but may be via cGMP, which has been shown as a direct inhibitor of renin secretion. We call attention to the lack of information about the potential action of the AT_2 receptor on sodium excretion. Finally, we report on functional magnetic resonance studies suggesting that cardiac AT_2 receptor over-expression is protective against LV remodeling post-MI. During the past several years, the AT_2 receptor has been characterized as a functional component of the RAS, mediating important actions in the cardiovascular system.

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Chapter 5
REGULATION OF RENIN IN JGA AND TUBULES IN HYPERTENSION

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INTRODUCTION

Although the main focus of this symposium is on the cardiac renin-angiotensin system (RAS), it is appropriate to consider developments in the renal RAS because of its pivotal role in the regulation of renal sodium and water excretion, and therefore, in maintaining body sodium and volume balance and in the long-term control of arterial pressure. Recent studies have raised awareness of the important and complex mechanisms that participate in the local regulation of the RAS within the kidney, which suggest differential and reciprocal control mechanisms that may also exist in other organs [1-3]. In the classical pathway, renin produced by the juxtaglomerular apparatus (JGA) cells in the kidney cleaves liver-derived angiotensinogen (AGT) to form angiotensin I (Ang I) within the circulation. Subsequently, Ang I is converted into Ang II, the main effector peptide of the RAS, by angiotensin converting enzyme (ACE) located at the luminal side of the endothelium throughout the vasculature. Ang II exerts its pleiotropic effects via stimulation of Ang II receptors (AT), of which at least two types have been described AT_1 and AT_2 [4].

While the systemic RAS is certainly important, many recent studies have focused on the existence of local RAS systems in the

brain, heart, adrenal glands, vasculature, as well as in the kidney [5,6]. The RAS in the kidney is of general significance because many forms of hypertension are characterized by an inappropriate activation of the intrarenal RAS that limits the capability of the kidney to maintain sodium balance [6 1,7]. This leads to hypertension which, in turn, affects other organ systems and tissues throughout the body. In Ang II-dependent hypertension, Ang II renal content is much higher than can be explained simply on the basis of equilibration with the circulating concentrations and often remains elevated even after circulating renin and Ang II concentrations have returned to normal or near normal levels [8]. Thus, it has become apparent that the changes in the intrarenal Ang II levels can not be explained simply by the contribution of the circulating RAS [9-12].

INTRARENAL ANG II IN HYPERTENSION

One model of hypertension that has provided significant insights is the two-kidney, one-clip (2K1C) Goldblatt hypertensive rat [8,13-19]. The hypertension that develops after moderate constriction of one renal artery has remained intriguing because of the functional alterations that occur in the contralateral kidney. As the stenosis is present in only one kidney, and because one normal kidney is sufficient to maintain fluid and sodium balance and arterial pressures at normal levels, it was unclear for many years why the nonclipped contralateral kidney fails to protect against the development of hypertension following unilateral arterial clipping. Studies from various laboratories demonstrated that, while the nonclipped kidney is not the initial causative factor, it also exhibits altered function resulting in reduced sodium excretion for any given arterial pressure [14,20-22]. The functional derangements include impaired renal autoregulatory capability, altered microvascular responses to vasoactive stimuli, increased activity of the tubuloglomerular feedback mechanism and enhanced fractional sodium reabsorption [17,18,20,23-29]. These alterations prevent the nonclipped kidney from re-establishing sodium balance except at elevated arterial pressures. Following stenosis, there is a marked increase in renin synthesis and release from the clipped kidney which leads to increased local and circulating Ang II levels [8,24,30-35]. During the initial phase of hypertension, the Ang II dependent alterations in renal hemodynamics and tubular reabsorption of the nonclipped kidney may be due, in part, to the elevations in circulating Ang II [20,32]. Importantly, the nonclipped kidney is highly responsive to

pharmacological blockade of the RAS [14,18-20,23,36]. Administration of ACE inhibitors or Ang II receptor antagonists increase renal blood flow (RBF) and glomerular filtration rate (GFR), reduce fractional sodium reabsorption, and decrease the sensitivity of the tubuloglomerular feedback mechanism [18,28,36]. Interestingly, the responsiveness of the nonclipped kidney to blockade of the RAS persists even during the maintenance phase of hypertension when plasma renin and Ang II concentrations return back towards normal suggesting a dissociation between the circulating Ang II levels and the renal Ang II dependency [8,26].

As depicted in Figure 1, it has been shown that the Ang II content of the nonclipped kidney is elevated even at a time when JGA renin content and mRNA levels are markedly decreased [8,37,38]. Thus, while the nonclipped kidney exhibits reduced JGA renin, it is clearly not depleted of Ang II [8,29,35,38-40]. The renal tissue Ang II content and ACE activity in the nonclipped kidney remain elevated during the maintenance phase of 2K1C hypertension [8,35]. Tokayama et al [41] extended earlier findings and demonstrated that different mechanisms are responsible for the enhanced intrarenal Ang II in clipped and nonclipped kidneys. In the clipped kidney, chymase levels are elevated while the nonclipped kidney has increases in ACE activity.

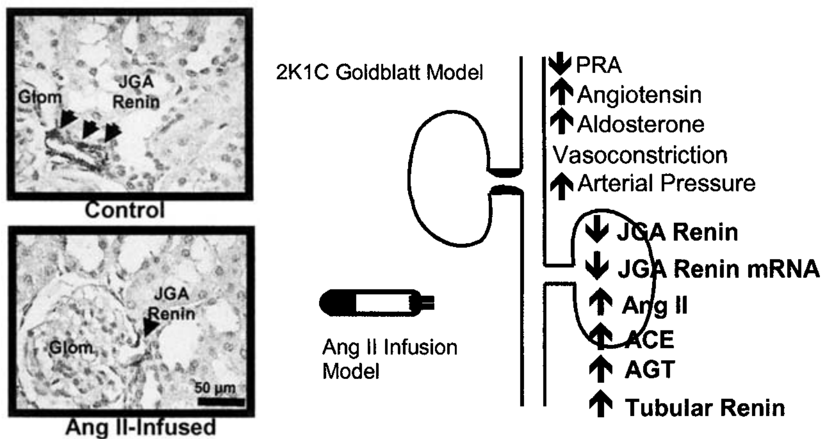

Figure 1. In 2K1C Goldblatt hypertension, the contralateral nonclipped kidney exhibits reductions in JGA renin and renin message but increases in intrarenal Ang II and ACE activity 8. Similarly, the kidneys in the Ang II infusion model have reduced JGA renin and renin message, but increased intrarenal Ang II levels, ACE activity, AGT protein and message, as well as tubular renin 11,56,59,60.

Studies demonstrating that low subpressor Ang II infusions mimic the pattern of hypertension observed in 2K1C rats provided further insights into mechanisms responsible for regulating intrarenal Ang II [35,42-44]. In the Ang II infused model, renal Ang II levels are also increased to levels above those that can be explained on the basis of circulating Ang II [11,12,35]. Renal ACE activity is also increased, but the plasma and JGA renin levels and renin mRNA become markedly suppressed [40]. These findings suggested that subtle elevations in circulating Ang II lead to increases in intrarenal Ang II content independent of the classical renin driven pathway. It was also shown that AT_1 receptor blockade led to reductions in intrarenal Ang II levels even though plasma Ang II concentrations increased indicating that part of the increase in intrarenal Ang II is due to receptor mediated uptake [11,12,45,46]. The TGR (mRen2) 27 rat model; in which an extra mouse renin (Ren-2) gene is present in the genome of the Sprague-Dawley rat, is also characterized by suppression of renal renin and renin mRNA content but have increased plasma levels of active renin (4.5-fold) and *prorenin* (300-fold) [47]. Using this animal model, Mitchell et al. [48], reported that the Ang II concentrations in plasma, total kidney and proximal tubular fluid are not suppressed compared to normotensive control rats.

The findings that in the nonclipped kidney of 2K1C rats [8,48] and in kidneys of Ang II infused rats [28,49-51] and of Ren2 transgenic rats [11,52], there is an augmented intrarenal Ang II content helps explain the enhanced sodium reabsorptive capability, maintained tubuloglomerular feedback responsiveness and augmented microvascular responsiveness that exists in kidneys of hypertensive models. Collectively, these derangements mediated by the diverse actions of Ang II on various renal structures (Figure 2) impair the kidney's ability to achieve normal rates of sodium excretion at normotensive pressures and attenuate the magnitude of the natriuretic response to increases in arterial pressure and thereby render the kidneys unable to protect against the development of hypertension.

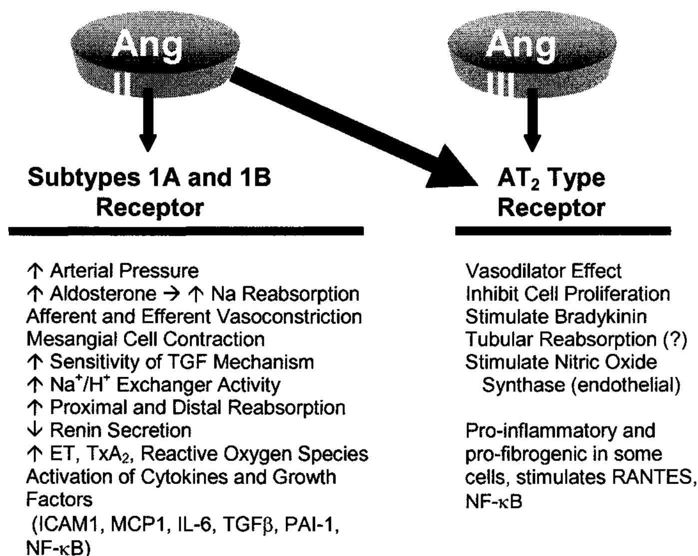

Figure 2. Action of intrarenal Ang II on renal hemodynamics and tubular function. TGF – tubuloglomerular feedback mechanism, ET – endothelin, TxA_2 – thromboxane.

Tubular Components of Renin-Angiotensin System

Ang II dependent models of hypertension are also characterized by an enhanced AGT mRNA and protein production produced primarily in proximal tubules. Chronic Ang II infusions exert a positive amplification of intrarenal AGT mRNA and protein levels [53-56]. The increased AGT production rate by the proximal tubules leads to increased secretion into the tubular fluid which spills over into the distal nephron segments and into the urine [57,58]. The presence of AGT throughout the nephron segment is important in the light of recent findings that renin is present in principal cells of collecting ducts [53,59,60]. The findings for components of the RAS associated with tubules have led to increased emphasis on the role of Ang II dependent alterations in tubular transport function as well as on hemodynamics (Figure 2). This emphasis has been stimulated further by the remarkable findings that proximal tubular fluid concentrations of Ang I and Ang II are in the nanomolar range and several times greater than can be explained on the basis of either the circulating or tissue levels of Ang II [61-63]. The nanomolar concentrations of tubular Ang II are thought to exert a major

influence in regulating proximal reabsorption rate [64-68]. Studies by Quan and Baum [69-71] provided evidence for an important role of luminal Ang II in regulating proximal reabsorption rate. Intraluminal Ang II also stimulates distal tubule sodium and bicarbonate reabsorption by increasing the activity of the Na^+/H^+ exchanger as well as augmenting sodium influx through an amiloride sensitive Na^+ transport pathway in the collecting tubule which is also an aldosterone sensitive segment [68,72-75]. These findings suggest a unique synergism between Ang II and aldosterone in regulating sodium transport in distal nephron segments.

The proximal tubular Ang II concentrations in the nonclipped kidneys of 2K1C rats, in the kidneys of Ang II infused rats and in hypertensive Ren2 transgenic rats are also maintained in the nanomolar range even though these kidneys are renin depleted [36,48,51]. The high Ang II levels in proximal tubules are thought to be due to the augmented AGT in proximal tubules. Importantly, there appears to be an association between the AT_1 mediated internalization of Ang II and the enhancement of intrarenal AGT such that when the AT_1 receptor is blocked, the Ang II mediated augmentation of AGT is prevented [56] (Figure 3). Furthermore, only JGA renin is downregulated in Ang II dependent hypertension. Recent studies summarized in Figure 4 indicate that renin in principal cells of connecting tubules and collecting ducts is augmented in Ang II induced hypertension [60]. The finding that principal cells of connecting tubules and collecting ducts express renin mRNA and renin protein provides a functional implication for the distally delivered AGT to form Ang I in response to renin provided by distal nephron segments [60,76,77]. ACE is also present in collecting duct segments [75], providing support for the formation of Ang II in distal nephron segments and further explaining the marked reductions in fractional sodium excretion that occur during conditions of chronically elevated intrarenal Ang II [50,51].

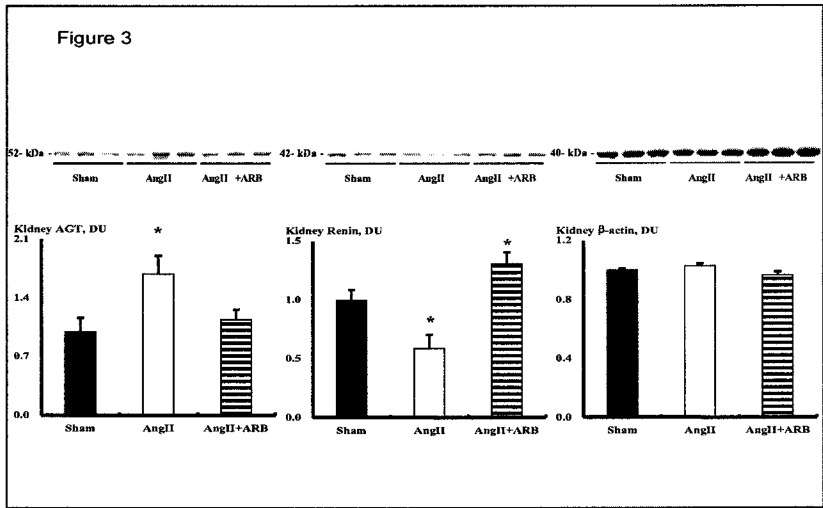

Figure 3. Effects of chronic Ang II infusions and AT1 receptor blockade (ARB) with olmesartan on renal cortical angiotensinogen (AGT) and renin levels [56].

Regulation of Tubular Renin

Renin gene expression is regulated at the posttranslational and secretory levels. The translational product of renin mRNA is preprorenin, which is cotranslationally cleaved to prorenin, an inactive precursor of renin [78]. Ang II inhibits basal renal renin secretion by JGA cells even when renal arterial pressure is maintained constant.[79,80]. While ACE inhibition stimulates JGA renin mRNA and JGA renin-specific immunostaining, Ang II attenuates the accumulation of renin mRNA without alteration of JGA renin distribution [81] suggesting that the lack of effect of Ang II on JGA renin distribution may be due to the length of turnover time for stored protein. The net result is that, Ang II exerts a direct inhibitory effect on renin gene expression in JGA cells.

As mentioned earlier, renin protein and message have also been reported in tubular structures. Renin immunoreactivity has been detected at the apical side of proximal tubule cells and in principal cells of connecting tubules and collecting ducts [82-84]. Originally, these findings were interpreted as being due to tubular pinocytosis of the filtered renin. However, recent studies have demonstrated the presence of renin mRNA in proximal tubular cells [85] and in connecting tubule cells, in which renin secretion assessed by immunoblotting has also been shown [76]. This evidence supports the hypothesis of local synthesis of renin in distal as well as proximal tubule cells. Indeed the immunostaining data indicate that renin abundance is much greater in the principal cells [60]. The

intriguing localization of renin in the principal cells of connecting tubule nephron and collecting ducts of rat, mouse and human kidney [76,86] supports the likelihood of generation of Ang I in the distal tubule from AGT secreted into the lumen of the proximal tubule. Furthermore, the intrarenal AGT upregulation elicited by Ang II [54] could help to maintain or even increase the production of intrarenal and intratubular Ang II in Ang II-dependent hypertension [55,59]. Under conditions of augmented intrarenal production of AGT, the increased AGT secretion would lead to increased passage of AGT into distal nephron segments where they may then be cleaved to Ang I by the enhanced tubular renin [58]. Importantly, while the chronic Ang II infusions clearly inhibit renin production and secretion by JGA cells, there is a reciprocal action on renin in tubular cells in that Ang II stimulates distal nephron renin mRNA and protein levels via an AT_1 receptor mediated mechanism [60,87] (Figure 4). The mechanism responsible for this reciprocal action of Ang II on JGA and collecting duct cell renin has not been determined but it is now clear that both Ang II as well as AT_1 receptor blockers have opposite effects at these two sites. Enhanced tubular renin immunostaining and mRNA, localized by in situ hybridization, have also been observed in the remnant kidney model [88]. In this study, administration of ACE inhibitors eliminated mRNA signals and attenuated renin immunostaining in the distal tubules indicating another model of reciprocal regulation since ACE inhibitors stimulate JGA renin [89]. Because the effects of Ang II on JGA renin production is inhibitory, it is difficult to explain how Ang II could exert a direct stimulatory effect on renin production in tubules. Several possibilities occur including an effect mediated by aldosterone, or other factors or paracrine agents activated by elevated Ang II levels.

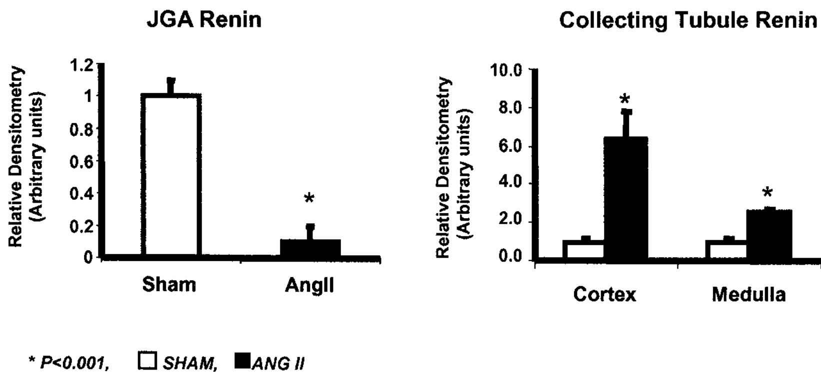

Figure 4. Quantitation of JGA and collecting tubule renin immunoreactivity in control and Ang II infused rats [60].

Once Ang I is formed as a consequence of increased AGT delivery and increased renin production by principal cells, increased intratubular Ang II formation becomes feasible in the light of increased evidence that ACE is present in distal tubular segments as well as on the brush border of proximal tubule cells. Casarini and co-workers [90] found that ACE activity decreases from the initial portion of the proximal tubular to the early distal tubule, but increases again in the urine. As depicted in Figure 5, intratubular Ang II formation may occur not only in the proximal tubules but also in the connecting tubules and collecting ducts. The increased intratubular Ang II formation may partially lead to augmented Ang II renal content and to enhancement of fractional sodium reabsorption rate and suppression of pressure-natriuresis characteristic of the Ang II-infused rat model [50,51]. Indeed, Ang II directly stimulates epithelial sodium channel (ENaC) activity in cortical collecting duct cells [74] and intraluminal conversion of Ang I to Ang II can occur in cortical collecting ducts [75]. Thus, the presence of renin in distal nephron segments may synergistically contribute to distal apical sodium transport. Because of the reciprocal regulation of tubular renin in Ang II-dependent hypertension, distal nephron renin may play a crucial role in maintaining the sustained high intrarenal and intratubular Ang II levels, and hence contribute to the progressive hypertension observed in Ang II-dependent hypertension.

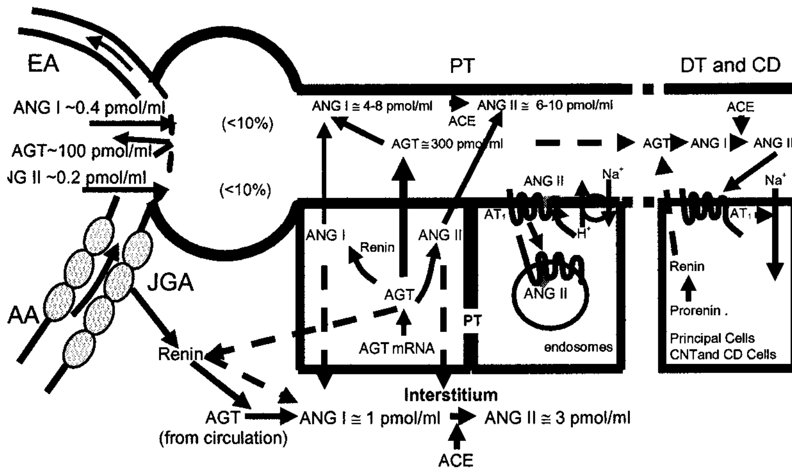

Figure 5. Intratubular/interstitial RAS and distal tubular formation of Ang I and Ang II.

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Chapter 6

SALT LOADING: A PARADIGM FOR A LOCAL CARDIAC RENIN-ANGIOTENSIN-ALDOSTERONE SYSTEM

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Considerable evidence derived from many major epidemiological, interventional, and experimental studies directly relates salt intake and blood pressure (1-10). The effects of dietary salt on exacerbation of hypertension and further increase in left ventricular (LV) mass have been also well-established. Interestingly, a growing body of evidence suggests that dietary sodium excess exerts a potent trophic effect on LV mass that is blood pressure-independent (5,7-9,11-16). This may be very important, since in addition to height of arterial pressure, LV hypertrophy (LVH) is an independent cardiovascular risk factor in hypertension (17-19). However, the mechanisms underlying the risk associated with LVH have been incompletely elucidated. It is likely that a number of factors may contribute, among them ventricular fibrosis, dysfunction and ischemia importantly participate (17-19). The most recent studies from our and other laboratories have shed more light on the relationship between these cardiac structural and functional changes and dietary sodium. Various mechanisms have been suggested to explain the detrimental cardiovascular effects of dietary salt excess besides its effect on hemodynamic load (6-8, 20-23). In response to high sodium intake a suppression of circulating renin-angiotensin-aldosterone system (RAAS) occurs (7,24,25); in contrast an inadequate suppression or even paradoxal activation of local RAAS has been described (24-29). Thus, a pathophysiological role for the local tissue RAAS components in salt induced-cardiomyocyte hypertrophy, exaggerated extracellular matrix protein accumulation as well as ventricular functional disturbances have been increasingly appreciated in recent years. Therefore, this chapter will summarize the cardiovascular structural and functional alterations related

to the dietary salt excess and available evidence supporting the role of tissue RAAS in their development.

SALT EXCESS AND LV STRUCTURE AND FUNCTION

During the last two decades meticulous attention has been paid to the interaction of high dietary sodium intake and LV mass that is independent of hemodynamic load. This consideration is extremely important since only small fraction of hypertensive patients demonstrate sodium-sensitive hypertension. Thus, a growing body of clinical evidence points to salt as a critical independent predictor of LV enlargement in patients with essential hypertension (12-14). Similarly, reports from our laboratory were among the earliest to address this question in spontaneously hypertensive rats (SHR), an excellent experimental model for naturally occurring hypertension. After metabolic and hemodynamic evidence for salt-sensitivity in SHR was shown (6), we determined the cardiac structural response to different levels of dietary salt excess (5). In that study arterial pressure, total peripheral resistance and LV mass progressively increased with higher sodium intake. However, the correlation between the level of sodium intake and cardiac mass was much stronger than the correlation between the arterial pressure and cardiac mass. Equally important, under the same experimental condition, LV mass was increased in WKY as well, although hemodynamics remained unchanged. These results, along with other reports from different laboratories, have added to the concept that salt excess exerts an additional cardiovascular effects besides it effect on blood pressure (7,8,30,31).

More recently, the profibrotic effect of increased sodium intake has been recognized as well (7). Thus, 8% salt loading increased LV collagen accumulation interstitially and perivasculary in both SHR and WKY. In both strains elevated arterial pressure was also demonstrated. However, the results pointed to the clear pressure-independent effects of high dietary salt intake. Indeed, the Wistar Kyoto rats receiving salt excess developed cardiac fibrosis to a similar extent as the untreated spontaneously hypertensive rats, and their systolic pressure was much lower (7). In yet another study, a calcium antagonist reduced the degree of salt-induced collagen deposition in stroke-prone spontaneously hypertensive rats in the absence of blood pressure changes, providing further evidence for the pressure-independent effects of salt (32).

Only recently the functional significance of salt-induced

myocardial fibrosis has been appreciated. The salt-sensitive hypertension of Dahl rats was associated with an extensive LV remodeling, collagen accumulation and severely impaired systolic and diastolic function (33). In our most recent study we particularly examined cardiac functional response to salt excess in SHR and how these changes relate to the LV hydroxyproline concentration as an estimate for collagen content (10). In that study the young and old adult SHR received 8 % salt in diet for the period of 8 and 52 weeks, respectively. During the course of that study some of the young salt-loaded rats developed evidence of heart failure that was confirmed by echocardiography and necropsy examination. These rats developed systolic heart failure manifested by decreased fractional shortening and maximal rate of myocardial contractility (dP/dTmax). Interestingly, all salt-loaded rats developed diastolic dysfunction. The rats that preserved systolic function, either young or old, for the most part, developed ventricular relaxation impairment associated with increased hydroxyproline concentration. Those rats developing systolic failure had impaired ventricular relaxation as well; and they also demonstrated "stiffer" chamber associated with even more pronounced increase in LV mass and collagen deposition. These results identified salt-induced myocardial fibrosis as an important contributor to the observed significant LV functional impairment. Worth noting is that salt excess moderately increased arterial pressure in both ages of SHR but dramatically increased LV mass index and fibrosis only in younger adult rats. The results of this study confirmed our previous findings that development of LVH in response to high dietary salt intake did not reflect solely the pressure overload underlining that some nonhemodynamic factors also participate importantly. Furthermore, these results also suggest that SHR was more susceptible to cardiac damage when high dietary salt is introduced earlier in life. Along with some clinical studies pointing out to the significant association between sodium intake and arterial pressure in newborns (34), these results underline necessity for careful examinations of potential detrimental cardiovascular effects of high dietary salt intake in early life as well.

EFFECTS OF HIGH SODIUM INTAKE ON CORONARY HEMODYNAMICS

Impairment in coronary hemodynamics associated with hypertension has been well-recognized (17). In addition to structural changes (including medial wall thickening with increased wall:lumen ratio, periarterial fibrosis, and a decreased number of small arterioles and

capillaries), endothelial functional abnormalities significantly contribute to the deterioration of coronary hemodynamics associated with hypertension. Extravascular factors such as myocardial hypertrophy and interstitial fibrosis may be responsible as well. It appears that salt excess, through its hemodynamic, but also non-hemodynamic effects, may be associated with each of them. For example, it has been demonstrated that elevated sodium concentration in culture medium induced hypertrophy of both cardiac myoblasts and vascular myocytes by promoting protein synthesis and impeding protein degradation (23) emphasizing blood-pressure independent effects of salt excess. In our recent study chronic salt excess to young SHR impaired significantly coronary vasodilatory response to dipyridamole infusion (35); perivascular and interstitial collagen volume fraction were a astolic function in SHR given salt excess may be, at least in part, due to lso increased. Thus, significant impairment in LV systolic and di joined effects of salt-induced increased collagen deposition and alteration in myocardial perfusion.

DIETARY SALT PROMOTES ARTERIAL STIFFNESS

Central artery stiffness represents an independent risk factor for the development of cardiovascular disease (36). Hypertrophy of large conduit arteries is common consequence of long-standing hypertension and it is frequently associated with accumulation of extracellular matrix protein followed by increase in arterial stiffness. Reduced compliance of large arteries, in turn, precipitates increase in pulse pressure and increases hemodynamic burden to the LV.

It is important to recognize that dietary salt excess plays a significant role in the development of structural and functional abnormalities in large arteries through its hemodynamic, but also non-hemodynamic mechanisms (37). One of the more recent clinical studies conducting in older adults with systolic hypertension, suggested importantly that reduction in dietary sodium intake by improving large elastic artery compliance reduced systolic arterial pressure in these patients. Since the improvement was achieved within first two weeks after the onset of reduced dietary sodium regiment, possible mechanisms were related to the favorable effects of reduced salt intake on augmented bioavailability of different vasoactive peptides and hormones (38).

In addition, strong interaction between high dietary salt intake and structural changes within large arteries such as increased collagen and fibronectin accumulation has been confirmed. Thus, in salt-loaded animals thickening of the large arteries and collagen accumulation

determined increased arterial stiffness (39-42); in some studies the effect of salt on vascular structure was independent of the hemodynamic load (43). Furthermore, structural changes in carotid artery could be prevented in stroke-prone hypertensive rats by lowering sodium intake without any effects on systemic arterial pressure; survival was prolonged as well (44). All of these findings underline the role of dietary salt excess in the pathogenesis of reduced arterial distensibility and its potential involvement in increased risk of target organ injuries and death related to them.

A POTENTIAL ROLE OF LOCAL RAAS IN PATHOGENESIS OF SALT-RELATED CARDIOVASCULAR DISEASE

As it was mentioned above, although an increased hemodynamic load (either volume or pressure) associated with higher dietary sodium intake has been considered as a primary factor responsible for the development of target organ injuries, various nonhemodynamic mechanisms may also contribute significantly. Thus, the function of sympathetic nervous system, the interaction between different vasoactive hormones and growth factors, even the direct effect of sodium per se have been extensively studied in various experimental models of salt-sensitive hypertension (7,16,20-23).

The contribution of endocrine renin-angiotensin system (RAS) comprising angiotensin synthesis in circulating blood is less likely since excessive salt ingestion suppresses plasma renin activity (PRA) and circulating angiotensin II level in most studies (7,24,26). The classic concept of endocrine RAS includes renin, released from kidney, that catalyzes the production of angiotensin I from liver-derived angiotensinogen in circulating blood, which in turns becomes the substrate for angiotensin-converting enzyme (ACE) in the pulmonary circulation converting to an octapetide angiotensin II. This powerful peptide exerts its effect (including aldosterone releasing effect) by binding to specific receptors. However, a large body of recent evidence testifies that each and every component of the RAS has been also identified throughout various tissue and organs (45). Moreover, the most recent studies have challenged the old concept that renin and ACE dependent angiotensin II production solely exists. Identification of new peptides (Ang 1-7 and Ang 1-9), enzymes (cathepsin, chymase, ACE-2) as well as novel receptors (renin receptor, AT1a, AT1b, AT2 receptors) and their functions has enriched our knowledge adding a new insight into

the pathophysiology of RAS in various diseases (for review see 46). Therefore, along the way that existence of a local functioning vs circulating RAS has shifted traditional paradigm in the regulation of arterial pressure as well as fluid and electrolyte balance, the question has been raised regarding deleterious interplay between high dietary salt intake and tissue generated components of this powerful system in the pathophysiology of salt-related cardiovascular and renal disease.

Relatively short-term salt loading induced cardiac ACE mRNA expression and stimulated its enzyme activity that was independent of elevated arterial pressure and changes in angiotensin II plasma levels in SHR and WKY (27). However, arterial pressure and LV mass were increased in SHR, but not in WKY. Neither in SHR (5) nor in Dahl salt-sensitive rats (26) cardiac angiotensin II level changed in response to high dietary salt intake. Although a subtle increase in angiotensin concentration is necessary to elicit its paracrine or autocrine action, further studies are clearly warrant to confirm biological significance of salt-induced upregulation of LV ACE.

Additionally, an increased cardiac AT_1 receptor expression has been described in WKY on high dietary salt intake (28); no change was reported in Dahl salt-sensitive rats (29). Although one can argue that an increase or no suppression in tissue AT_1 receptor expression may be a compensatory response to reduced renin release from juxtaglomerular cells elicited by salt excess, the different regulation of angiotensin receptor expression in different tissue have been described. Thus, it has been shown that sodium load-induced downregulation of vascular AT_1 receptor expression in both Dahl salt-sensitive as well as salt-resistant rats (47), suggesting that vascular AT_1 receptor regulation may not be involved in salt-sensitive hypertension in this strain. In contrast, the greater increase in AT_1 receptor expression in brain in response to salt excess in Dahl salt-sensitive when compared with Dahl salt-resistant rats may drive, at least in part, salt-sensitive hypertension in this strain. Therefore, it seems that under the condition of dietary salt excess local angiotensin effects may as well vary from one tissue to another by differences in the expression of its receptors.

Development of specific angiotensin receptor antagonists provides an excellent tool in examining the role of RAS in the pathogenesis of various diseases. They have been also extensively used in an attempt to verify the contribution of local RAS in salt-related cardiovascular alterations in the face of suppressed circulating RAS. Indeed, the growing body of evidence has shown that AT_1 receptor blocade given concomitantly with high dietary sodium intake ameliorated or even prevented abnormal LV relaxation and compliance

in different models of salt-sensitive hypertension, interestingly without reduction in blood pressure. Suppressed gene expression of collagen I as well as reduced cardiac collagen content and collagen cross-linking were associated with observed attenuation in myocardial stiffening (48, 49). It should thus not come with surprise that treatment with AT_1 receptor antagonist improved coronary resistance in DOCA/salt treated rats without any antihypertensive effects (50). Our preliminary results demonstrated as well that the AT_1 receptor antagonist candesartan, ameliorated salt-related structural and functional cardiovascular abnormalities in SHR without preventing the rise in arterial pressure (Figure 1) implying the role of cardiac RAS in these deleterious effects of salt excess.

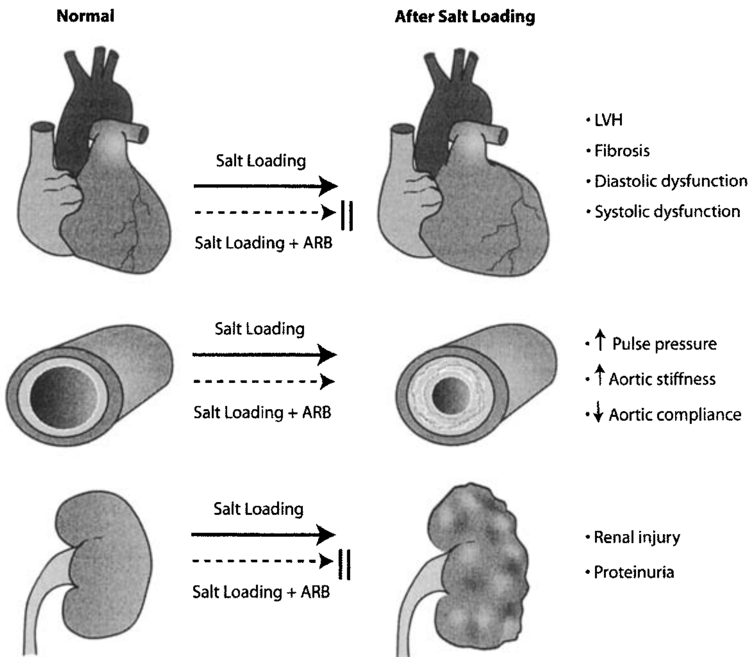

Figure 1. Effects of high salt diet (8%) and AT_1 receptor blockade (ARB) on the heart, large arteries and kidney. ARB given concomitantly with high dietary salt excess ameliorated salt-related structural and functional cardiovascular and renal abnormalities in SHR without preventing the rise in arterial pressure implying the role of tissue RAS in these deleterious effects of salt excess.

Angiotensin II exerts also aldosterone stimulating effects through its binding to specific receptors in the target organ cells that may include adrenal cortex but also extra-adrenal sites (51-53). Beside the well-known role of aldosterone in the regulation of sodium homeostasis and blood pressure control its growth promoting effects have been extensively investigated as well (54). It has also been shown that dietary salt excess decreased plasma aldosterone; however it also stimulated cardiac aldosterone synthesis associated with moderate cardiac hypertrophy in normotensive rats (25). On the other hand, treatment with an aldosterone antagonist reversed cardiac hypertrophy and fibrosis and improved cardiac function in some experimental models of salt-sensitive hypertension (55-57). It also prevented the development of malignant nephrosclerosis and cerebrovascular lesions in saline-drinking stroke prone SHR in the absence of blood pressure lowering effects (58). It seems, therefore, that salt excess increases local aldosterone production, which plays an important role in pathogenesis of associated cardiovascular lesions independently of the circulating RAAS.

CONCLUSION

A growing body of evidence suggests that dietary sodium excess, besides its effect on arterial pressure, exerts potential to affect target organs in blood pressure-independent manner. Although higher sodium intake reduces the activity of circulating RAAS, inadequate suppression or even activation of the local, tissue RAAS may significantly contribute to the salt-related cardiac structural and functional disturbances.

Acknowledgments

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Chapter 7
LESSONS FROM EXPERIMENTAL GENERATION OF INTRACELLULAR ANGIOTENSINOGEN AND AII

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INTRODUCTION

A large body of evidence has accumulated, principally over the last decade, favoring the existence of both intracellular AT_1 receptors (AT_1R) and angiotensin II (AII).[3-5] However, the function and consequence of intracellular receptor or receptor-ligand complexes has, until recently, remained ambiguous. Using two alternative approaches, we have, of late, shown that AII, generated in an intracellular fashion, can enhance proliferation of cells that express the AT_1R.[2,3] AII generated in an intracellular manner can also alter the distribution of the AT_1R. In several cell types that we have investigated, the AT_1R (visualized as a fluorescent fusion protein) is present in the endoplasmic reticulum and Golgi, and on the plasma membrane. Intracellular generation of AII is accompanied by a redistribution of the cognate AT_1 receptor; plasma membrane associated receptor is diminished and receptor accumulates in the nucleus. The following reviews the approaches that we have used to (1) generate intracellular AII and AT_1R, (2) establish that the intracellular AII is retained and is functionally active within cells that express the corresponding receptor, (3) demonstrate ligand-mediated receptor trafficking, and (4) explore signaling mechanisms involved in altered growth kinetics.

MATERIALS AND METHODS

pPreAogen/ pPreAogen$_{ATC}$

Rat preangiotensinogen was PCR amplified using upstream 5'-GATCGACTCGAGGCCACC**ATG**ACTCCCACGGGGGCAGGC-3' (ATG start site underlined), downstream primer [Ang(-S)D3] 5'-ACCTATCGAGACCGAGGTACCTGCACCACATTTTGGGGGTTAT C-3' and the rat Aogen clone, gift of K. Lynch.[6] ATG^1-to-ATC mutant preangiotensinogen was PCR amplified using upstream 5'-GATCGACTCGAGGCCACC**ATC**ACTCCCACGGGGGCAGGC-3' and downstream Ang(-S)D3. Products were digested with Xho I/Kpn I and ligated to Xho I/Kpn I-digested pEGFP-N1 (Clontech, Palo Alto CA) in order to generate pPreAogen/EGFP and pPreAogen$_{ATC}$/EGFP.

pAT$_1$R/DsRed2

An ~1100 bp fragment containing the AT$_{1a}$ receptor (Genbank accession #NM_030985) was RT/PCR amplified from rat liver RNA using upstream primer 5'- GATCGAAAGCTTGCCACC**ATG**GCCCTTAACTCTTCTGCT -3' and downstream primer 5'- CGAGACCGAGGATCCTGCTCCACCTCAAAACAAGACGC – 3'. The product was 1) digested with Hind III/Bam HI, ligated to Hind III/Bam HI-digested pDsRed2-N1 and designated AT$_1$-R/DsRed2, and 2) digested with Hind III/Bam HI, ligated to Hind III/Bam HI-digested pEYFP-N1 and designated AT$_1$R/EYFP.

pFarn/DsRed2

pEGFP-F encodes a farnesylated EGFP (Clontech, Palo Alto CA). It contains the 20 amino acid farnesylation signal from c-Ha-Ras fused to the C-terminus of EGFP and binds to the inner face of the plasma membrane [7,8]. To generate pFarn/DsRed2, the farnesylation sequence was amplified from pEGFP-F using upstream primer 5'- GATCGAAAGCTTGCCACC**ATG**AAGCTGAACCCTCCTGAT – 3' and downstream primer 5'- CGAGACCGAGGATCCTGGGAGAGCACACACTTGCAGCT-3'. The PCR amplification product was digested with Hind III and Bam HI and ligated to Hind III/Bam HI-digested pDsRed2-N1. The resulting pFarn/DsRed2 possesses the farnesylation peptide fused to the N-terminus of DsRed2.

pNrf2-FL/EGFP

The complete coding region of the mouse Nrf2 cDNA [9] was ligated downstream of the EGFP gene in the vector pEGFP-C3 to generate pNrf2-FL/EGFP

Enzyme Immunoassay

Angiotensin II levels were measured using a competitive enzyme immunoassay (Phoenix Pharmaceuticals Inc., Belmont CA) in which a biotinylated AII peptide competes with peptide in standard solution or samples as described.[2]

RESULTS AND DISCUSSION

I. Intracellular AII: Is AII produced within cells or transported into cells?

A number of studies suggest that intracellular AII, AT_1R and/or AII:AT_1R receptor:ligand complexes exist and may be functionally active.[3-5] In the conventional paradigm, angiotensinogen (Aogen), generated from preangiotensinogen, is channeled through the secretory pathway and processed to AII extracellularly. Since receptors for uptake of AII have not been identified, the existence of intracellular AII necessitates an alternative paradigm. Three possible paradigms follow.

(1) Paradigm 1: Intracellular AII is generated from an alternative form of non-secreted angiotensinogen. Alternative translation products, resulting in extracellular and intracellular active peptides exist for many different proteins. Often these are generated through alternative translation start site use.[10-14]

The AI/AII encoding portion of preangiotensinogen lies immediately downstream of the signal peptide-encoding region. Signal peptides contain hydrophobic core domains and, acting as biological addresses, direct proteins to the ER translocon.[15,16] Signal peptide truncation by alternative translation start site selection could produce a non-secreted form of angiotensinogen. Indeed Corvol and colleagues[17-19] have found cell-free translation of rat liver RNA in a rabbit reticulocyte lysate sytem to yield two products (around 52,500 and 55,700). These

products are reduced to a single electrophoretic band upon treatment with renin. *Therefore, the investigators have suggested that the size difference must reflect a difference in the pre-region (signal peptide).* Examination of the DNA sequence between the transcription start site and the AUG(Met[1]) translation start site, reveals the existence of no additional in-frame AUGs. Since there also exist no in-frame AUG codons within the signal peptide (excepting, of course, the classically identified AUG at position 1), we asked whether translation might be initiated at a non-classical or non-canonical codon. *Our inspection of the signal sequence of the Aogen precursor indicates the existence of three in-frame CUGs (Fig. 1). A number of studies show that non-canonical CUG sequences may function as alternative translation start sites.* All three of the CUG codons in the Aogen signal-encoding sequence are present in the context of a perfect Kozak consensus sequence [PuNN<u>AUG</u>Pu, where conservation of the purines (Pu) is critical,[20]]. This context has been found to be crucial for non-AUG as well as AUG start site codons.[21]

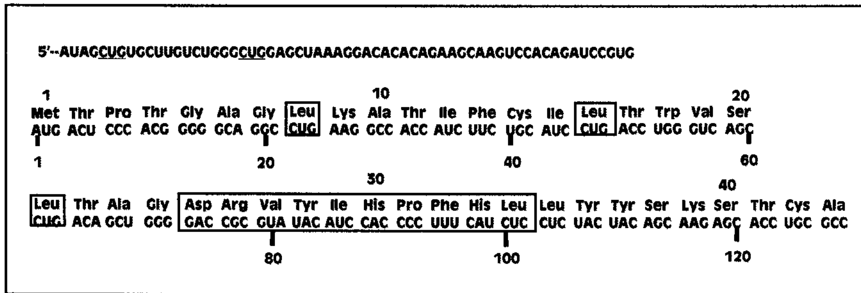

Figure 1. The 5'-end of the rat Aogen transcript. AI/AII-encoding region and downstream in-frame CUG(Leu) codons are indicated in boxes. Upstream in-frame CUGs are underlined.

Non-AUG translation start codons have been found to generate proteins of bFGF, FGF-3, c-myc 2/c-myc 1 among others.[10-12,14] Translation of these mRNAs can start at AUG or an alternative codon (the most commonly used of which is CUG) and the choices often determine the subcellular localization of the resulting proteins. Most notably, the parathyroid hormone-related peptide transcript, the protein of which may be secreted or localized to the nucleus, possesses in-frame non-AUG (CUG and GUG) codons within the signal peptide (as does the pre-Aogen transcript).[13] Use of these alternative translation start sites results in loss of the signal peptide and retention of the translated PTHrP. Since a nuclear localization signal lies mid-region in the PTHrP protein, those peptides that are not secreted are necessarily localized to the nucleus. Therefore, PTHrP is either secreted or translocated to the

nucleus based on translation start site choice. By comparison, human bFGF is either *retained in the cytoplasm* or transported to the nucleus based upon alternative translation start site usage.[10]

We performed theoretical translations of the Aogen precursor from each of the three in-frame CUGs within the signal peptide. Theoretical translation from the first (most 5') in-frame CUG [CUG(Leu^8)] results in elimination of only seven amino-terminal amino acids. A cleavable signal peptide is still recognizable in this protein using the internet search tool, PSORT (http://us.expasy.org/). The second in-frame CUG [CUG(Leu^{16})] results in elimination of the first 15 amino acids and the theoretical translation product shows no N-terminal signal peptide by either of two different signal prediction methods (PSG and GvH:von Heijne's method). Indeed, this translation product is predicted to be primarily cytoplasmic by Reinhardt's method and the k-NN prediction. The predicted size of the full-length pre-Aogen product is 52 kDa and that translated from the second CUG of the signal peptide is 50.5 kDa (using Peptide Mass program, Expasy).

We asked, therefore, whether any of the alternative in-frame CUGs in the signal peptide might be used to generate a non-secreted angiotensinogen. **A common method for investigating potential alternative translation start sites which might be active in specific cell types and/or under particular conditions is mutation of the primary translation start site or candidate alternative sites followed by examination of the transfected DNA translation products.** Therefore, we mutated the archetypal ATG at position 1 to a non-initiator ATC in order to select for translation from alternative potential start sites. PreAogen$_{ATC}$ was then ligated upstream of EGFP in an EGFP expression plasmid. A major alternative protein product (~ 68 kDa) is generated from expression of this construct in COS cells (Figure 2). As the EGFP moiety is 30 kDa, the Aogen moiety is necessarily ~38 kDa and is generated from a start site considerably downstream from the AI/AII encoding portion of the mRNA. ATG(Met^{99}) is present in the context of a Kozak consensus sequence and theoretical translation of this product (Aogen $^{99\text{-}477}$) is consistent with the size of our major protein product by Western blot. However, a minor product of 80 kDa is also generated. Deduction of the 30 kDa GFP moiety size, provides for an Aogen moiety of 50 kDa, a size consistent with generation of an alternative product from the second in-frame CUG. This product would, in theory, both be retained intracellularly and possess the AI/AII-encoding region. **Note that we do not know whether either of these products is formed in native cells.** *However, the larger of these products is consistent with intracellular Aogen and AII generation*

through alternative start site use. It is also possible that the alternative translation product Aogen$^{99\text{-}477}$ is generated under some conditions and may have functional relevance; since this product does not contain the AI/AII sequence, however, any function would necessarily be independent of intracellular AII.

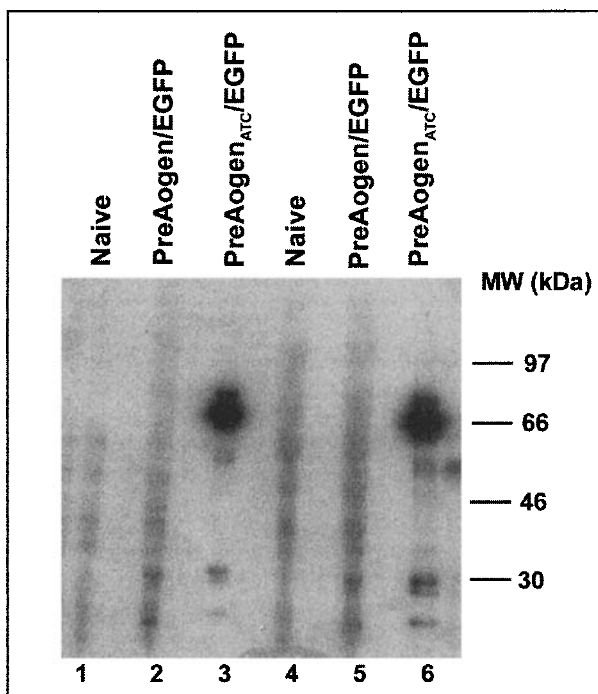

Figure 2. Western blot of COS cell extracts 48 h post-transfection with indicated plasmids. Blots were screened with anti-GFP antibody (Santa Cruz Biotech. Inc.) and HRP-conjugated rabbit anti-IgG (ECL kit). The band sizes of the preAogen$_{ATC}$/EGFP products, by regression analysis are 80 and 68 kDa. Assuming a GFP moiety size of ~30 kDa, these correspond to Aogen sizes of 50 and 38 kDa. Note that no product is observed in cells transfected with pPreAogen/EGFP. Indeed, we find preAogen/EGFP to be rapidly trafficked through the secretory pathway; Aogen/EGFP is not strongly detectable in COS cells using fluorescence microscopy either, except following application of a cold block.[1] Lanes 1, 2: 50 ug; Lanes 3, 6: 1 ug; Lane 4: 100 ug; Lane 5: 90 ug protein.

Do we have any evidence for intracellular retention of a native or experimentally unaltered form of Aogen? Our prior published studies show that Aogen/EGFP is not visible within cells at *24 h post-transfection* except upon application of a cold block. Cold-induction slows the rate of transport of Aogen through the secretory pathway

permitting accumulation to visible levels. However, at *48 h post-transfection*, even in the absence of a cold-block, a low-level of diffuse cytoplasmic fluorescence is observed in COS and CHO-K1 cells. This may argue for the presence of an alternative, intracellular form of Aogen in these cells; this form of Aogen could accumulate to higher levels depending upon the cell type and culture or *in vivo* conditions.

(2) Paradigm 2: Intracellular AII is generated from angiotensinogen after internalization of angiotensinogen from the extracellular space. The recent report of the existence of an Aogen receptor on the placenta-derived cell line, CRL-7548,[22] provides evidence in favor of this model. Furthermore, it has been shown that AII and renin coexist in the storage granules of kidney juxtaglomerular cells. These cells do not express angiotensinogen and an AT_1 receptor blocker does not block intracellular AII accumulation; Mercure and colleagues,[23] therefore, suggest that angiotensinogen may be internalized into JG cells. In addition, Peters and associates[24] have demonstrated that adult cardiac myocytes internalize unglycosylated prorenin after which intracellular angiotensins are generated. In corresponding control experiments they show that angiotensins supplied in the culture medium are not internalized; intracellular angiotensins are likely generated from intracellular angiotensinogen. This is certainly an area that merits further investigation.

(3) Paradigm 3: Intracellular AII is present and active in cells after receptor-mediated internalization and escape from the endosomes. Endosomal escape is a well-established but poorly understood concept. There exists a general consensus that polycation-DNA complexes enter cells via endocytotic pathways and that endosomal escape into the nucleus precedes DNA expression, but the technicalities are yet undefined. Presumably, receptors or receptor:ligand complexes can similarly escape endosomes and enter the nucleus and, indeed, the mechanisms by which this might occur have been discussed at length (for review[25-27]). A nuclear localization signal (NLS) consensus homology, which appears to be functional by competitive peptide studies, has been identified in the cytoplasmic tail of the AT_1 receptor and may play a role in nuclear localization of the receptor and/or receptor:ligand complex.[28]

Regardless of the mechanism by which AII is generated or imported, it does accumulate to significant levels within cells. We sought, therefore, to identify the functional significance of intracellular AII by devising experimental methods for generation of intracellular AII

exclusive of extracellular AII.

II. Strategies for experimental generation of intracellular AII

a. Ang(-S)Exp/pSVL, Ang(-S)Cntr/pSVL

To investigate the potential for (and subsequent effect of) Ang II generation within cells, we mutated a rat Aogen cDNA and ligated it into an expression plasmid (pSVL) to produce a nonsecreted (-S) form of Aogen (Figure 3A).[3] Ang(-S)Exp lacks the N-terminal signal sequence required for secretion. Ang II produced from this protein is, in theory, generated through an intracrine mechanism and processing of Ang(-S)Exp to AII requires intracellular renin and ACE or equivalent enzymes. The corresponding control Ang(-S)Cntr lacks both the signal sequence and part of the AI/AII-encoding portion. We have shown that the fluorescent fusion products of both of these proteins are retained intracellularly and accumulate to high levels.[1]

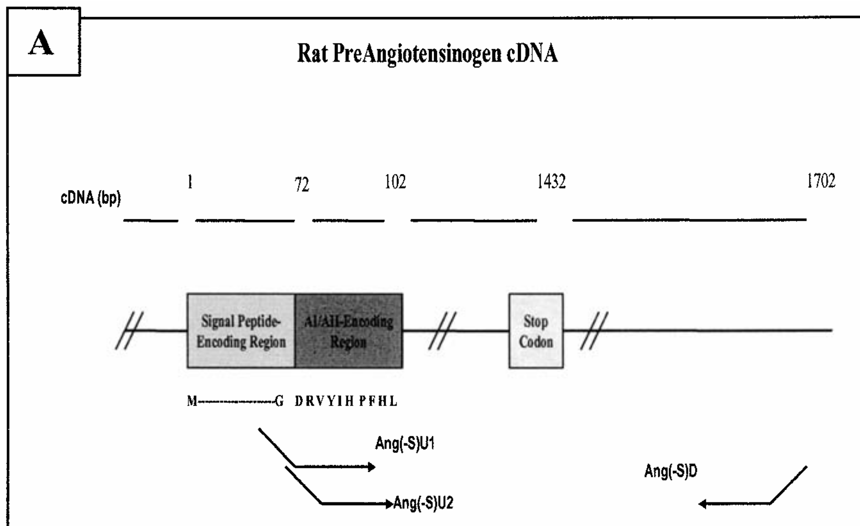

Figure 3. (A) Illustration of the rat preAogen cDNA, corresponding protein domains/features and relative positions of amplification primers used to generate Ang(-S)Exp and Ang(-S)Cntr products. [Upstream primer = Ang(-S)U1, downstream primer = Ang(-S)D, PCR product = Ang(-S)Exp]. [Upstream primer = Ang(-S)U2, downstream primer = Ang(-S)D, PCR product = Ang(-S)Cntr]. Ang(-S)Exp represents a mutated Aogen which lacks a signal sequence (non-secreted) but possesses an intact AI domain. Ang(-S)Cntr represents an Aogen clone with a mutated AI domain. The signal peptide extends from M (methionine at amino acid position 1) to G (glycine at amino acid position 24).

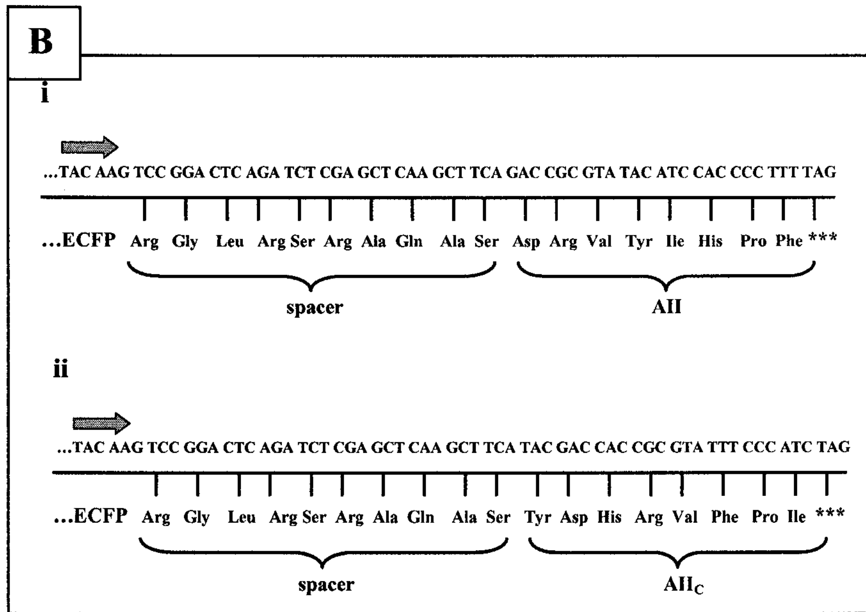

Figure 3. (B) Illustration of expression plasmids encoding fluorescent fusion proteins. pECFP/AII (i) and pECFP/AII$_C$ (ii) (encodes scrambled control of AII).[2]

b. ECFP/AII

In order to determine the effects of intracellular AII on COS-7 and CHO-K1 cells we designed and generated a fusion construct of the AII peptide-encoding sequence with enhanced cyan fluorescent protein (blue fluorescence) (see Figure 3B for plasmid illustration). As a control, we prepared a similar plasmid encoding a peptide of scrambled AII sequence fused to ECFP (Figure 3B). In each case, the encoded peptide is separated from ECFP by a ten amino acid spacer arm. ECFP/AII proved to be reactive to both anti-AII and anti-EGFP antibodies as determined by western blot confirming the presence and integrity of both domains in the expressed fusion protein.[2]

Three-dimensional models for AT_1R in complex with AII[29-31] suggest that the peptide ligand is at least partially buried in the receptor binding pocket suggesting that the ligand might function poorly as a fusion protein. However, Hunyady and colleagues[32] have shown that an AII-labeled fluorophore which possesses, at the amino-terminus, the bulky multi-aromatic ring structure, rhodamine, retains functional integrity. In addition, Yadav and associates[33] have investigated at length the specific properties of rhodamine conjugated at the Lys^3 (substituted for Val) position of AII and show it to be biologically active, to bind to

the receptor and to be internalized efficiently. Consistent with these observations, in the present study, N-terminal ECFP-conjugated AII appears to retain intracellular biological activity.

While a number of studies have utilized AT_1R fusion proteins with variable results, to our collective knowledge, this is the first study to employ an angiotensin II fluorescent fusion protein and to monitor its effects upon receptor distribution and signal transduction.

III. Growth kinetics of transfected cells

a. Expression of intracellular AII in cells which express native receptor

We have shown that intracellular AII expressed as Ang(-S)Exp is growth stimulatory for cells which possess native AT_1R and enzymes required to process Aogen to AII.[3] Expression plasmids *Ang(-S)Exp/pSVL* and *Ang(-S)Cntr/pSVL* were transiently transfected into H4-II-E-C3 rat hepatoma cells and mitotic indices measured at 48 h post-transfection. *Ang(-S)Exp/pSVL* consistently increased nuclear labeling by approximately 20% ($p < 0.05$) in six separate experiments. The control plasmid, *Ang(-S)Cntr/pSVL*, had no effect upon nuclear labeling. Stably integrated *Ang(-S)Exp/pSVL* increased labeling an average of 33% ($p < 0.001$) over *Ang(-S)Cntr/pSVL*.

Anti-AII antibodies completely suppressed exogenous AII-induced proliferation of H4-II--E-C3 naive cells but failed to inhibit growth of *Ang(-S)Exp/pSVL* stably-transfected cells verifying that Ang II generated by the transgene is not exported and does not act through an extracellular mechanism. Furthermore, we show by AII EIA that Ang(-S)Exp expression in H4-II-E-C3 cells substantially enhances intracellular AII levels while no increase is detectable in the medium (Figure 4).

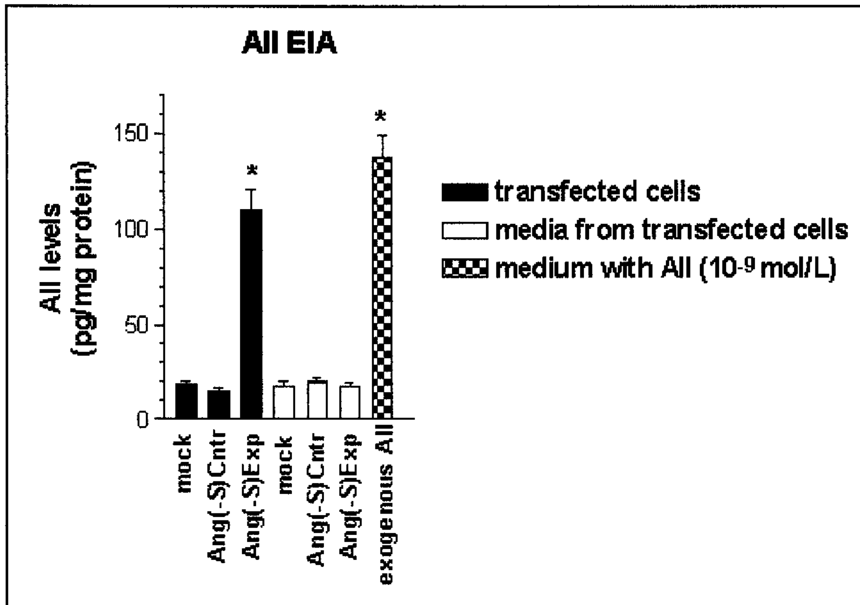

*Figure 4. Cellular AII levels were determined in mock-H4-II-E-C3 cells and those stably transfected Ang(-S)Exp/pSVL or Ang(-S)Cntr/pSVL using Phoenix Pharmaceutical EIA kit (linear range of 0.1 – 0.8 ng/ml) with human antibody. Percent cross-reactivity to AI = 1.7. *p < 0.0001 compared to mock-transfected. Note that corresponding media possess no detectable AII. AII is, however, detected when added exogenously.*

b. Co-expression of intracellular AII with transfected receptor

In order to both identify biological effects of intracellular AII:ligand receptor interactions and to visualize trafficking of the receptor, we constructed and compared expression plasmids encoding the AT_1R fused to either the red fluorescent protein, DsRed2 (*Discosoma* red gene from the IndoPacific sea anemone) or the yellow fluorescent protein, EYFP [enhanced yellow fluorescent protein (a variant of EGFP)]. We found the red fusion protein to be inaccurately targeted. Rather than targeting the ER, golgi and plasma membrane, as does the AT_1R/EYFP, the DsRed2 fusion protein appears to be trapped within the perinuclear ER and golgi (Figure 5a). DsRed2 is inherently capable of navigating to the plasma membrane; we show that farnesylated DsRed2 (the c-Ha-Ras farnesylation sequence targets fused moieties to the plasma membrane) advances to the plasma membrane efficiently in CHO and COS cells (Figure 5b). The erroneous trafficking of GPCR:DsRed2 complexes or aggregates is clearly a function of the GPCR moiety or a property of the fusion moiety. This suggests that DsRed2 may be limited in its general usefulness for GPCRs.

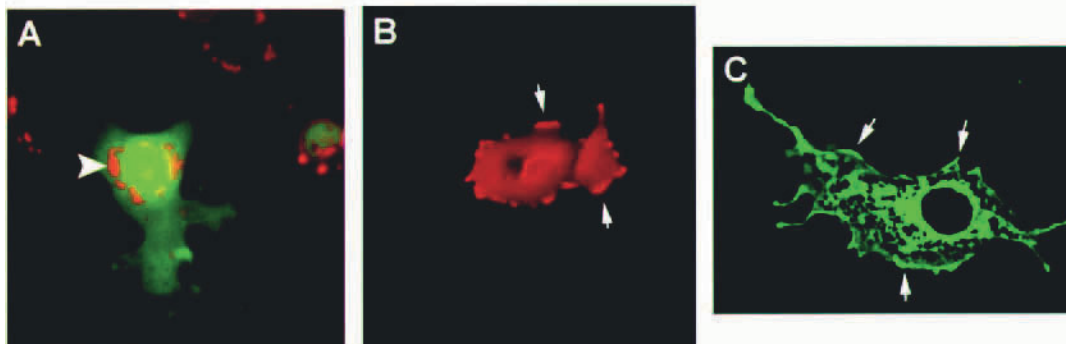

Figure 5. DsRed2 fusion protein of the AT_1R is inaccurately targeted following transfection. Fluorescence microscopy of COS-7 cells, 96 h post-transfection with pAT_1R/DsRed2 (arrowhead) and pNrf2-FL/EGFP (nuclear marker) (A), or pFarn/DsRed2, a plasma membrane marker (arrow) (B). Deconvolved image of COS-7 cells, 96 h post- transfection with pAT_1R/EYFP (arrow) (C). Arrows show fusion proteins associated with the plasma membrane.

AT_1R/EYFP, in contrast, does show a biologically authentic distribution (Figure 5c). Moreover, intracellular expression of ECFP/AII leads to intracellular retention of AT_1R/EYFP.[2] Co-expressed ECFP/AII causes a change in the steady-state distribution of the receptor fusion protein; specifically, we find co-localization of AT_1R/EYFP and ECFP/AII in the nuclei of COS cells.[2] pECFP/AII cotransfected with pAT_1R/EYFP increases proliferation as determined by BrdU incorporation into nuclei by >35% in COS-7 and CHO-K1 cells ($p < 0.001$). Anti-AII antibodies added to the culture medium have no effect verifying that growth stimulation is not caused by increased AII-antibody immunoreactive material in the culture media. The control plasmids, pECFP, pEYFP had no effect upon growth. Similarly, pECFP/AII, pECFP/AII_C, pAT_1R/EYFP, transfected independently, had no effect upon nuclear labeling.

In a comparable fashion, COS-7 and CHO-K1 cells were stably transfected with pAT_1R/EYFP and then treated with exogenous AII, AII + losartan or AII + anti-AII antibody for comparison.[2] AII increases the percentage labeled COS-7 and CHO-K1 cells by 56% and 70% respectively ($p < 0.001$ compared to vehicle), the increases of which were completely inhibited by anti-AII antibodies and losartan.

IV. Downstream targets of intracellular AII.

a. PDGF

We have shown that intracellular AII mediates its growth stimulatory effect in COS and CHO-K1 cells, at least in part, through stimulation of PDGF A-chain transcription and upregulation of PDGF secretion;[1] anti-PDGF antibodies partially inhibit growth of Ang(-S)Exp transfected rat hepatoma cells. Moreover, while H4-II-E-C3 rat hepatoma cells possess mRNAs for both long and short PDGF A-chain peptides, the long form is specifically upregulated in cells that express intracellular Aogen/AII. A number of studies collectively suggest that PDGF long isoform is upregulated in mitogenesis, acute wounds, tumorigenesis and other disease processes.[34-36]

b. cAMP regulatory element-binding protein

Intracellular co-expression of ECFP/AII with AT_1R/EYFP alters receptor fusion protein distribution and enhances cell proliferation. We investigated, therefore, the effects of co-expression of these proteins upon downstream signaling pathways. Classic AII/AT_1R signaling is known to occur through a number of pathways. Since extracellular AII is believed to promote phosphorylation of the transcription factor, CREB, in some systems,[37-39] we compared CREB phosphorylation activation in COS-7 and CHO-K1 cells transiently transfected with *pECFP/AII* and *pAT_1R/EYFP*.[2] We also compared CREB activation in COS-7 and CHO-K1 cells stably transfected with *pAT_1R/EYFP* and treated with exogenous AII. Coexpression of *pECFP/AII* and *pAT_1R/EYFP* in COS or CHO-K1 cells, activates CREB 5 - 6 -fold ($p < 0.001$) compared to control (*pECFP* or *pEYFP*). Extracellular AII treatment similarly activates CREB phosphorylation 4 – 5-fold ($p < 0.001$) in AT_1R stably-transfected COS-7 and CHO-K1 cells. These results suggest that both endogenous AII (as ECFP/AII) and exogenous AII, in CHO-K1 cells and COS-7 cells, possess the ability to activate AT_1R/EYFP and stimulate CREB phosphorylation.

SUMMARY AND FUTURE DIRECTIONS

The tools that we describe in this report are particularly useful in determining the effects of intracellular AII activity in an array of native physiologically relevant cell types and in transgenic animals. Presently, a more detailed understanding of the mechanisms by which intracellular AII accumulates and the circumstances by which it accumulates, as well

as identification of the signaling pathways involved in intracellular AII-stimulated cell growth may be useful in clinical intervention. Further information regarding intracellular trafficking pathways for AII and its cognate receptor, and nuclear functions may be useful in recognizing tissues and conditions in which intracellular AII may be active, and in disrupting intracellular AII-mediated functions.

It also should be noted that these studies support the proposition that angiotensin II, like many other peptide hormones and growth factors, acts in an intracrine fashion. By intracrine is meant the intracellular functioning of an extracellular signaling peptide, whether that intracellular action occurs following internalization from the extracellular space or retention in the cell which synthesizes the peptide. Thus defined, intracrine action is displayed by a wide variety of peptide hormones and growth factors. Indeed, various enzymes (for example, phosphoglucose isomerase, and renin) and transcription factors (for example, engrailed) also display intracrine functionality, meaning they demonstrate the capacity to act as intracellular signaling molecules. We have proposed that intracrine action serves functions other than simply refining hormone action at a target cell. For example, intracrines often form intracellular feedback loops within cells and also frequently interact with ribosomal RNA. This led to the suggestion that peptide intracrines developed to coordinate trophic cellular events with ribosomal biology and to produce a memory of the original trophic stimulus through the production of intracellular feedback loops. A second hypothesis suggests that early in metazoan evolution, intracrines exited cells to signal at nearby cells, thereby coordinating tissue-wide differentiation. Thus modern intracrines evolved to serve roles in cellular differentiation, memory, and hormonal responsiveness.[4,40,41] The intracrine view thus described leant itself to a variety of predictions. One was that the tumor suppressor protein p53 could function as an intracrine.[4,41] Recently developed evidence dealing with the activity of extracellular p53 fragments supports this hypothesis.[42] Similarly, the intracrine view suggested that the homeotranscription factor PDX-1 could function as an intracrine, as is the case with other homeoproteins.[4,41] The recent demonstration that the application of PDX-1 to pancreatic duct cells mimicked the intracellular action of PDX-1 to induce islet cell differentiation is consistent with this suggestion.[4,41] Moreover, the recent demonstration of angiotensinogen trafficking to glial cell nuclei is consistent with the suggestion that angiotensinogen is an anti-angiogenic serpin intracrine.[40,43] These notions regarding an expanded view of intracrine action notwithstanding, our data along with recent evidence indicating a direct effect of intracellular angiotensin II to induce

hypertrophy in cardiac myocytes and the demonstration of likely intracrine actions of renin and angiotensinogen strongly support the relevance of intracrine action in cardiovascular and other tissues.[4,40,41,43,44]

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Chapter 8
ON THE CARDIAC RENIN ANGIOTENSIN SYSTEM: THE HEART AS A SOURCE OF ANGIOTENSIN II

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Evidence is available that angiotensin II (Ang II) regulates heart contractility (Koch Weser, 1995), cell coupling and impulse propagation (De Mello, 1994; De Mello, 1996; De Mello et al, 1997; De Mello, 1999) and is responsible for remodeling and the induction of apoptosis (Horiuchi et al, 1999). Moreover, local renin angiotensin systems have been described in heart and other tissues (Dzau 1987).

The synthesis of some of the RAS components inside the heart cell is however, controversial. The levels of cardiac renin are extremely low in nephrectomized animals (Katz et al.1997) suggesting that cardiac renin is dependent upon uptake from plasma at least in the normal heart (Danser et al, 1994; Campbell, Valentin 1994). Furthermore, the source of angiotensinogen in the heart, for instance, is not known. Although angiotensinogen is shown in the heart, no angiotensinogen is released from the perfused rat heart (de Lannoy et al, 1997) and no angiotensinogen is present in the supernatant of serum deprived neonatal cardiac cells (van Kesteren et al, 1999) what suggests that cardiac angiotensinogen is coming from the circulation. Similar observations have been published with respect to cardiac renin. Indeed, renin and renin mRNA levels in normal heart tissues are very low or even undetectable (de Lannoy et al, 1997; van Kestern et al, 1999) suggesting that, under normal condictions, cardiac renin is coming from plasma through diffusion in the interstitial space. Recently, renin receptors have been described in several tissues (Nguyen et al 2004).

The contribution of plasma renin to cardiac renin was tested by

using transgenic mice expressing human renin in the liver (TTRhRen-A3) which were mated to mice expressing human angiotensinogen exclusively in the heart (MHChAgt-2). The results indicated low or undetectable angiotensin peptide in the heart of single transgenic animals while double transgenic mice showed a remarkable increase in cardiac levels of Ang I and Ang II, indicating that plasma renin is able to act on its substract within the heart (Prescott et al, 2000).

However, it has been demonstrated that in some tissues a second renin gene transcription start site can be utilized leading to the synthesis of renin but lacking the secretory signal peptide. Initially found in the brain, the non-secreted transcript was found also in the myocardium, particularly during myocardium infarct (Clausmeyer et al 2000), when the cardiac renin angiotensin system is activated. Indeed, the expression of exon 1A-renin mRNA in the left ventricle was found to be stimulated 4-fold during myocardial ischemia supporting the view of an intracellular function of renin (Clausmeyer et al, 2000).

Other studies indicated that overexpression of angiotensinogen gene in normal heart muscle cells of mice, for instance, caused an increase of angiotensin II concentration in the right and left ventricles and elicited hypertrophy of both ventricles without any change in arterial blood pressure (Mazzolai et al, 1998). These observations substantiate the notion of a cardiac renin angiotensin system at the level of cardiac myocytes. Upregulation of cardiac ACE mRNA as well as protein has been reported in salt-sensitive types of hypertension supporting the view that the cardiac RAS is in parte responsible for the detrimental action of salt-overload (Zhao et al 2000).

Evidence of an intracrine renin angiotensin system has been previously described (De Mello 1994; De Mello, Danser 2000; De Mello, Re 2003; Cook et al 2001) and is particularly important under pathological conditions when an increased expression of renin and angiotensinogen genes seems to occur in cardiac muscle. Evidence has been presented, for instance, that stretch of myocardial fibers enhanced the expression of theses genes (Malhotra et al 1999; Tamura et al 1998). On the other hand, it is known that ACE and Ang II, at cardiac sites, are increased after myocardial infarction and ventricular hypertrophy induced by pressure-overload (Yamada et al, 1991). Cardiac Ang II generation increases the expression of renin, AOGEN, AT1 and AT2 forming a positive feedback loop (Tamura et al 1998). The left ventricular hypertrophy induced by aortic coarctation leads to an increase of renin and AOGEN mRNA in the left ventricle while the renin levels in plasma are only transiently elevated (Baker et al, 1990). The major question remains: which is the physiological or physiopathological

meaning of a cardiac renin angiotensin system and particularly of an intracellular system?

It is known that intercellular communication is impaired in the failing heart (De Mello, 1999). Indeed, the values of gap junction conductance measured in cell pairs isolated from the failing heart of cardiomyopathic hamsters, showed areas in which the gap junction conductance was extremely low (0.8-2.5 nS) and incompatible with impulse propagation (De Mello, 1996).

The activation of the plasma renin angiotensin system during the process of heart failure, is largely responsible for the impairment of heart function and the remodeling of the ventricle (Dzau, 1987; Lindpaintner et al, 1990). Furthermore, the activation of a local renin angiotensin in the failing heart (see for review De Mello, Danser 2000), might be implicated in cell abnormalities seen during this condition.

IS ANGIOTENSIN I CONVERTED AT HEART CELL MEMBRANE?

ACE is expressed in many cells especially in endothelial cells and consists of a cytosolic domain, a hydrophobic transmembrane domain and an extracellular domain subdividied in two homologous domains: a residue N and a domain called C (Soubrier et al 1988). Although molecular cloning has provided details about the function and structure of ACE, several questions remain about the functions of the enzyme in the heart.

Previous observations of de Lannoy et al (1994) showed that Ang I is converted to Ang II at the interstitium of cardiac tissue. Recently, we found that Ang I is converted to Ang II at the level of cardiac cell membrane. Indeed, administration of Ang I to the extracellular fluid causes an appreciable increase of cardiac excitability of and generation of cardiac arrhythmias in the rat ventricle (De Mello 2004a).This effect of Ang I is related to its conversion to Ang II because in enalapril maleate suppressed this effect of Ang I. The possibility that Ang I can be converted to Ang II at the level of cell membrane was studied in myocytes isolated from the same preparation. The results indicated that Ang I reduced the inward calcium current-an effect abolished by enalaprilat (De Mello 2004a).

Previous studies showed that Ang II, per se, reduced the inward calcium current in rat cardiomyocytes and increased it in hamsters cells (De Mello 1998). These findings support the view that Ang II can be formed at the level of cardiac cell membrane making possible a fast

interaction of the peptide with AT1 receptors. The generation of Ang II at the surface cell membrane provides a mechanism of paracrine and autocrine action of the peptide and indicates that the heart is a source of Ang II.

RENIN ANGIOTENSIN SYSTEM AND INTERCELLULAR SIGNALING

Although contraction is the most important function of the heart we cannot forget that the cardiac muscle is a complex electrochemical machine in which the generation of electrical propagated responses and its propagation from cell-to-cell is essential for the trigger of the contractile process. An increase in resistance at the level of the gap junctions, for instance, results in impairment of impulse propagation and cardiac arrhythmias (De Mello, 1999). Moreover, the suppression of cell communication reduces the heart contractions by sequestering a large number of cells which are not able to participate in the contraction process. Histologic studies indicated interstitial fibrosis, necrosis and calcifications in the failing heart (see De Mello 1999). The renin angiotensin aldosterone system is involved in the decline of cell communication and the generation of fibrosis. Both processes contribute to the sequestration of large number of heart cells (De Mello 2004b). Several observations indicated that at an advanced stage of heart failure, Ang II added to the bath caused cell uncoupling abolishing intercellular communication and impulse propagation (De Mello 1996).This effect of the peptide is related to the activation of PKC and consequent phosphorylation of gap junction proteins.The possibility exists that tyrosine kinase is also involved in the Ang II- mediated signal transduction (see Hunter 1996).

ON THE INTRACRINE RENIN ANGIOTENSIN SYSTEM

The possible internalization of prorenin and the formation of renin inside the cardiac myocyte under pathological condictions might lead to two possible consequences: formation of Ang II inside the cell (Peters et al 2002) with consequent intracellular action of the peptide or release of renin to the extracellular medium and formation of Ang II outside the cell with activation of AT1 receptors. Although intracellular Ang II can be the result of synthesis or internalization of the peptide, the presence of an intracrine RAS has been supported experimentally.

Indeed, it was found that intracellular administration of Ang I abolished cell communication an effect suppressed by intracellular enalaprilat or losartan (De Mello 1996; De Mello Danser 2000).

Recently, it has been shown that intracellular dialysis of Ang I in cells of 2 month-old cardiomyopathic hamsters in which the ACE activity is not increased, caused a small decline of junctional conductance (33 %) while in 6 month-old animals in which the ACE is enhanced Ang I abolished cell coupling (De Mello 2003b). In these animals in which the ACE activity is enhanced the intracellular dialysis of Ang II also caused cell uncoupling, an effect not abolished by losartan added to the extracellular fluid.

The possibility that endogenous Ang II contributes to the regulation of cell communication was investigated dialyzing enalaprilat inside the cell. In 2 month-old animals, for instance, in which the ACE is not increased, enalaprilat caused no change of gj while in 6 month-animals the increase of gj was of 72% (De Mello 2003b). These observations suggest that there is ACE in the cytosol. The presence of a soluble form of ACE inside the cell might explain these results. Evidence exists that a post-translational proteolytic cleavage of membrane bound ACE by secretases, release the protein in a soluble form (see Hooper, Turner 2000). It is conceivable that the transport of the soluble form into the cell might represent an important source of intracellular ACE but further studies are needed to confirm or discard this hypothesis.

INWARD CALCIUM CURRENT IS MODULATED BY EXTRACELLULAR AND INTRACELLULAR ANGIOTENSIN

Further support for the existence of an intracrine RAS system is the finding that intracellular Ang II injection increases I_{Ca} density in the failing heart (De Mello, Monterrubio 2004). Althouth similar results were found with extracellular Ang II, only intracellular Ang II increased I_{Ca} inactivation.This effect on I_{Ca} inactivation might be related to the release of Ca by the SR through an activation of ryanodine receptor. Indeed, thapsigargin which depletes the SR of Ca ions, abolished the effect of intracellular Ang II on I_{Ca} inactivation. Moreover, intracellular Ang I also increased I_{Ca} density and enhanced the rate of I_{Ca} inactivation (De Mello, Monterrubio 2004) an effect abolished by intracellular enalaprilat. The effects of intracellular Ang II are not the same found when Ang II is added to the extracellular fluid because only intracellular Ang II increased the rate of I_{Ca} inactivation. Although intracellular Ang II can be the result of internalization it is unlikely that this be the case in

our experimental conditions. It is important to emphasize that the overexpression of renin and angiotensinogen genes during the process of heart failure can provide the elements for the synthesis of intracellular Ang II. An alternative hypothesis is that intracellular Ang II might be synthesized by internalization of both renin and angiotensinogen.

The role of a possible intracellular ACE on the intracellular conversion of Ang I remains to be determined. Internalization of the enzyme associated with a monoclonal antibody has been described in endothelial cells (Muzykantov et al 1996) rising the possibility that similar phenomenon occur in cardiac myocytes.

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Chapter 9

THE HEMATOPOIETIC SYSTEM: A NEW NICHE FOR THE RENIN-ANGIOTENSIN SYSTEM

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ABSTRACT

The role of the renin-angiotensin system was previously thought to be restricted to the cardiovascular system. It now appears that this system plays also an important role in hematopoiesis. The different elements of the renin-angiotensin system are expressed early during embryogenesis. The renin system plays a role at different steps of hematopoiesis, notably during the first wave of hematopoiesis in the chick embryo (primitive hematopoiesis), and during the adult phase of hematopoiesis in the fetus (definitive hematopoiesis). In addition, the renin-angiotensin system is involved in the reconstitutive hematopoiesis following experimental irradiation in mice: renin-angiotensin system antagonists improve the hematopoietic recovery in this situation.

INTRODUCTION

The hematopoietic system consists of a complex network of hematopoietic organs from which early progenitors arise and progressively differentiate into intermediate and mature cells of at least eight hematopoietic lineages (Cross and Enver 1997). In humans, bone marrow is the main site of the definitive hematopoiesis in adults whereas in rodent spleen is also involved. The hematopoietic early progenitors are

maintained in specific compartmentalized niches where they interact with other cell types (Watt and Hogan -2000). Within the bone marrow, stroma cells of at least four distinct types (osteoblasts, macrophages, fibroblasts and endothelial cells) regulate the maturation and differentiation of hematopoietic stem cells. The regulation of hematopoiesis is the result of multiples processes involving cell-cell or cell-extracellular matrix interactions, with the participation of specific hematopoietic growth factors, various cytokines and intrinsic modulators (Barreda and Belosevic -2001). The specific expression and combination of these factors, which can act as positive or negative regulators, determine the survival, proliferation, commitment, and differentiation of hematopoietic progenitors (Tenen et al. -1997; Morrison et al. -1997).

The renin-angiotensin system (RAS) appears to be one of these factors, and is involved in both the primitive and definitive hematopoiesis. The RAS is well known for its role in the regulation of blood pressure and fluid homeostasis. In addition to its systemic effect, evidence for an autocrine/paracrine role of this system has been well documented (Dzau and Pratt -1986; Dzau et al. -2001). The angiotensin peptides may act as locally active growth factors or inhibitors and are able of exerting effects in various tissues, similarly to cytokines (Dostal and Baker -1999). There are emerging new data suggesting a role of the RAS in the regulation of hematopoietic stem cells (Haznedaroglu and Ozturk -2003). Our laboratory has recently studied the involvement of the different RAS components in primitive and definitive hematopoiesis in two animal models, the chick embryo and the adult mouse, respectively. Our results show that this system is indeed involved in these two phases of hematopoiesis.

RAS INHIBITORS AND HEMATOPOIESIS IN HUMAN PATIENTS

Early on after the introduction of ACE inhibitors in clinics, it has been noted that a small reduction of hematocrit could be observed in enalapril-treated patients (Griffing and Melby - 1982). In two clinical conditions it has been reported anedocticallythat RAS inhibitors (ACE inhibitors and angiotensin receptor blockers) could decrease hematocrit levels: patients with chronic renal failure or patients experiencing erythrocytosis after renal transplantation (Vlahakos et al. -1991; Conlon et al. -1996; Julian et al. -1998; Vlahakos et al. -2003). It has even been proposed that such inhibitors could be useful for the treatment of post-renal transplantation erythrocytosis.

Several hypotheses could account for this effect: an interaction between the RAS and erythropoietin, an effect mediated by the hemoregulator peptide AcSDKP which is a substrate of angiotensin I-converting enzyme (ACE), and a direct effect of angiotensin peptides on hematopoiesis.

It has been shown that the RAS is associated with the production of endogenous erythropoietin from the peri-tubular fibroblasts of the kidney. Activation of the RAS enhances erythropoietin production (Vlahakos et al. -1995). Similarly, administration of ACE inhibitors reduces plasma erythropoietin levels, exacerbating anemia (Walter -1993). However, this hypothesis has not been investigated further and the role of the RAS in erythropoietin production or function remains uncertain.

THE TETRAPEPTIDE ACETYL-N-SERYL-ASPARTYL-LYSYL-PROLINE (ACSDKP) AND ANGIOTENSIN CONVERTING ENZYME

The protection of hematopoietic progenitors during a cytotoxic treatment could improve bone-marrow regeneration and induce a favorable outcome. Stem cell inhibitors have been shown to inhibit the proliferation of normal hematopoietic progenitors by maintening them in a quiescent state whereas the leukaemic progenitors are sensitive to cytotoxic S-phase specific agents. The use of inhibitory factors by reducing stem-cell depletion and improving neutrophil recovery could have therefore a therapeutic benefice (Parker-1995). AcSDKP is a new ACE physiological substrate, which has been discovered independently of the RAS for its property to regulate negatively hematopoietic precursors. As it will be shown below, ACE is the major enzyme implicated in AcSDKP inactivation and ACE inhibitors increase plasma and urinary AcSDKP levels. Potentiation of AcSDKP by ACE inhibition could theorically lead to a negative effect on hematopoiesis.

AcSDKP was originally isolated from calf fetal bone marrow (Lenfant et al. -1989) by its property to inhibit *in vitro* the proliferation of hematopoietic pluripotent stem cells and by blocking the response of hematopoietic cells to proliferative stimuli (Monpezat and Frindel -1989; Robinson et al. -1992). *In vivo,* the deprivation of endognous AcSDKP by injection of an AcSDKP antibody in mice induces an imbalance of normal hematopoiesis (Frindel and Monpezat -1989). During aggressive chemotherapy, previous administration of AcSDKP was able to protect the spleen colony-forming units (CFU-S) compartment from depletion by

preventing the quiescent primitive hematopoietic cells to enter into S-phase and consequently rendering them less vulnerable to S-phase-specific drugs (Lenfant et al. -1989). The recovery of high proliferative potential colony-forming cells (HPP-CFC), colony-forming units-granulocyte-macrophage (CFU-GM) and burst forming units-erythroid (BFU-E) cells was faster and improved in AcSDKP-pretreated mice (Masse et al. -1998). AcSDKP inhibits the generation of granulopoietic and erythroid colony formation, this inhibition is influenced by the stroma compartment (Cashman et al. -1994) or the addition of cytokines (Jackson et al. -1996).

ACE cleaves AcSDKP by its dipeptiyl carboxypeptidase activity *in vitro* and *in vivo,* and generates the C-terminal dipeptide Lys-Pro (Rieger et al. -1993). Somatic ACE is derived from a duplicated ancestral gene (Hubert et al. -1991; Kumar et al. -1991) and is composed of two highly homologous domains called N-and C-domains, each comprising a catalytically active site (Soubrier et al. -1988). Although highly similar in terms of structure and function, the two sites display some differences. In particular, the N-active site is 50-fold more efficient than the C-active site for AcSDKP hydrolysis (Rousseau et al. -1995).

The hypothesis that specific ACE inhibitors could influence AcSDKP metabolism, increase its stability in plasma and ultimately potentiate its ability to reversibly block the hematopoietic proliferation was investigated. AcSDKP is a natural substrate of ACE *in vivo* as a single oral dose of captopril administered to healthy volunteers increases steeply plasma AcSDKP levels (Azizi et al. -1996). In another study, plasma and urinary AcSDKP concentrations of volunteers treated by enalapril for 15 days increased 2- to 5-fold (Comte et al. -1997). In this study, enalapril induced a decrease in the number of the circulating CFU-GM and BFU-E (Comte et al. -1997).

We tested whether ACE inhibition could protect stem cells from lethal or sublethal irradiation in mice. When the ACE inhibitor perindopril was administered for 4 days, 48 hours prior to irradiation, plasma ACE activity was totally inhibited and the survival of mice was significantly improved. This was correlated with an accelerated hematopoietic recovery, and a significant increase in platelet and red cell counts (Charrier et al. -2004). Pre-treatment with perindopril increased bone marrow cellularity and the number of hematopoietic progenitors (CFU-GM, BFU-E and CFU-MK) from day 7 to 28 after irradiation. The increase of AcSDKP plasma concentration by the selective N-domain ACE inhibitor (Dive et al. -1999) did not have any effect in this protocol whereas the angiotensin II receptor antagonist telmisartanhad the same effect as perindopril, showing that ACE inhibitors exert their radio-and

hemo-protective effects through an angiotensin-dependent mechanism and not through potentiation of AcSDKP (Charrier et al. -2004).

A genetically engineered mouse model was created in which the ACE N-terminal catalytic site was selectively inactivated by site directed mutagenesis (Fuchs et al.-2004). In these mice lacking N-terminal ACE activity, plasma and urinary AcSDKP levels were significantly higher than in wild type mice. As AcSDKP was presumed to affect hematopoiesis, the hematocrit of these mice was analyzed. No significant difference was observed between the two strains of mice. Mice without the functional N-domain of ACE recovered from phenylhydrazine-induced anemia as well as the wild type mice (Fuchs et al. -2004).

Although altogether these experiments do not support an important physio-pathological role of AcSDKP in hematopoietic cell proliferation, one must consider that only plasma and urinary AcSDKP levels were monitored in these studies. It is possible that plasma AcSDKP levels obtained during by ACE inhibitor treatment are not sufficiently high to affect hematopoietic stem cells. The local AcSDKP concentration in the bone marrow should be considerated as ACE is present in bone marrow and may influence local concentrations of AcSDKP. Thymosin β4, thought to be the precursor of AcSDKP, is expressed in bone marrow endothelial cells and study of its gene expression should contribute to understand the local regulation of AcSDKP (Huang and Wang -2001; Huff et al. -2001)

IMPLICATION OF ANGIOTENSIN II IN ADULT HEMATOPOIESIS

ACE knock-out mice have been enginereed in order to explore the structure/function of ACE. Unexpectedly, these mice had a normocytic anemia (about 25 % reduction in hematocrit level) associated with elevated plasma erythropoietin levels (Table I). Mice in which the ACE gene had been totally deleted (ACE.1 knockout mice) had no ACE expression and a severe renal insufficiency. Mice in which a partial deletion of the ACE gene had been made (ACE.2 mice) had a lack of tissue ACE, 35% of the normal plasma ACE activity and showed no evidence of renal insufficiency. From the comparison of these two strains, it seemed very unlikely that anemia was the consequence of the renal failure. The anemia was not due to a volume expansion but actually to a reduction of red cell mass. The degree of anemia in these two strains of mice was equivalent, despite a significant difference between plasma AcSDKP levels, suggesting that AcSDKP was not the primary cause of

anemia. The plasma levels of angiotensin II in these two strains of mice reached only 10 to 20% of angiotensin II level in wild type mice. To evaluate the role of angiotensin II *per se* in hematopoiesis, hematocrit was measured after an infusion of angiotensin II for two weeks. The hematocrit level was corrected in ACE-deficient mice to near wild-type levels, suggesting strongly that the lack of angiotensin II in these mice was the direct cause of the anemia (Esther et al. -1996; Cole et al. -2000).

Table I. Parameters of the anemia observed in homozygous mice for total ACE gene inactivation (ACE.1) or partial ACE gene inactivation (ACE.2) vs wild type mice (WT) (from J. Cole et al.)

	WT	**ACE.1**	**ACE.2**
Hemoglobin (g/dl)	15.0	12.0	11.7
MCH[(1)]	15.3	15.8	16.0
Reticulocytes (%)	4.4	3.1	2.6
Indirect bilirubine (mg/dl)	0.43	0.48	0.44
Serum iron (µg/dl)	133	139	ND[(1)]
Transferrin saturation (%)	32.5	32.8	ND

(1) MCH: mean corpuscular hemoglobin ; ND : Non determined

To study the mechanism of action of angiotensin II on hematopoiesis, the proliferation of erythroid progenitors was studied in healthy male volunteers and in hemodialysed patients (Naito et al. - 2003). Peripheral blood mononuclear cells were isolated and cultured. The BFU-E-derived colonies were counted and revealed that patients under hemodialysis generated fewer BFU-E than healthy volunteers. In both groups, BFU-E growth was stimulated by the addition of high doses of angiotensin II (10^{-4} M) in the culture medium, an effect, which was inhibited by losartan.

In *in vitro* models, angiotensin II alters the proliferation and differentiation of erythroid and myeloid precursors from human $CD34^+$ stem cells (Mrug et al. -1997; Rodgers et al. -2000; Brunet et al. -2002; Peng et al. -2003). However, opposite effects have also been reported,

which may be due to the difference in experimental procedures (presence or not of serum in the culture medium, different angiotensin II concentrations used, etc.). Confounding factors must be controlled in these experiments such as the use of serum, which can be a source of cytokines, and of proteolytic activity, leading to the generation of angiotensin metabolites. Angiotensin II increased proliferation of BFU-E colonies, which at this stage expressed the AT1 receptor. The effect was abolished by losartan (Mrug et al. -1997).

It has been reported recently that another angiotensin peptide, angiotensin (1-7), may contribute to hematopoietic cell proliferation. Ang (1-7) can be generated by several enzymatic pathways from angiotensin I, angiotensin II or angiotensin (1-9) (Cesari et al. -2002; Ferrario -2002; Leung -2004). Ang (1-7) shares some of the properties of angiotensin II, but can also oppose to some of them. The recovery of GM-CFU, GEM-CFU and BFU-E progenitors from irradiated mice as well as from mice treated with 5-fluorouracil, a chemotherapeutic drug, was improved after Ang (1-7) administration, suggesting that Ang (1-7) had an effect on all hematopoietic lineages (Rodgers et al. -2002; Rodgers et al. -2003).

Primitive hematopoiesis

The role of the components of the RAS (ACE and angiotensin II) in adult hematopoiesis led our laboratory to investigate the possible role of these components in another important hematopoietic event, the primitive hematopoiesis. The establishment of hematopoiesis can be studied in the chick embryo, which is a convenient model for its easier accessibility compared to mammalian embryos. In the chick embryo, the primitive hematopoiesis, produced only in the yolk sac, is restricted to the erythroid and macrophage lineages and occurs in blood islands prior to embryonic hematopoiesis (Lassila et al. -1982). In the extra embryonic area, the blood islands differentiate from the mesoderm where two lineages arise: the endothelial cells and the hematopoietic cells (Caprioli et al. -1998). The expression of ACE was detected by *in situ* hybridization and immunohistochemistry before the beginning of blood island differentiation in the extra embryonic mesoderm, at stage HH6 (24 hours of development) when the circulation is not yet established between the yolk sac and the embryo (Savary et al. -2004). At 30 hours of development angiotensinogen was detected in the extra-embryonic endoderm and angiotensin II receptor in both the embryonic and extra-embryonic mesoderm.

The RAS may be of critical importance in the microenvironment of the blood islands. To evaluate a putative functional role of this system during the differentiation of blood islands, fosinoprilate, an ACE inhibitor, was administrated to two-day-old embryos. 48 hours later, the hematocrit was measured in the circulation and was significantly lower in the treated embryos than in control embryos. To further determine if angiotensin II has an effect on lowering the hematocrit, the Ang II receptor antagonist Sar^1 -Ile^8 -Ang II was added at the same stage. A similar 15% significant decrease of the hematocrit was found, showing that the RAS had an effect on the primitive hematopoiesis. The embryos were no more sensitive to the application of either the ACE inhibitor or the Ang II receptor antagonist if they were treated 6 hours later, indicating that the effect of the RAS on erythropoiesis probably occurs in a short time window. Based on the spatio-temporal pattern of ACE distribution in the endoderm in close contact with the blood islands and its effect on the regulation of the hematocrit, these results show for the first time that the RAS modulates blood island differentiation during the primitive yolk sac erythropoiesis (Savary et al. -2004).

PERSPECTIVES

RAS blockade with angiotensin II antagonists or ACE inhibitors has been used to treat patients with post transplantation erythrocytosis or *polycythemia vera* to reduce hematocrit levels. Results described above suggest that ACE inhibitors and angiotensin II receptor antagonists could be used to decrease the hematopoietic toxicity of irradiation and possibly of aggressive chemotherapy. However, no mechanistic explanation for these observations has been generally accepted. The precise cellular localization of the RAS molecules in the hematopoietic precursors and in the different cellular components of the stroma of the bone marrow should be investigated to determine the putative autocrine-paracrine interactions between these different compartments. The stromal cells present in the bone marrow constitute a cell population that attends the hematopoietic stem cell and its progeny. This set of cells contributes to quiescence, self-renewal and commitment of stem cells and proliferation, maturation and apoptosis of more mature hematopoietic cells. Angiotensin peptides may act directly as growth factors on hematopoietic precursors but could also act indirectly by modifying the commitment or the fate of these precursors by acting on the bone marrow stromal cells. The signaling pathway involved in the effect of the RAS on hematopoiesis has to be investigated as several signaling pathways

can be activated by angiotensin II.

Finally, these experimental observations may have clinical importance: the use of RAS blockers could be considered for protecting hematopoietic stem cells during irradiation and aggressive chemotherapy.

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Chapter 10
MECHANICAL SIGNALING AND THE CARDIAC RENIN-ANGIOTENSIN

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SUMMARY

Cardiac hypertrophy is a common outcome of hypertension or myocardial infarction and a major contributor to cardiovascular morbidity and mortality. Under conditions of increased hemodynamic load, the heart compensates by undergoes compensatory hypertrophy, a response that restores lost function and normalizes wall stress. It is thus very important to understand the molecular mechanisms responsible for the development of cardiac hypertrophy. In isolated cardiac myocytes, mechanical stretch induces activation of several protein kinases, fetal gene expression, upregulation of renin-angiotensin system components and cellular hypertrophy. Pretreatment with an angiotensin II (Ang II) type II receptor blockers significantly attenuates all of the mechanical stretch induced events. Many animal and clinical studies have shown that blockade of the RAS with AT_1 receptor blocker or angiotensin converting enzyme inhibitor induce regression of cardiac hypertrophy and prevent progression of heart failure, resulting in a reduction in cardiac morbidity and mortality. These studies indicate that the local RAS is activated by hemodynamic overload and that the AT_1 receptor has a crucial role in the development of load-induced cardiac hypertrophy. Although it is evident that mechanical stress is the primary trigger of cardiac hypertrophy, it is not clear how mechanical stimuli is sensed and converted into intracellular signals. Integrins and associated signaling machinery have been reported to be sensors for mechanical

stress. Below, we focus on evidence for integrins as the mechanosensors and mechanotransduction systems responsible for cardiac hypertrophy and activators of the cardiac RAS.

MECHANICAL LOAD AND CARDIAC HYPERTROPHY

Cardiac hypertrophy, an important compensatory mechanism in response to chronic increases in hemodynamic load, is defined as an increase in heart size resulting from an increase in cardiac myocyte cell volume. The hypertrophic process is initially beneficial because there is an increase in the number of contractile units and a reduction in ventricular wall stress. However, sustained hemodynamic overloading of the myocardium eventually results in heart failure, which is characterized by chamber dilatation, contractile dysfunction, and impaired survival. Considerable research efforts have focused on molecular mechanisms responsible for transducing hemodynamic load into myocardial growth and the transition to terminal heart failure. Mechanical stretch activates signal transduction pathways resulting in reprogramming of gene expression and production of factors that initiate myocardial growth. A growing number of intracellular signaling pathways have been identified as important transducers of the hypertrophic response in cardiac myocytes. A link between mechanical load and the cardiac renin-angiotensin system (RAS) is evidenced by upregulation of the system in failing hearts and isolated cardiac cells exposed to mechanical stress. Studies have demonstrated that mechanical stretch causes a rapid secretion of angiotensin II (Ang II) by cardiac myocytes.[1-4] The prevention of stretch-induced hypertrophic response by Ang II receptor blockade suggests that autocrine production of Ang II plays a critical role in cardiac growth.[3-7] Although it is evident that mechanical stress is the primary stimulus, the mechanosensor and associated transduction systems remains to be elucidated for regulation of the cardiac RAS and cardiac hypertrophy.

Expression of the Renin-Angiotensin System in Cardiac Tissue

Renin, angiotensinogen (Ao), and angiotensin converting enzyme (ACE) have been localized in all chambers of the heart in several

species, including humans. In neonatal and adult rat hearts, renin, Ao, angiotensin I (Ang I), and Ang II are expressed by ventricular myocytes and fibroblasts at the mRNA and protein levels.[8-14] Ang I and Ang II have been localized in atria and/or ventricles of several species, [15-17] including rat.[18-20] The presence of Ang II and its precursors in cultured cardiac myocytes and fibroblasts,[6, 8-11, 21] suggest that these cell types contribute to cardiac Ang II levels. The presence of Ang II receptors on both cell types suggests that the cardiac RAS can regulate/modulate myocardial function and growth. In cardiac tissue, the Ang II type I receptor (AT_1) couples to intracellular calcium mobilization, phospholipid metabolism and growth stimulatory pathways, whereas the Ang II type II receptor (AT_2) couples negatively to growth, presumably by activation of tyrosine, serine and threonine protein phosphatases.[22-24]

Mechanical Load Stimulates Growth of Cardiac Tissue via Angiotensin II

We and others have reported that mRNA expression of cardiac renin, Ao, ACE and AT_1 and AT_2 receptors are upregulated in response to pressure overload or after myocardial infarction in various animal species. Changes in the cardiac RAS suggests that locally produced Ang II could act in an autocrine and/or paracrine fashion to mediate hypertrophic growth of cardiac myocytes, proliferation of fibroblasts and ventricular remodeling.[7, 25, 26] Mechanical stretch stimulates several growth-related processes in ventricular myocytes linked to activation of the cardiac RAS.[4] Passive stretch of cardiac myocytes cultured on deformable membranes activates phosphorylation cascades of many protein kinases and induces the expression of specific genes observed in hypertrophic growth of the heart.[27-29] Increased production of Ang II plays a critical role in the induction of many of these hypertrophic responses. The pathological role of the cardiac RAS has been assessed using transgenic animal models. Targeted overexpression of Ao in hearts of transgenic mice causes myocyte hypertrophy, fibrosis and increased cardiac mass.[30] When AT_1 expression was targeted to ventricular myocytes in transgenic rats,[31] the hypertrophic response to pressure overload was increased compared to control animals, suggesting that synergy occurs between mechanical load and AT_1 activation in inducing cardiac growth. These studies underscore the importance of understanding cross-talk between mechanical load and regulation of the cardiac RAS.

Mechanical Regulation of the Cardiac Renin-Angiotensin System

In neonate and adult myocytes, acute exposure (5 - 30 min) to mechanical stretch causes autocrine release of Ang II[1, 2, 4, 5, 32] and longer exposures (8 - 48 h) increase mRNA expression of AT_1 and AT_2 receptors, renin and Ao.[21, 32-34] A portion of the stretch induced increase in Ao gene expression occurs via AT_1 coupled mechanisms in neonatal and adult rat ventricular myocytes.[33] In primary cultures of neonatal rat cardiac myocytes, the AT_1-dependent increase in Ao gene expression occurs primarily via activation of the JAK-STAT pathway.[35-38] Acute pressure overload of the myocardium also activates the JAK-STAT system in the adult heart,[39] suggesting that this pathway may be an important regulator of Ao gene expression in the pathological heart. In primary cultures of adult rat ventricular myocytes, mechanical stretch also increases p53 binding to the promoter regions of genes for Ao and AT_1.[40] The mechanosensor, proximal signaling mechanisms, and associated autocrine secretory pathways remain to be elucidated.

Integrins as Mechanoreceptors

Mechanisms responsible for converting mechanical stretch into biochemical signals are poorly understood. Integrins have recently been recognized as important for the transduction of positional cues from the extracellular matrix (ECM) to intracellular signaling machinery. Integrins are composed of noncovalently associated α and β subunit heterodimers that consist of a large extracellular domain, a transmembrane region and a short cytoplasmic domain.[41] The integrin family consists of 18 α and 8 β subunits which dimerize (α/β heterodimers) to form over 24 pairs of nontyrosine kinase receptors with distinct and often overlapping specificity for ECM proteins. In heart, the system is also regulated by spatial and temporal expression patterns and shifts in subunit isoforms.[42] Integrin signaling enables cells to integrate information from external stimuli, such as the ECM, soluble factors and mechanical forces. Although cytoplasmic domains lack intrinsic kinase activity, integrins can recruit from diverse signaling and cytoskeletal molecules. Ligand binding to integrins triggers inositol lipid metabolism and calcium ion fluxes, activate tyrosine and serine/threonine protein kinases and modulate signal transduction of other receptors. The intracellular tails of integrins can also modulate binding affinity via

inside-out signaling activating ectodomains by inducing ligand competent conformations.[43] The bi-directional signaling enables cells to interact with the environment and mediate vital processes such as adhesion, differentiation, migration, proliferation and apoptosis.

Integrin Expression in Cardiac Tissue

Of the large possible number of integrin receptors, only a few specific α- and β-chains are expressed in the myocardium.[44] There are α (1, 3, 5-7, 9-11, v) and β (1, 3-5, 7) subunits in the heart. Integrin expression is developmentally regulated[45-50] and shifts during physiologic/pathologic remodeling.[51, 52] For example, α_1 and α_5 subunits are downregulated postnatal, but reappear in the pressure overloaded myocardium.[53, 54] In cardiac myocytes, the α subunits predominantly associate with splice variants of β_1. The splice variant β_{1D} is the major isoform and exclusively expressed in skeletal muscle and cardiac myocytes.[49, 55] Adult myocytes primarily express $\alpha_3\beta_1$, through which these ligands adhere to laminin and type IV collagen, but poorly bind to other collagens and fibronectin.[53, 56] In neonatal rat cardiac myocytes and fibroblasts, β_3 and β_5 subunits also form heterodimers with α subunits to evoke functional responses.[29, 57] Cardiac fibroblasts express many of the same α-subunits as myocytes, except α_6 or α_7, as this cardiac cell type lacks a laminin containing basement membrane. Cardiac fibroblasts express α_v and the collagen-specific α_2 subunits are absent in adult myocytes.[44]

Integrins have been demonstrated to play vital roles in cardiac health and disease. The differentiation and function of cardiac myocytes and other cell types in the heart, fibroblasts[58] and endothelial cells[59] are greatly influenced by integrins. In this regard, integrins mediate differentiation of precursor cells into mature cardiac myocytes,[60] act as stretch receptors,[61, 62] and mechanotransducers[63] and modulate electrical activity[64, 65]. Disruption of integrin expression or function has marked pathologic consequences.[66] Ablation of α_4,[67], α_5,[66] or β_1[48, 60] yields lethal cardiac defects. β_1 overexpression induces hypertrophy. Loss of β_1 disrupts adrenergically mediated hypertrophy.[68] Overexpression of β_1 induces hypertrophy and augments hypertrophy due to adrenergic agents, which themselves increase β_{1D} expression.[68] Given that adrenergic stimulation contributes to the progression of heart failure this data implicate β_1 integrins in this process. α_6 is vital to myocyte function, prevalent on cardiac myocytes, and not found on cardiac fibroblasts[44]. Isoform (α_{6A}, α_{6B}) levels shifts during cardiac development[45, 47, 69-71] and

are abundant in adult hearts.[45] Integrin β_3 is critical to focal adhesion complexes in adult cardiac myocytes and function in cardiac hypertrophy.[57, 72, 73] Cardiac myocyte survival depends upon the integrity of myocyte-integrin interactions, the loss of which triggers anoikis.[74] Integrins have been implicated as important mediators of myocyte hypertrophy[53, 54, 57, 68, 72, 75-77] myocardial infarction,[78-81] transplantation responses[82, 83] and heart failure.[84-88] Since the cardiac RAS is upregulated in these various pathologies, there is circumstantial evidence to suggest that integrins also regulate the cardiac RAS. Howevever, the specific integrin subunits and associated signaling pathways remain to be determined.

Integrin Signaling in Cardiac Tissue

In the last decade, some of the basic mechanisms by which integrins couple to cellular function have been unraveled. Since the cytoplasmic tails of integrins lack enzymatic activity, integrins transduce signals by associating with adapter proteins that connect to the cytoskeleton, cytoplasmic kinases, and transmembrane growth factor receptors.[41] The gathering of activated signaling proteins with cell-matrix adhesions results in the focal amplification of signal transduction.[89] The β-subunit links the integrin with the cytoskeleton and signal transduction apparatus of the cell. Ligand binding initiates integrin clustering and promotes assembly and reorganization of actin filaments by activation of small GTPases (Cdc42, Rac, RhoA) and cytoskeletal organizers (paxillin, talin, vinculin). The clustering is regulated by signaling enzymes, such as PI-3K (phosphatidylinositol 3-kinase), protein kinases C (PKCs), and Ras/Rap GTPases,[90, 91] creates a signaling complex in which ECM and cytoskeletal proteins to assemble into multi-protein aggregates on each side of the membrane. Activated integrins can transmit signals directly through nonreceptor tyrosine kinases, including FAK (focal adhesion kinase) involving Src and p130^{Cas} and Shc involving Fyn and caveolin.[93] This results in activation of downstream signaling cascades, including PI-3K/Akt; MAPKs (mitogen activated protein kinase), such as ERKs (extracellular signal-regulated kinase), JNK (c-Jun N-terminal kinases) and MEK (MAPK/ERK Kinase) and downstream effectors Fos and Jun.[41, 44, 94-100] In cardiac myocytes and fibroblasts, β-subunits have been shown to couple to FAK, MAP kinase cascades, gene expression, and hypertrophic growth.[48, 60, 68, 75, 80] In neonatal rat cardiac myocytes, stretch-induced expression of brain natriuretic peptide has been shown to require coordinate activation of β_1,

β_3 and $\alpha_v\beta_5$ integrins and MAP kinases (ERK, JNK and p38-MAP kinase).[29, 101] This is consistent with the observation that overexpression of β_{1D}-integrin stimulates atrial natriuretic peptide expression in myocytes.[75] Although several integrins are expressed by cardiac fibroblasts, the role of these receptors in mediating mechanical effects on cellular function is poorly understood. In adult rat cardiac fibroblasts, mechanical stretch activates ERK and JNK, but not p38-MAP kinase via β_1 integrin,[98] suggesting that integrin signaling differs in myocytes and fibroblasts.

Regulation of Integrin Signaling by Angiotensin II

A critical role for the underlying mechanisms of Ang II-induced fibrosis can be attributed to adhesion, an essential cellular function that involves interactions of ECM proteins to integrins. Consequently, integrin expression on the cardiac fibroblast surface, as well as ECM–integrin–cytoskeleton coordination must be modified to contribute to maintenance of the tissue structure and function in diseased hearts. Several studies have demonstrated the profibrotic effects of Ang II in cardiac fibroblasts to be linked to alterations in ECM and integrin expression.[58, 102-104] In vitro administration of Ang II has been shown to regulate α_8, α_v, β_1, β_3, and β_5 integrins,[58, 103, 104] osteopontin[105-107] and α-actinin expression[102] through AT_1 receptor activation. In the rat, in vivo administration of Ang II has also been to enhance matrix production in the left ventricle and thoracic aorta, which was paralleled by increased $\alpha_8\beta_1$ integrin expression by myofibroblasts.[108] The coordinate regulation of $\alpha_8\beta_1$ integrin on fibroblasts and ECM proteins implicates this integrin in the deposition of matrix components leading to fibrosis. Integrins α_v, β_5 and α-actinin have also been shown to be upregulated in hypertrophied ventricles of spontaneously hypertensive rats (SHR), but not in normal Wistar-Kyoto rat ventricles.[102] In this study, expression was attenuated by the AT_1 receptor blocker losartan, but not by the vasodilator hydralazine despite similar lowering of blood pressure in SHR. These studies underscore the role of Ang II in adhesion by demonstrating that Ang II not only regulates the production of the extracellular adhesion molecule osteoponin, but also regulates the expression of cell surface integrins that bind to osteoponin and to other ECM proteins, as well as the expression of the cytoskeletal protein α-actinin, which is intimately connected to integrins at the site of focal adhesions. Thus, Ang II regulates proteins that mediate cell attachment at all locations of the cell (e.g., secreted matrix proteins, cell membrane receptors, and intracellular strut proteins),

suggesting that Ang II orchestrates a coordinated series of cellular events to enhance cell adhesion, as well as signaling pathways that are associated with adhesion such as focal adhesion kinase.

The AT_1 Receptor as a Mechanosensor

The AT_1 receptor is a guanine-nucleotide-binding protein-coupled receptor (GPCR), a member of a large family of cell-surface receptors that contain common structural features characterized by seven transmembrane domains. It is of interest to note that integrins and the AT_1 activate many of the same pathways, suggesting that cross-talk occurs between these two systems. It has been recently demonstrated that in cardiac myocytes, the AT_1 receptor can serve as sensor and transducer of mechanical stress through an Ang II-independent mechanism.[109] This work implies that mechanical regulation of the cardiac RAS is regulated/modulated by the AT1. It was demonstrated tht mechanical stress not only activated ERKs and phosphoinositide production in vitro, in also induced cardiac hypertrophy in vivo.[109] Mechanical stress activated ERKs in cardiac myocytes prepared from neonatal and adult mice lacking the Ao gene (ATG-/-). In AGT-/- mice, pressure overload induced cardiac hypertrophy was substantially attenuated in animals receiving AT_1 receptor blocker. However, mechanical stretch did not activate ERKs in cells expressing the ET_{1A} or β_2-adrenergic receptors in a ligand independent manner,[109] which suggests that not all GPCRs that couple to cardiac hypertropy[110, 111] are necessarily a mechanical sensor. Interestingly, blocking the AT_1 receptor has differential effects on expression of RAS components in stretched cardiac myocytes. Stretch-induced upregulation of AT_1 has been shown to be inhibited by AT_1 receptor blocker, whereas upregulation of renin, Ao and ACE were not suppressed by the AT_1 receptor antagonist,[21] suggesting that the AT_1 is not the only mechanotranduction system that couples to RAS regulation in cardiac myocytes.

The mechanism by which the AT_1 serves as a mechanosensor/mechanotransducer is unclear. In growth factor receptor systems, integrins are a key component of the activation process. Integrin-mediated adhesion transactivates several receptor tyrosine kinases, including platelet-derived growth factor receptor, epidermal growth factor receptor, and vascular endothelial growth factor receptor in the absence of the growth factors.[43] Integrin clustering and association with the cytoskeleton appear to give rise to integrin- growth factor receptor complexes.[112] The aggregation of growth factor receptors results

in their partial activation, thus bringing growth factor signaling closer to a threshold of activity and enabling cross talk between integrins and growth factor receptors. Recently, it has been demonstrated that β_{1D} integrin serves as an upstream activator of the AT_1 in cardiac myocytes.[64] Activation of β_1 integrin, using paramagnetic beads coated with monoclonal antibody, resulted in activation of the stretch-activated chloride ion channel. Treatment of cardiac myocytes with an AT_1 receptor antagonist prevented activation of the chloride channel, suggesting β_1 integrins activated AT_1 by Ang II release and/or transactivation. Because of the demonstrated physical association of integrins with a variety of receptors, this mechanism for cooperation may be of general importance.

Evidence is emerging that significant interaction between integrins and GPCR signaling cascades occurs within lipid rafts and caveole.[113, 114] These lipid structures in the plasma membrane contain a high concentration of various cytoplasmic membrane-associated signaling molecules, termed supramolecular complexes. The caveole are specialized types of lipid rafts containing caveolins, which are proteins that form the coat material and decorate caveolar necks.[115] Caveolae are postulated to play a role in dynamic trafficking and activation/deactivation of GPCRs. Caveolin binding is mediated by a "membrane-proximal region" of caveolin, which has been termed the caveolin scaffolding domain. Through this domain, caveolins bind to many classes of signaling molecules: integrins, heterotrimeric G-Protein α-subunits, Ras, Src family kinases, receptor tyrosine kinases and PKC isoforms.[116] In addition to concentrating signal transducers within a distinct region of the plasma membrane, caveolin binding may functionally regulate the activation state of caveolae-associated signaling molecules, as signaling proteins associated with caveolin are maintained in an inactive state. This may prevent inappropriate activation by gathering components of signal transduction in a spatially defined compartment. Once activation of a given signaling pathway occurs and proper signaling ligands are available, the sequestered molecules dissociate from caveolin and leave the caveolae.[117] It is therefore conceivable that AT_1 localized within rafts or caveole could interact with integrins and signaling molecules, not available in other regions of the plasma membrane. Although AT_1 has been reported to have a dynamic association with caveolin,[118, 119] electron microscopy of plasma membrane sheets showed that AT_1 was not concentrated in caveolae, but clustered in cholesterol-independent microdomains and upon activation it partially redistributed to lipid rafts.[120] Although AT_1 was reported to interact with caveolin-3, it appeared to only act as a chaperone for newly

synthesized AT_1.[120] This suggests that lipid rafts may serve the primary site for integrin and AT_1 cross-talk. Although activation of tyrosine kinases by both integrins and growth factor receptors without physical association is well established, it is interesting that there is no evidence for integrin mediated activation of heterotrimeric G-protein signaling cascades outside membrane supramolecular complexes.

CONCLUSION

Myocardial stress is associated with alterations in gene expression that reflect the nature of the initial event, as well as in compensated and decompensated stages of cardiac failure. It is well documented that cardiac RAS is activated by hemodynamic overload and that the AT_1 receptor has a crucial role in the development of load-induced cardiac hypertrophy. Further study of the basic cellular mechanisms underlying load-induced mechanotransduction and regulation of the cardiac RAS will continue to provide insight into approaches to target cardiac hypertrophy. Integrins, focal adhesion molecules and the AT_1, have been reported to act as sensors and transducers of mechanical stress in the myocardium. However, elucidation of the molecular basis of stress-induced regulation of the cardiac RAS remains a challenge due to the complex temporal and spatial interactions that occur between integrins, the AT_1 and signaling cascades activated by growth factors.

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Chapter 11
ANGIOTENSIN CONVERTING ENZYME 2: A CRITICAL REGULATOR OF THE RENIN-ANGIOTENSIN SYSTEM

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As reviewed elsewhere,[1] the Danish investigators Tigerstedt and Bergman demonstrated in 1890 that infusion of a rabbit kidney extract into the circulation of a rabbit produced a potent pressor response, establishing a direct connection between the regulation of blood pressure and the kidney.[2] Based upon the origin of this pressor compound, they named this endocrine substance "renin". However, the importance of this discovery was not appreciated until decades later when landmark studies by research groups around the world, including the laboratories of Page, Braun-Menendez, Skeggs, Vane, Erdos, Bumpus, and many others, firmly established the primary components and enzymatic reactions that participate in the classical renin-angiotensin system.

The renin-angiotensin system is a key player in the physiological regulation of arterial pressure, homeostasis, and cell function acting both as a circulating hormone and a paracrine/autocrine regulator[3, 4]. The production of the major bioactive components of the renin-angiotensin system is initiated by the conversion of angiotensinogen to the decapeptide angiotensin I (Ang I), an inactive peptide, as shown in Figure 1. The cascade diverges at this point with the processing of Ang I to the physiologically active peptide hormones, Ang II and Ang-(1-7). Ang II and Ang-(1-7) have different carboxy termini as well as contrasting biological actions which are mediated by specific, high-affinity receptors. Ang II activates two pharmacologically distinct classes of seven transmembrane, G-protein coupled receptors-the

angiotensin type 1 (AT_1) and angiotensin type II (AT_2) receptors.[5, 6]

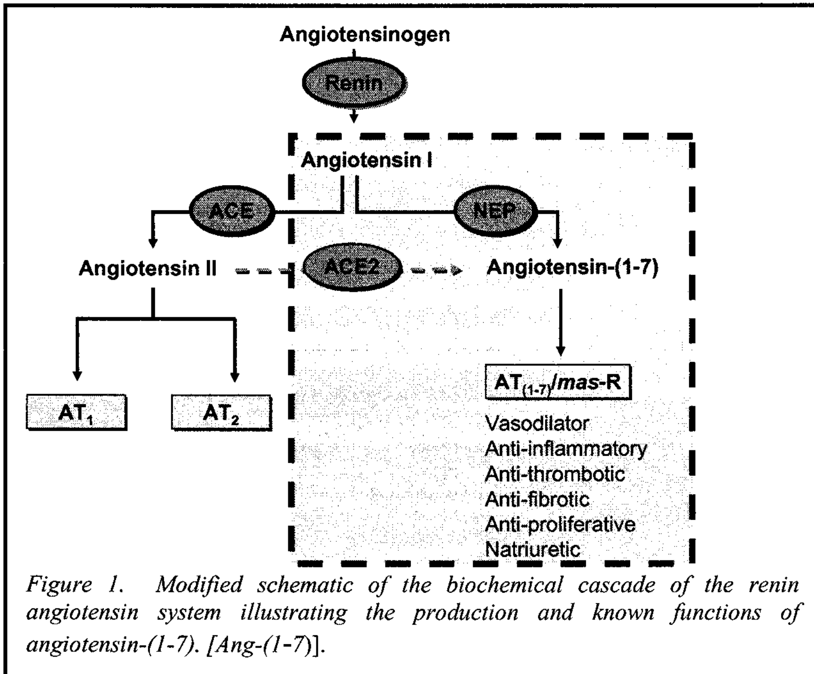

Figure 1. Modified schematic of the biochemical cascade of the renin angiotensin system illustrating the production and known functions of angiotensin-(1-7). [Ang-(1-7)].

In contrast, the G protein-coupled, orphan receptor *mas* was recently identified as a functional Ang-(1-7) receptor ($AT_{(1-7)}$),[7, 8] demonstrating that the actions of the two divergent branches are mediated by distinct receptors and their associated physiological responses. Although the renin-angiotensin system was classically characterized as an endocrine system, the identification of all of the components (angiotensinogen, renin, angiotensin converting enzyme and angiotensin peptide receptors) within select tissues provides clear evidence for local or tissue renin-angiotensin systems.[9-11] The demonstration of tissue renin-angiotensin systems, including the existence of tissue bone marrow renin angiotensin system,[12] further compounds the complexity of the pathway, providing evidence not only for the local synthesis, release, and action of angiotensin peptides, but tissue-specific regulation of peptide production or effect.[13]

Studies conducted by our group as well as others showed that Ang-(1-7), present in the circulation at concentrations similar to Ang II, produces unique physiological responses which are often opposite to those of Ang II.[9] Ang II is a potent vasoconstrictor, it stimulates thirst and aldosterone release, and inhibition of its production or effect using

ACE inhibitors or AT_1 receptor antagonists reduces mean arterial pressure.[14] In contrast, Ang-(1-7) reduces the blood pressure of hypertensive dogs and rats,[15, 16] alters renal fluid absorption,[17-21] causes vasodilation,[16, 22,23-26] and participates in the antihypertensive responses to ACE inhibition or AT_1 receptor blockade in hypertensive rats.[27, 28] While Ang II is a potent mitogen, stimulating vascular growth as well as hypertrophy in terminally differentiated cells,[29, 30] Ang-(1-7) inhibits growth of vascular smooth muscle cells, cardiomyocytes and cardiac fibroblasts.[31-33] Thus, a growing body of evidence indicates that Ang-(1-7) acts as a physiological modulator of Ang II, with opposing actions on body fluid volume, blood pressure, and cell growth.

Ang-(1-7) is derived from Ang I by tissue-specific endopeptidases, including neprilysin, thimet-oligo peptidase and prolyl endopeptidase (as reviewed in [9]). Although circulating Ang I is primarily converted into Ang II by angiotensin converting enzyme (ACE) localized on pulmonary vascular endothelial cells, Ang I present in various tissues can be converted into Ang-(1-7) by these tissue endopeptidases. In addition, ACE degrades Ang-(1-7) to the inactive angiotensin peptide, angiotensin-(1-5).[34] Thus, treatment of animals or patients with ACE inhibitors results in an increase in both circulating and urinary Ang-(1-7), due to blockade of the conversion of Ang I to Ang II and the degradation of Ang-(1-7).[35] Although neprilysin, thimet-oligo peptidase and prolyl endopeptidase were thought to be the major enzymes responsible for the generation of Ang-(1-7), the recent discovery of a novel enzyme, angiotensin converting enzyme 2 (ACE2), and the demonstration that it has high catalytic efficiency for the conversion of Ang II to Ang-(1-7) suggest that ACE2 may be a major enzyme for Ang-(1-7) production.

ACE2, the newest member of the renin-angiotensin system, was discovered by parallel, independent investigations using genome-based strategies to probe for either proteins with functions similar to that of ACE[36] or proteins involved in cardiac function.[37] ACE2 exhibits a high catalytic efficiency for the conversion of Ang II to Ang-(1-7)--almost 500-fold greater than that for the conversion of Ang I to Ang-(1-9).[38] From an array of over 120 peptides, only dynorphin A and apelin 13 were hydrolyzed by ACE2 with comparable kinetics to the conversion of Ang II to Ang-(1-7). ACE2 thus provides the missing connection between Ang II and Ang-(1-7), leading to a regulatory balance between the pressor and depressor arms of the renin-angiotensin system.

ACE2 shares about 42% nucleotide sequence homology with ACE, conserving critical active-site residues, and both enzymes are metallopeptidases, containing the typical HEXXH zinc-binding motif.

Similar to ACE, ACE2 is present in a wide variety of cells and tissues with high expression in the heart and kidney, as documented by us in the Sprague-Dawley rat (Figure 2). A study of 72 human tissues showed high ACE2 mRNA in cardio-renal and gastrointestinal tissues with limited expression in the central nervous system and lymphoid tissues.[39] Despite shared similarities, there are notable differences between the two enzymes. ACE has two catalytic sites, while ACE2 only has one. ACE2 is a carboxy-monopeptidase with a preference for hydrolysis between a proline and carboxy-terminal hydrophobic or basic residues, differing from ACE which cleaves two amino acids from its substrate. This reactivity divergence is due to amino acid substitutions in ACE2, causing changes in the substrate-binding subsite.[40] Important clinically, ACE inhibitors, central to the treatment of cardiovascular disorders, have no direct effect on ACE2 activity.[36, 37] However, we found a marked up-regulation of ACE2 mRNA in Lewis rats treated with either lisinopril (Figure 3) or Ang II receptor blockers[41] showing that blockade of Ang II synthesis or receptor activity indirectly affect ACE2 expression by a transcriptional regulatory mechanism. These results support a role for Ang-(1-7) in the cardioprotective effects of ACE inhibitors.

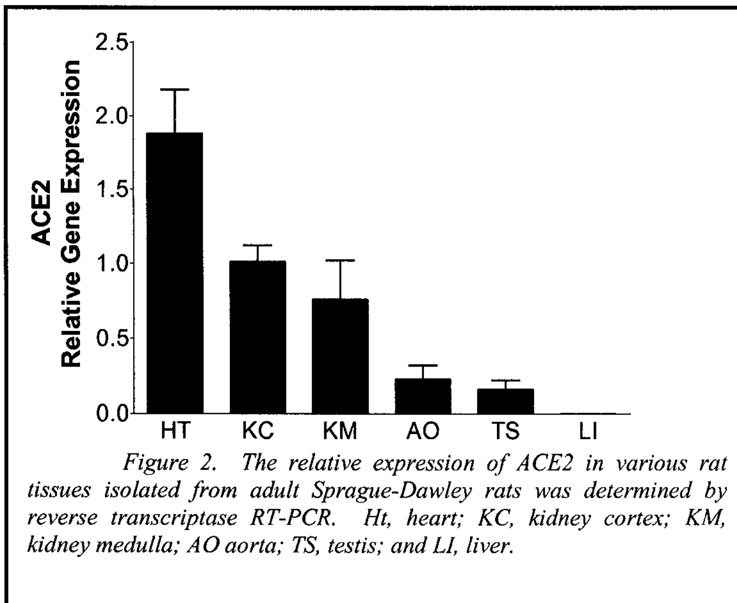

Figure 2. The relative expression of ACE2 in various rat tissues isolated from adult Sprague-Dawley rats was determined by reverse transcriptase RT-PCR. Ht, heart; KC, kidney cortex; KM, kidney medulla; AO aorta; TS, testis; and LI, liver.

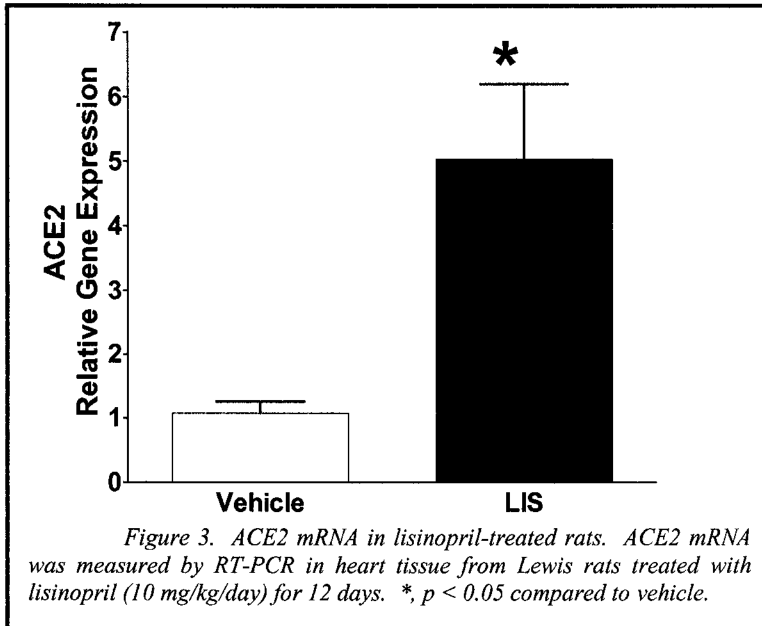

*Figure 3. ACE2 mRNA in lisinopril-treated rats. ACE2 mRNA was measured by RT-PCR in heart tissue from Lewis rats treated with lisinopril (10 mg/kg/day) for 12 days. *, $p < 0.05$ compared to vehicle.*

The ACE2 gene is located on the X chromosome and rat *ace2* maps to a quantitative trait locus with a significant logarithm-of-the-odds (l.o.d.) score for hypertension in three models of hypertension - the Sabra salt-sensitive rat, the spontaneously hypertensive rat (SHR) and the stroke prone SHR (SHRSP).[42] In these three rat strains, both ACE2 mRNA and protein were significantly reduced, suggesting that ACE2 is a candidate gene for this quantitative trait locus. More important, however, the elevated blood pressure in these three strains of rats may result from the increase in Ang II and reduced Ang-(1-7) as a result of decreased ACE2 activity. These studies suggest that ACE2 maintains the delicate balance between the pressor peptide Ang II and the depressor Ang-(1-7). Pathophysiological conditions that alter ACE2 activity will tip this equilibrium, leading to hypertension or hypotension. The discovery and characterization of ACE2 quells any doubts about the importance of the Ang-(1-7) arm of the renin-angiotensin system.

ROLE OF ANG-(1-7) AND ACE2 IN HEART

The majority of studies of the physiological role of Ang-(1-7) first focused on its effects on blood pressure and cellular growth.

However, emerging evidence suggests a role for the heptapeptide in cardiac function. We showed intense Ang-(1-7) immunoreactivity in rat heart, both before and after myocardial infarction.[43, 44] Ang-(1-7) immunoreactivity was found in myocytes of the left and right ventricles as well as the interventricular septum of sham and coronary artery ligated rats. The region surrounding the ischemic zone in the ligated rats (the penumbra) had more intense Ang-(1-7) staining. While positive Ang-(1-7) immunoreactivity was found throughout the cytoplasm of cardiac myocytes, no staining was found in interstitial cells, the stroma outside myocytes, and intramyocardial vessels. Figure 4 shows strong staining for Ang-(1-7) in myocytes of the left ventricle of a rat heart; the Ang-(1-7) staining co-localized with immunoreactive ACE2. Although not shown, we also detect immunoreactive *mas*, the Ang-(1-7) receptor, in myocytes of the left ventricle. The identification of immunoreactive Ang-(1-7) in rat heart correlates with previous observations showing the presence of Ang-(1-7) in the venous effluent from the canine coronary sinus[45] and the production of Ang-(1-7) from Ang I and Ang II in the interstitial fluid collected from microdialysis probes placed in canine left ventricle.[46] Collectively these data demonstrate that Ang-(1-7), the enzyme that generates Ang-(1-7), and the Ang-(1-7) receptor is present in the heart, suggesting a role for the heptapeptide in cardiac function.

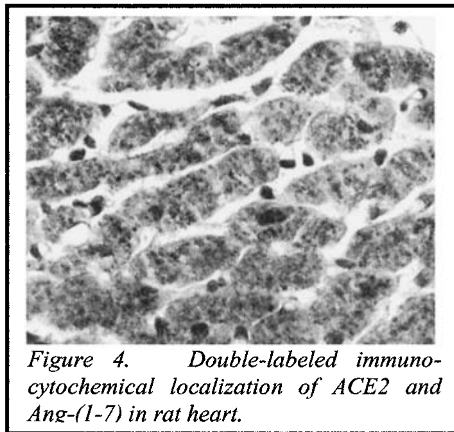

Figure 4. Double-labeled immunocytochemical localization of ACE2 and Ang-(1-7) in rat heart.

Recent evidence also demonstrates that Ang-(1-7) participates in the regulation of cardiac function. Ang-(1-7) treatment of isolated rat hearts following ischemia/reperfusion reduced the incidence and duration of arrhythmias.[47] The cardioprotective effects of Ang-(1-7) on the isolated ischemic heart were blocked by a selective $AT_{(1-7)}$ receptor antagonist or a cyclooxygenases inhibitor, suggesting that they were

mediated by the release of endogenous prostaglandins. In addition, Loot et al. [48] showed that an 8-week infusion of Ang-(1-7) following coronary artery ligation prevented the deterioration of cardiac function, as indicated by a 40% reduction in left ventricular end-diastolic pressure. The Ang-(1-7)-mediated improvement in cardiac function was associated with a significant decrease in myocyte size. Taken together, these results suggest a role for Ang-(1-7) in the regulation of myocyte growth and an impact on cardiac dynamics.

Studies with ACE inhibitors or AT_1 receptor antagonists indicate a role for the renin-angiotensin system in heart function and cardiac hypertrophy. Mice deficient in *ace* or *angiotensinogen* exhibit normal cardiac development or function, demonstrating that ACE and angiotensinogen are not critical in these processes. In contrast, ACE2-knockout mice have severely impaired heart function, including mild thinning of the left ventricle and a severe reduction in cardiac contractility.[42] Loss of ACE2 was associated with an increase in tissue and plasma Ang II, providing further evidence of a role for ACE2 in the hydrolysis of Ang II. No evidence of cardiac hypertrophy or fibrosis was observed in the 6-month old ACE2 deficient mice, suggesting that more time may be needed for significant progression of these pathologies. Generation of double mutant *ace/ace2* knockout mice completely abolished the cardiac dysfunction of the ACE2 knockout mice and caused a decrease in blood pressure.[42] In addition, disruption of ACER, the Drosophila ACE2 homolog, results in severe defects in heart morphogenesis.[42] These observations suggest that ACE2 counter-balances the function of ACE in the heart and provide strong support for the physiological interplay between the effector molecules, Ang II and Ang-(1-7).

In contrast, over-expression of ACE2 in mouse heart resulted in premature and sudden death, which was associated with ACE2 gene expression in a dose-dependent fashion.[49] In mice with moderate ACE2 expression, 50% of the animals succumbed to sudden death by 23 weeks of age, while 50% of the mice with higher expression (a 2.9-fold increase) were dead by 5 weeks of age. Although the hearts of these mice were normal by gross examination, they had severe and progressive conduction and rhythm disturbances. These abnormal electrophysiological responses correlated with a loss of connexins 40 and 43. The effect of ACE2 over-expression on connexin 40 and 43 and the resulting abnormal heart function may result from increased production of Ang-(1-7) or decreased Ang II. Although these studies show that over-expression of ACE2 contributes to cardiac pathology, they emphasize that ACE2 plays a critical role in cardiac development and

function.

Zisman et al. [50] showed that Ang-(1-7) is made in the intact human heart, as measured by the production of the heptapeptide in four heart transplantation recipients. The production of Ang-(1-7) decreased when Ang II formation was suppressed by an ACE inhibitor, suggesting that a major pathway for the formation of Ang-(1-7) was directly dependent upon the availability of Ang II as a substrate. The production of Ang-(1-7) from Ang II was inhibited by the selective ACE2 inhibitor from the Millenium Corporation (C16), decisively proving a role for ACE2 in Ang-(1-7) production in the heart.[51] In this same study, Ang-(1-7) was generated by infusion of Ang I, which was reduced by the neprilysin inhibitor thiorphan, confirming our initial studies showing that neprilysin is an additional Ang-(1-7)-forming enzyme.[52] Both of the Ang-(1-7) forming activities identified in the human heart were increased in failing human heart ventricles, providing evidence that Ang-(1-7) serves a cardioprotective role in heart failure.[50] This is in agreement with Goulter et al.,[53] who showed that ACE2 was up-regulated in human idiopathic dilated cardiomyopathy and ischemic cardiomyopathy and our recent studies in normotensive rats post-myocardial infarction.[42]

We investigated the effect of coronary artery ligation (CAL) on the expression of cardiac ACE2 in rats following a myocardial infarction (MI), to further explore the role of ACE2 in cardiac function following injury.[41] In addition, either losartan or olmesartan were administered to other rats via osmotic mini-pumps, to determine whether blockade of AT_1 receptors alters cardiac ACE2. Twenty-eight days after CAL, the heart weight to body weight ratio was 21% higher in vehicle-treated rats as compared with vehicle-treated sham-operated controls ($p < 0.001$). Loss of myocardial contractile mass was accompanied by significant increases in left ventricular end-diastolic pressure (LVEDP), increased cardiac rate, and reduced left ventricular $+dP/dt_{max}$. Rats medicated with either of the Ang II receptor blockers had a LVEDP lower than that found in vehicle-treated CAL rats. Plasma concentrations of Ang I, Ang II, and Ang-(1-7) were also significantly elevated above values determined in sham-operated rats and immunoreactive Ang-(1-7) was increased in the myocardium of CAL rats, as described above. Rats given losartan showed further increases in plasma levels of Ang I (71%), Ang II (216%), and Ang-(1-7) (632%) while similar findings were also obtained in rats medicated with olmesartan. As shown in the bottom panel of Figure 5, cardiac ACE2 mRNA was not altered by CAL but was increased significantly in rats treated with losartan for 28 days after CAL. In contrast, cardiac ACE mRNA was not changed by either CAL or treatment with the AT_1 receptor blockers (as shown in the upper panel of

Figure 5). Changes in cardiac ACE2 mRNA among all groups studied correlated directly with plasma levels of Ang-(1-7) and inversely with plasma levels of Ang II. In addition, plasma Ang-(1-7)/Ang II ratios were significantly augmented in the AT_1 receptor-treated groups, a finding that suggests increased formation of Ang-(1-7) from Ang II. These results suggest a differential effect of Ang II receptor blockade on the expression of cardiac ACE and ACE2 mRNAs and strongly support a role for the heptapeptide in the cardio-beneficial effects of AT_1 receptor blockade, since Ang-(1-7) markedly increased following blockade of AT_1 receptors. Since concurrent experiments showed that co-administration of the AT_2 receptor antagonist (PD123319) did not suppress the increase in cardiac ACE2 mRNA, these data confirmed that the effect was mediated by the AT_1 receptor.[41]

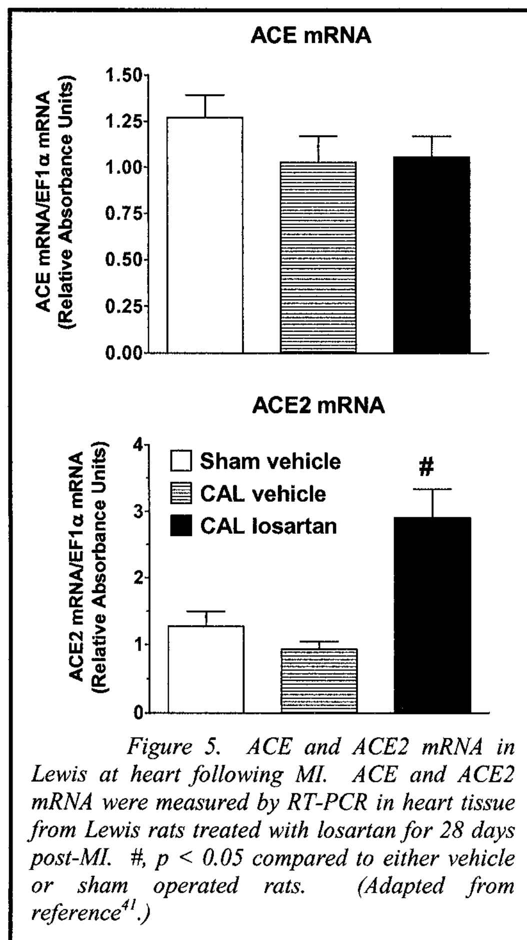

Figure 5. ACE and ACE2 mRNA in Lewis at heart following MI. ACE and ACE2 mRNA were measured by RT-PCR in heart tissue from Lewis rats treated with losartan for 28 days post-MI. #, $p < 0.05$ compared to either vehicle or sham operated rats. (Adapted from reference[41].)

The increase in ACE2 mRNA in ligated rats treated with Ang II receptor blockers suggests that AT_1 receptor blockade prevents a down-regulation of ACE2 by Ang II. Alternatively, the increase in Ang-(1-7) by losartan treatment may up-regulate ACE2. We isolated myocytes and cardiac fibroblasts from neonatal rats and measured ACE2 mRNA following treatment with Ang II, in the presence or absence of losartan, to directly determine whether angiotensin peptides regulate cardiac ACE2. Ang II caused a significant reduction in ACE2 mRNA in cultured myocytes and fibroblasts, an effect blocked by the AT_1 receptor antagonist.[54] These results suggest that Ang II elevation following CAL reduces ACE2 by a transcriptional regulatory mechanism, an effect which is reversed by blocking the AT_1 receptors. In agreement with these studies, we showed that ACE2 is increased in renal tubules of rats treated with omapatrilat, a combined ACE/neprilysin inhibitor [55]. Thus, we hypothesize that increased concentrations of Ang II down-regulate ACE2 to prevent its conversion to Ang-(1-7), thereby blocking Ang-(1-7)-mediated effects.

The studies performed thus far indicate that ACE2 is highly regulated at transcription, a characteristic not unexpected for a rate-limiting enzyme that maintains the equilibrium between opposing arms of a biochemical pathway. It would not be surprising that multiple layers of regulation, including translational and post-translational modifications, are involved in determining the tissue-specific concentrations of the enzyme. It is the modulation of this cellular control that must be exploited, if ACE2 is to serve as an important therapeutic target. We showed that ACE inhibition or AT_1 receptor blockade caused a marked increase in ACE2 mRNA in the heart, providing a mechanism for the cardioprotective effects of these drugs, specifically hydrolysis of the vasoconstrictor, mitogenic Ang II to the vasodilator, anti-proliferative Ang-(1-7).

The emergence of ACE2 as a critical regulator of the renin-angiotensin system emphasizes the need for the development of novel therapies that specifically regulate this enzyme, not through the blockade or inhibition of other components of the pathway. Only time will tell whether we are entering a new phase of drug development, where we design therapies to precisely control the transcription of a target. While the emphasis to date was placed on the regulation of Ang II as the key determinant in cardiovascular physiology, the discovery of ACE2 highlights the need to account for the action of both opposing arms of the renin-angiotensin pathway, specifically the biological effects of Ang-(1-7), when elucidating mechanisms of normal heart function and the development of drug regimes to combat cardiac pathologies.

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Chapter 12
DYSLIPIDEMIA AND ANGIOTENSIN II AND ATHEROGENESIS

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INTRODUCTION

Hyperlipidemia and hypertension are major risk factors for atherosclerosis. They are often present in the same patient. It is thought that an interaction between hyperlipidemia and neurohumoral systems, such as the renin-angiotensin system (RAS), not only explains the co-existence of hypertension and dyslipidemia but also suggests a major role in the pathogenesis of atherosclerosis. Data from various studies have suggested that the effects of RAS and hyperlipidemia are not independent and the underlying mechanisms by which both initiate and accelerate atherosclerosis may overlap. Treatment directed at lowering total cholesterol, low-density lipoprotein (LDL) cholesterol, triglycerides and raising high-density lipoprotein (HDL) cholesterol levels have resulted in a reduction of cardiovascular events. Angiotensin-converting enzyme (ACE) inhibitors and angiotensin type 1 (AT1) blockers modulate the RAS and have been shown to reduce cardiovascular events in patients with vascular disease. There is a suggestion that treatment directed at lowering cholesterol along with agents that modulate RAS may have added benefit in the prevention, progression and treatment of coronary artery disease, hypertension and heart failure.

OXIDATIVE STRESS IN ATHEROSCLEROSIS

Increased oxidative stress plays an important role in various steps in hypercholesterolemic atherosclerosis (Figure 1). Oxidative stress activates endothelial cells, results in the expression of adhesion molecules leaving an inflammatory state, and facilitates oxidation of LDL-cholesterol and its uptake in vascular components of vessel wall. Oxidative stress is also responsible for the release of metalloproteinases (MMPs) which cause disruption of atherosclerotic plaque, and activation of platelets and formation of the occlusive thrombus in the narrowed lumen. Both dyslipidemia and angiotensin II facilitate this process of oxidative stress in the vessel wall (1). Angiotensin has been shown to induce NADH oxidase activity (2) and AT1 receptor blockers reduce superoxide anion production and NADH oxidase activity in aortas from rabbits fed a normal diet (controls) or a high-cholesterol diet and reduce atherosclerosis (3). NADH oxidase represents a major vascular source of superoxide anions, and increased tissue levels of angiotensin II may cause its increased activity. The improvement of endothelial dysfunction, inhibition of the NADH oxidase, and reduction of early plaque formation by AT1 receptor antagonists suggest a crucial role of angiotensin II–mediated superoxide anion production in the early stage of atherosclerosis (3).

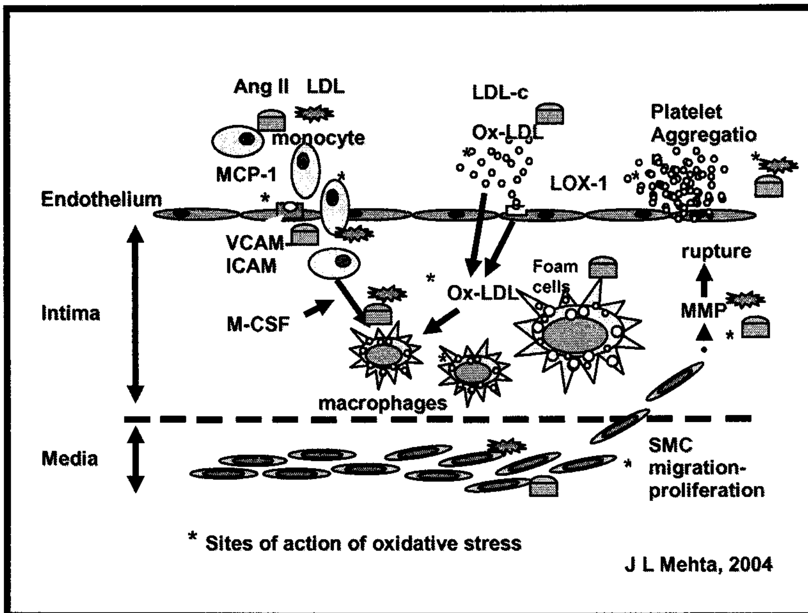

Figure 1. Sites of oxidative stress in atherosclerosis.

Clinical and experimental studies have reported a marked decrease in endothelium-dependent vasodilation as one of the early stages of atherosclerosis (4). In some cases, it has been shown due to increased inactivation of endothelium-derived nitric oxide by superoxide anions rather than a consequence of decreased nitric oxide production (5).

FACILITATIVE ROLE OF ANGIOTENSIN II ON OXIDATION OF LDL CHOLESTEROL AND ITS UPTAKE IN VESSEL WALL

Oxidized low-density lipoprotein (ox-LDL) is more important than native LDL-cholesterol in atherogenesis (6). Cholesterol accumulation in the macrophages and their transformation into foam cells are major events in the development of atherosclerosis. This process results from increased uptake of ox-LDL (7) and enhanced macrophage cholesterol synthesis. Angiotensin II has been shown (8) to increase s macrophage cellular cholesterol biosynthesis with no significant effect on blood pressure or plasma cholesterol levels (8). These effects have been shown to be reversed by ACE inhibitors like fosinopril and the AT1 receptor blocker losartan. Nickenig et al (9) have sh own that LDL-cholesterol accumulates in cultured vascular smooth muscle cells via AT1 receptor activation. The relevance of AT1 receptor became evident in experiments wherein angiotensin II was unable to increase cholesterol synthesis in macrophages that lack the AT 1 receptors.

Angiotensin II facilitates macrophage cholesterol influx, perhaps by enhancing LDL oxidation in arterial wall components (10). Angiotensin II can bind to LDL and form modified lipoprotein, which is taken up by the scavenger receptors on the macrophages leading to cellular cholesterol accumulation (11). We examined the kinetics of ox-LDL uptake in endothelial cells and observed that angiotensin II enhanced the uptake of ox-LDL in these cells (12). This effect was blocked by the AT1 receptor blocker losartan, but not the angiotensin II type 2 (AT2) receptor blocker PD 123319. Angiotensin II has been shown to up-regulate macrophage messenger RNA (mRNA) for 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. Fluvastatin, a competitive inhibitor of HMG-CoA reductase, blocked the stimulatory effect of angiotensin II on macrophage cholesterol biosynthesis in one study (13). This demonstrates that the probable biochemical site for the action of angiotensin II along the cholesterol

biosynthesis pathway is HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis. Further, the stimulation of cholesterol biosynthesis in macrophages and ox-LDL uptake in smooth muscle cells, macrophages and endothelial cells requires or is at least facilitated by AT1 receptor activation. HMG-CoA reductase expression may play an important role in this process.

DYSLIPIDEMIA ACTIVATES RENIN-ANGIOTENSIN SYSTEM

Hypercholesteremia has been shown to increase the expression of ACE and AT1 receptors in the atherosclerotic lesions in experimental animals (14, 15). Studies in human atherosclerotic tissues have confirmed the upregulation of RAS, particularly in regions prone to plaque rupture (16, 17). Importantly, these same areas show extensive deposits of inflammatory cells, macrophages, and apoptosis.

Incubation of vascular smooth muscle cells with LDL cholesterol has been shown to increase AT1 reception expression (19). Li et al (20) observed that ox-LDL increases mRNA and protein for AT1, but not AT2, receptors in the human coronary artery endothelial cells, implying that AT1 expression is amplified at the transcriptional level. A ctivation of the redox-sensitive transcription factor NF-κB (nuclear factor kappa B) plays an important role in the process. To define the relationship of RAS and lipids in humans, Nickenig et al (9) administered angiotensin II to normocholesterolemic and hypercholesterolemic men and found that the increase in blood pressure was exaggerated in the latter group and was blunted by LDL-cholesterol lowering agents. A linear relationship between AT1 receptor density on platelets and LDL-cholesterol concentration in the plasma was observed. Down-regulation of AT1 receptor expression with statins has also been shown in vascular smooth muscle and endothelial cells (20, 21).

The expression of genes for chymases, enzymes by which angiotensin II can be formed independent of ACE activation has been shown to be increased in aortic atherosclerotic lesions of monkeys fed a high-cholesterol diet (22). The functional significance of chymase in the development of atherosclerosis, however, remains uncertain. These observations suggest a close interaction of dyslipidemia and RAS.

ANGIOTENSIN II, DYSLIPIDEMIA AND LOX-1

Atherosclerotic plaques are rich in ox-LDL, and patients with acute coronary syndrome have increased plasma levels of ox-LDL; however, the serum cholesterol levels in these patients are not significantly different from controls (23). We have identified high-affinity lectin like receptor for ox-LDL; LOX-1, in cultured human coronary artery endothelial cells by reverse transcriptase–polymerase chain reaction, Western blot testing, and radioligand binding (24). Native LDL does not bind to this receptor. Endothelial cells *in vitro* and *in vivo* internalize and degrade ox-LDL through this putative receptor-mediated pathway that does not seem to involve the classic macrophage scavenger receptor. Cytokine tumor necrosis factor α (25) and fluid shear stress (26) have also been shown to up-regulate LOX-1 gene expression. LOX-1 expression is involved in apoptosis (programmed cell death) in response to ox-LDL (27, 28), MAPK-1 (mitogen-activated protein kinase 1) activation, and expression of adhesion molecules and attachment of monocytes to activated endothelial cells (29). NF-κB plays a critical role in the effects of ox-LDL on endothelial cells (24). The pro-apoptotic effect of angiotensin II in human coronary artery endothelial cells and the role of AT1 receptor and protein kinase C activation have also been shown by our group (30).

We showed that angiotensin II upregulates LOX-1 expression and this effect can be blocked by the AT1, but not AT 2 receptor blockers. On the other hand, ox-LDL up regulates AT 1, but not the AT2, receptor expression in human coronary artery endothelial cells (31). These observations suggest a cross-talk between dyslipidemia and angiotensin II. The two systems act synergistically in inducing cell injury and initiating an inflammatory state, a prelude to atherosclerosis (Figure 2).

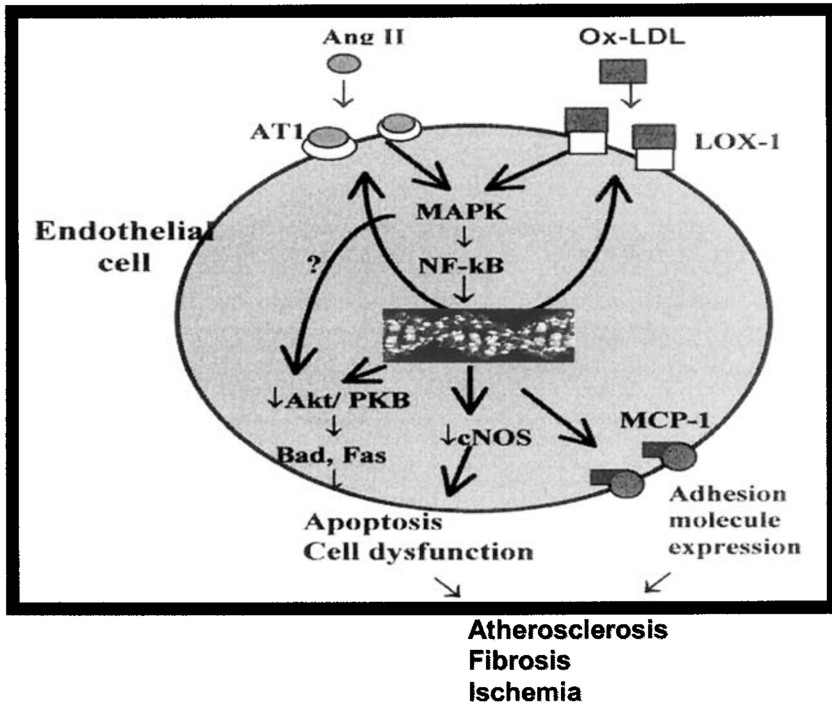

Figure 2. Upregulation of AT1 receptors by ox-LDL and of LOX-1 by Ang II.

Chen et al (32) from our laboratory showed intense immunostaining for and up-regulation of the gene for LOX-1 in the atherosclerotic tissue of rabbits fed a high-cholesterol diet. In addition to reducing atherosclerosis, losartan blocked LOX-1 up-regulation. Recent studies from our laboratory show marked up-regulation of LOX-1 in concert with apoptosis in human atherosclerotic plaques, particularly in the regions that are prone to rupture. This emphasizes the importance of redox-sensitive pathways in the cross-talk between ox-LDL and RAS.

It is noteworthy that statins have been shown to reduce atherosclerotic plaque formation (33), attenuate the reduction in endothelial NO synthase expression (34), and reduce the expression of leukocyte adhesion molecules (35). Statins have also been shown to reduce the transcription of LOX-1 as well as reduce the binding of ox-LDL to human coronary endothelial cells (35).

ROLE OF AT2 RECEPTORS IN THE PRO-ATHEROGENIC EFFECT OF ANGIOTENSIN II

Angiotensin II also activates a type 2 (AT2) receptor which is highly expressed in fetal tissues and decrease rapidly after birth (36). Under normal circumstances AT2 receptors are minimally expressed in adult tissues, but are still detectable in the pancreas, heart, kidney, adrenals, myometrium, ovary, brain and vasculature (36). However, these receptors are re-expressed in adults after vascular and cardiac injury and during wound healing, suggesting a potential role in tissue remodeling. The functional role of these receptors is unclear, but may relate to antagonize AT1 receptor mediated effects under physiological conditions. Although most studies have focused on the protective effects of AT2 receptor activation against cardiac ischemia-reperfusion injury and hypertension, one study has demonstrated that AT2 receptor activation triggers an anti-growth effect and modulates AT1 receptor-induced vascular smooth muscle cell proliferation and extracellular matrix accumulation, implying an anti-atherosclerotic effect (37). AT2 receptor expression may also be regulated by AT1 receptor blockers. It has been demonstrated that AT1 receptor blockade may activate AT2 receptors by increasing their expression, resulting in the cardioprotective effects against acute ischemia-reperfusion injury (33).

INHIBITORY EFFECT OF RAS INHIBITORS ON ATHEROSCLEROSIS

Both ACE and AT1 receptor blockers have been shown to reduce the progression of atherosclerosis (24-26). The AT1 receptor blocker losartan therapy has been shown to suppress the expression of adhesion molecules and NF-κB by activating its regulatory protein IκBα in rabbits fed a high cholesterol diet (26). To determine the specific role of RAS inhibitors (vs the blood pressure lowering effect), Leif et al (24) conducted a study with low doses of fosinopril or losartan. Control animals were given either placebo or a dose of hydralazine that lowered blood pressure. LDL oxidation as measured by levels of thiobarbituric acid reactive substances or by formation of conjugated dienes was suppressed by low-dose fosinopril, suppressed only modestly by losartan, and unaffected by placebo or hydralazine hydrochloride. Atherosclerosis was inhibited by fosinopril and losartan, and by hydralazine, suggesting that the antiatherosclerotic effects of RAS inhibitors may be due, at least in part, to direct inhibition of LDL

oxidation and other actions of angiotensin II in the vessel wall.

Bavry et al (27) in our laboratory showed that the ACE inhibitor quinapril decreased intra-arterial thrombus formation, whereas the AT1 receptor blocker losartan had a minimal effect. The inhibitory effect of ACE inhibitors on the generation of plasminogen activator $inhibitor_1$ may be relevant in this differential effect.

Endothelial dysfunction in hypercholesterolemic animals has been shown to be improved by ACE inhibitors (28). Bradykinin antagonists can diminish some of this effect, suggesting that the inhibition of bradykinin breakdown rather than angiotensin II formation may be important in this effect (35).

As discussed earlier, inhibition of angiotensin II–sensitive, NADH-dependent, superoxide-producing enzymes may be another mechanism responsible for the improvement in a reduction of NO inactivation (11).

Lauten et al (38) have suggested treatment with ACE inhibitor and AT1 receptor blockers have independent additive anti-inflammatory effects on vascular endothelium and smooth muscle cell layers, which may contribute to their anti-atherosclerosis effect.

MECHANISM OF PRO-ATHEROGENIC EFFECTS OF OX-LDL

Ox-LDL induces endothelial dysfunction (39), which involves decreased expression/activity of constitutive endothelial nitric oxide synthase (eNOS); increased expression of adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemoattractant protein-1 (MCP-1), which are responsible for the adhesion of monocytes to ECs; increased expression of inflammatory mediators such as matrix metalloproteinase-1 (MMP-1) and CD40L/CD40; and increased platelet aggregation and increased apoptosis (Figure 1). In endothelial cells, many of the pro-atherosclerotic effects of ox-LDL are mediated via LOX-1, whose activation induces the generation of intracellular reactive oxygen species (24), which in turn stimulate mitogen MAPK and transcription factor NF-κB, activation, leading to the regulated gene expression (40). As discussed earlier, ox-LDL is taken up by monocytes via a variety of scavenger receptors. The accumulation of ox-LDL in the macrophages with their transformation into foam cells is an early event in the development of atherosclerosis (40).

In addition to endothelial cells and monocytes/macrophages,

ox-LDL affects the biology of other vascular components. Ox-LDL induces the migration and proliferation of vascular smooth muscle cells, which is a hallmark in the pathogenesis of atherosclerosis (41). The effects of ox-LDL on fibroblasts have also been examined. One study showed that ox-LDL treatment stimulates the proliferation of vascular fibroblasts (42), and another study showed increased formation of collagen in LDL or ox-LDL treated fibroblasts (43). As further evidence for a role of ox-LDL in atherosclerosis, plasma levels of ox-LDL are increased in patients with atherosclerosis and in those at high risk of developing atherosclerosis related events (44).

Other studies have shown that a number of antibodies to ox-LDL are increased in plasma in patients with atherosclerosis. Recent studies from our laboratory show that Cu-LDL-IgG antibody levels parallel progression as well as regression of atherosclerosis (45).

ANIMAL STUDIES ON THE CROSS-TALK BETWEEN DYSLIPIDEMIA AND RAS IN ATHEROSCLEROSIS

Since hyperlipidemia and RAS activation are two well-known inter-related risk factors for atherogenesis (*in vitro* and *in vivo*), simultaneous blo ckade of hyperlipidemia and RAS (with statins) and AT1 receptor blockers may have synergistic anti-atherosclerotic effects. Both these groups of agents are independently effective in preventive atherosclerosis-related events. The effects of concurrent administration of these two therapies, therefore, would be of great interest.

In recent studies, we observed that the concurrent treatment of hyperlipidemia and inhibition of RAS had a synergistic anti-atherogenic effect in the apo-E deficient mice, which are well recognized as a standard animal model for the study of atherosclerosis and related diseases. In this study, C57BL/6J mice were studied as a negative control. As positive control, apo-E deficient mice were given regular chow supplemented with 1% cholesterol. For "hyperlipidemia treatment" group, apo-E deficient mice were given rosuvastatin (1mg/Kg/day) in addition to a high cholesterol diet. Rosuvastatin is a member of HMG CoA reductase inhibitor family of drugs. It is used as a lipid lowering drug. For "RAS inhibition" group, apo-E deficient mice were given the AT 1 receptor blocker candesartan (1mg/Kg/day) in addition to high cholesterol diet. For the "concurrent therapy" group, apo-E deficient mice were given the two therapies currently. All

mice were treated for 12 weeks, and the extent of aortic atherosclerosis was measured by Sudan IV staining. We found that the aortic atherosclerosis induced by high cholesterol diet in apo-E deficient mice was attenuated by rosuvastatin or candesartan given alone, but interestingly, the concurrent therapy had a synergistic anti-atherogenic effect (Figure 3).

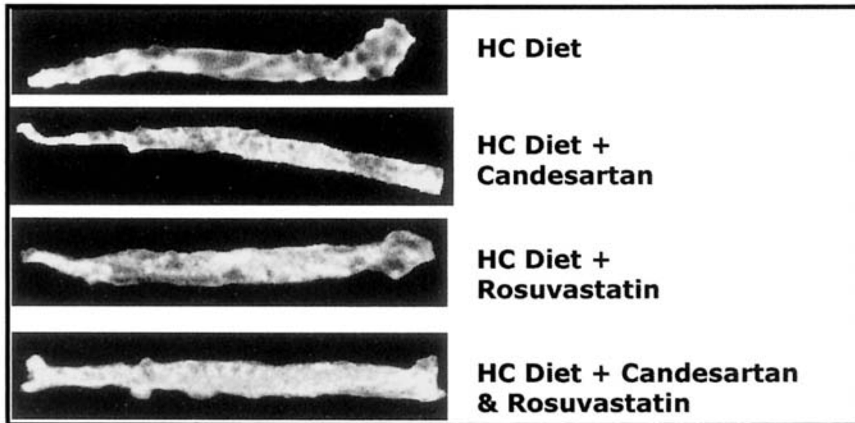

Figure 3. Representative examples of the extent of atherosclerosis in different groups of animals.

We also examined the potential mechanisms for the synergistic anti-atherogenic effects of current therapy. First, we observed the expression of LOX-1 in mice aorta from each group and found that both rosuvastatin and candesartan each decreased the expression of high cholesterol diet-induced LOX-1 expression (Figure 4), however, the concurrent therapy with rosuvastatin and candesartan decreased LOX-1 expression to a level even lower than the untreated negative control mice. Based on these observations, it is safe to state that LOX-1 is a critical player in atherogenesis, and it is targeted by anti-hyperlipidemia therapy as well as anti-RAS therapy, leading to the anti-atherosclerotic effects. These observations gain support from preliminary observations that deletion of LOX-1 almost totally blocks the evolution of atherosclerosis in LDL-receptor deficient mice (unpublished data).

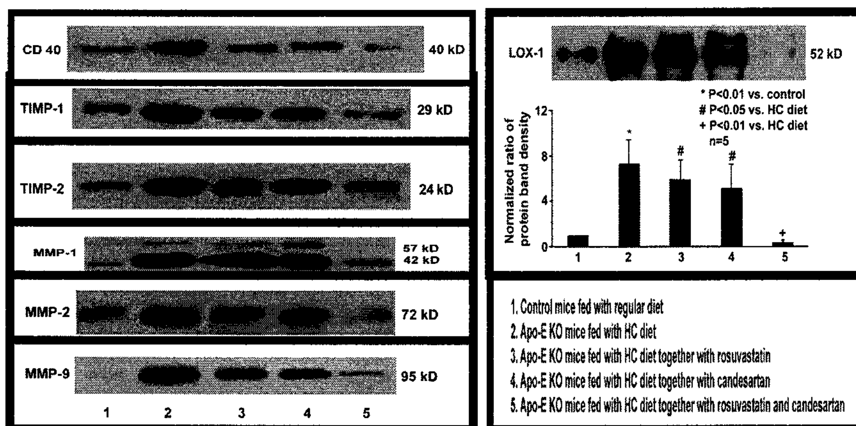

Figure 4. Different inflammatory markers and LOX-1 expression in different groups of animals.

A number of studies support the idea that atherosclerosis is a chronic inflammatory disease. Considerable evidence implicates inflammatory mediators such as CD40 (52), MMPs (53) and their endogenous tissue inhibitors TIMPs (54) are involved in the development of atherosclerosis. CD40 expression has been shown to be inhibited by AT1 receptor blockers (55) and statins (56). The expression of MMPs has also been reduced by both AT1 receptors blockers (57) and statins (58). We looked at the expression of these inflammatory mediators in the mice aorta from each group in our study. We found that all these pro-atherogenic inflammatory mediators were markedly upregulated in the apo-E deficient mice after 12 weeks feeding with high cholesterol diet. Although these hyperlipidemia-induced effects were modestly attenuated by rosuvastatin or candesartan alone, the combination therapy showed total blockade of these pro-atherogenic mediators (Figure 4).

We also studied the signal transduction pathway involved in the regulated expression of LOX-1 and CD40 as well as MMPs in the mice aorta. We focused on mitogen-MAPKs, because it has been shown that MAPK, especially the oxidative stress-sensitive p38 subtype, is involved in the hyperlipidemia-induced atherosclerosis (59). We identified that p38 MAPK (expression and phosphorylation) was upregulated in apo-E deficient mice fed with high cholesterol diet, compared with the control mice on regular diet. Both rosuvastatin and candesartan each had a moderate inhibitory effect on p38 MAPK expression and phosphorylation; however, the concurrent therapy dramatically reduced the expression as well as phosphorylation of p38

MAPK back to the control level. We also checked the expression and phosphorylation of p44/42 MAPK, but did not find any change among different groups (Figure 5).

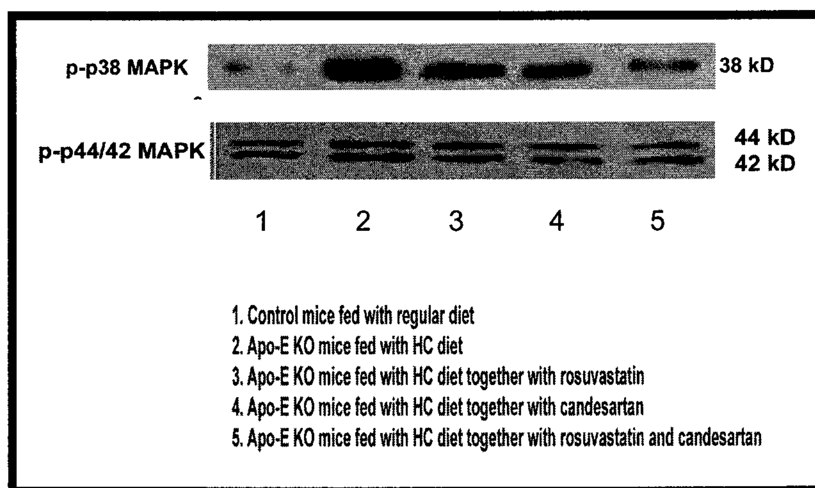

Fig. 5. Expression of activated p38 and p42.44 MAPK in different groups of animals.

This innovative study confirms the interaction between hyperlipidemia and RAS activation, and also implies that LOX-1 and inflammation mediators as well as p38 MAPK play a critical role in atherogenesis. More importantly, it provided a novel therapeutic strategy against atherosclerosis.

SUGGESTIVE EVIDENCE OF CROSS-TALK BETWEEN DYSLIPIDEMIA AND RAS IN HUMAN STUDIES

The prevalence of hypertension is more in populations with high levels of serum cholesterol. Dyslipidemia may be another metabolic factor influencing blood pressure. Lloyd-Jones et al (60) evaluated patients from the Framingham Heart Offspring Study and noted that on average more than 40% of men and 33% of women with blood pressures of 145/90 mm Hg or higher were also dyslipidemic. These two entities are frequently associated, even when current rigorous definitions are used. Hypertensive individuals are more likely to become dyslipidemic over time.

Sung et al (61) examined the blood pressure response to a

standard mental arithmetic test in healthy, normotensive patients with hypercholesterolemia (mean total cholesterol, 263 mg/dL) and 33 normotensive, normocholesterolemic patients. None of the hypercholesterolemic patients was receiving lipid-lowering therapy. They noted that the blood pressure response during the arithmetic test was significantly higher in the hypercholesterolemic group compared with the normocholesterolemic group (18 vs 10 mm Hg, respectively, $P < .005$). In a double-blind crossover design, hypercholesterolemic patients were then divided into 2 subgroups and received either 6 weeks of lovastatin or placebo. Treatment with statins reduced total cholesterol and LDL-C levels and was associated with lower mean systolic blood pressure. Diastolic blood pressure changes did not significantly correlate with lipid lowering suggesting that hypercholesterolemic patients have exaggerated systolic blood pressure and lipid-lowering therapy improves the response to stress.

Nazzaro et al (62) examined the effects of lipid lowering on blood pressure in hypertensive and hypercholesteremic patients. Subjects were given placebo for 4 weeks and then divided into 2 groups. Each group of 15 patients subsequently received simvastatin, 10 mg, or enalapril, 20 mg, for 14 weeks and combination of both drugs for an additional 14 weeks. Blood pressure was then measured during stressful stimuli such as the Stroop color test plus the cold pressor forehead test. Enalapril lowered blood pressure but interestingly simvastatin also lowered blood pressure (although to a lesser extent), and the combination had much higher impact.

Several human studies have shown that lipid lowering therapy with statins may have blood pressure lowering effect (Table 1) (ref. 63-69). Few other clinical trials have suggested interaction between RAS and dyslipidemia (Table 2) (ref. 70-73). In the TREND (Trial on Reversing Endothelial Dysfunction) trial quinapril hydrochloride was shown to improve endothelial dysfunction in patients with elevated LDL-C levels of ≥130 mg/dl (70). QUIET (Quinapril Ischemic Events Trial) studied the effect of ACE inhibition on CAD and showed less progression of disease in patients with LDL-C of ≥130 mg/dl (71). In LCAS (Lipoprotein and Coronary Atherosclerosis Study) trial change in lumen diameter as assessed by quantitative coronary angiography was noted in patients randomized to fluvastatin sodium. LDL-C reduction was also analyzed by Marian et al (73) according to the ACE insertion/deletion (I/D) phenotype. ELITE (Evaluation of Losartan In The Elderly) studied the effect of captopril and losartan in elderly pts with CHF and found greater survival benefit in patients taking statins in addition to captopril and losartan.

Table I. Blood pressure-Lowering Effect of lipid-Lowering Treatment

Source	Study Outline	Drug Used	Final Outcomes
O'Callaghan et al, [63]	Pts with hypertension and hyperlipidemia	Pravastatin sodium vs placebo for 12wk	Pravastatin did not lower blood pressure
Abetel et al, [64]	Pts with hypertension and hyperlipidemia	Fluvastatin sodium for 3mo	Fluvastatin lowered blood pressure by 8-16 mm Hg
Glorioso et al, [65]	Pts with hypertension and hyperlipidemia	Pravastatin sodium vs placebo for 32wk	Pravastatin decreased blood pressure by 8 mm Hg
Sposito et al, [66]	Pts with hypertension and hyperlipidemia	ACE inhibitor (enalapril maleate or lisinopril) alone or with statin (lovastatin or pravastatin)	Additive blood pressure-lowering effect of the combination compared with ACE inhibitor alone
Borghi et al, [67]	Pts with hypertension and hyperlipidemia	Statin (pravastatin or simvastatin) in addition to antihypertensive treatment	Additive benefit of statins in blood pressure lowering shown
Tonoto et al, [68]	Microalbuminuric hypertensive pts with DM type II	Simvastatin in addition to antihypertensive treatment	Simvastatin exert additional blood pressure-lowering effect and reduced 24hr urinary albumin excretion
Jonkers et al, [69]	Pts with hypertriglyceridemia and hypertension	Bezafibrate	Bezafibrate reduced systolic blood pressure by 5mm Hg

Abbreviations: ACE, angiotensin converting enzyme

Table 2. Summary of the Results of Clinical Trials Suggesting Interaction between Renin-Angiotensin System and Dyslipidemia

Clinical Trials	**Study Objective**	**Results**
TREND [70]	Effect of ACE inhibitor (quinaprine hydrochloride) in acetylcholine-induced endothelial response of coronary artery according to LDL-C level	Quinapril hydrochloride, 40mg/d, had a greater efficacy improving endothelial function in the group with LDL-C levels ≥130 mg/dl thanin the group with LDL-C levels ≤ 130 mg /dl.
QUIET [71]	Effect of ACE inhibitor (quinaprine hydrochloride) on ischemic events and angiographic progression of coronary disease assessed in pts who underwent percutaneous intervention	Overall effect on primary ischemic and angiographic end point was neutral; however pts with LDL-C levels ≥130mg/dl had significantly less disease progression compared with pts LDL-C levels ≤ 130 mg /dl.
LCAS [72]	Effect of statin fluvastatin sodium on minimal lumen diameter as assessed by quantitative coronary angiography according to ACE genotype; LDL-C reduction according to ACE genotype was analyzed	Change in lumninal diameter in the fluvastatin arm was noted. There was significant reduction in the LDL-C levels according to the ACE genotype
ELITE II [73]	Effect of captopril and losartan potassium on cardiac events in elderly pts with CHF	Both captopril and losartan decreased crude mortality similarly; however pts who were taking statins had additional reduction in mortality

Abbreviations: ACE, angiotensin converting enzyme; CHF, congestive heart failure; ELITE II, Evaluation of Losartan In The Elderly; LCAS, Lipoprotein and Coronary Atherosclerosis Study; LDL-C, low-density lipoprotein cholesterol; QUIET (Quinapril Ischemic Events Trial; TREND (Trial on Reversing Endothelial Dysfunction)

CONCLUSIONS

Hypertension and dyslipidemia, the two major risk factors for atherosclerotic disease, are frequently associated in patients with CAD. Data from clinical studies suggests the existence of lipoprotein-neurohormonal interactions that may adversely affect vascular

ultrastructure and function. On the other hand data from preclinical studies suggests up-regulation of RAS by dyslipidemia, most likely from increased ox-LDL production. Activation of RAS leads to release of superoxide anions, transcriptional upregulation of LDL and increased ox-LDL uptake in macrophages, smooth muscle cells, and endothelial cells. These findings broaden our vision about the interplay among different risk factors for atherosclerosis which can act synergistically to increase cardiovascular risk. It also extends our knowledge of reduction of cardiovascular risk by the anti-atherosclerotic effects of local ACE inhibition. Trials aimed at modifying RAS along with the drugs that reduce total cholesterol and LDL-C levels will address the clinical relevance of this biological interaction. In conclusion, findings from cellular, animal, and human experiments suggest a cross-talk between dyslipidemia and RAS relative to vascular dynamics.

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Chapter 13
ACE INHIBITORS DIRECTLY ACTIVATE BRADYKININ B_1 RECEPTORS TO RELEASE NO

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INTRODUCTION

Angiotensin I-converting (ACE; kininase II) inhibitors have been successfully used to treat millions of patients world-wide, but their cellular, subcellular and molecular modes of action are still being unraveled. The inhibition of ACE (kininase II) blocks angiotensin II release or bradykinin inactivation [1-3] but these alone do not fully account for the usefulness of ACE inhibitors. They also potentiate the effects of bradykinin and its ACE-resistant analogs on the B_2 receptors by inducing an enzyme/receptor interaction (ACE/B_2), heterodimer formation [4-7].We showed this by using atrial strips and ileal segments of guinea pigs in bio-assay and in cultured cells of human and animal origin.

Of the two bradykinin receptors B_1 and B_2, B_2 is ubiquitous as a first line, primary mediator of kinin action under physiological conditions [8, 9]. Normally, few cell types express B_1 receptors, but various pathologic conditions such as ischemia, myocardial infarction, atheromatous disease, or exposure to inflammatory cytokines rapidly induce its expression [9]. The elimination of the B_2 receptor gene in knockout mice also up-regulated the B_1 receptor [10]. The peptide agonists of the receptors differ, as plasma carboxypeptidase N or cell

membrane carboxypeptidase M cleave the C-terminal Arg of the B_2 receptor agonists kallidin (Lys-bradykinin) and bradykinin to generate B_1 agonists desArg-kinins (Fig.1) [9, 11, 12]. Both of these kininase I-type human carboxypeptidases have been cloned and the crystal structure of carboxypeptidase M was recently published by R.A. Skidgel and F. Tan with the collaboration of the Max Planck Institute in Munich [13].

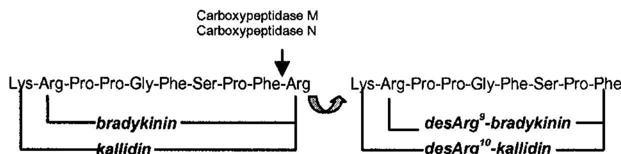

Figure 1. Schematic diagram that illustrates how carboxypeptidases generate specific B1 receptor agonists (desArg9-bradykinin, desArg10-kallidin) from the B2 agonists (bradykinin, kallidin).

Multiple contributions of the kinin B_2 receptors to the effects of ACE inhibitors have already been published [1, 7]. In contrast, little was known about the relationship between the B_1 and ACE inhibitors. We hypothesized an unexplored role for the B_1 receptors from description of conditions of many patients treated with ACE inhibitors that can also lead to B_1 receptor induction. Following this lead, we found that ACE inhibitors in nanomolar concentrations directly activate the bradykinin B_1 receptors in cells without an intermediate peptide ligand and in the absence of ACE [14]. This interaction leads to a prolonged NO release from endothelial cells. In subsequent studies we investigated further how the activation of B_1 leads to NO release [15]. We also tested whether the two B_1 receptor agonists, the peptide desArg^{10} -kallidin and the ACE inhibitor enalaprilat, acting on different domains on the receptor could initiate transduction through different signaling pathways.

METHODS

Cell Culture. Effects of ACE inhibitors were tested using several cultured cell types, which included:

A. CHO, HEK293 and COS7 cells that were transfected to express human B_1 receptors [14]
B. Bovine pulmonary artery endothelial (BPAE) cells and IMR-90 fibroblasts, which constitutively express B_1 receptors [14, 15]

C. Human pulmonary artery endothelial (HLMVE) cells, which were routinely treated with interleukin-1β (IL-1β) and interferon-γ (IFN- γ) for 16 to 18 h prior to the experiment in order to induce B_1 receptors [15]

Methods used. Measurement of $[Ca^{2+}]_i$ and detection of NO were performed as reported [14, 15]. Real time NO was measured using a porphyrinic microsensor. Current generated on the porphyrinic electrode was proportional to the NO released and was quantitated with a known standard NO solution.

Phosphoinositide hydrolysis was assayed as previously described [15]. Briefly, cells were labeled with $[^3H]$ inositol for 16-24 h, then washed with 5 mM LiCl. Cells were extracted and inositol phosphates were separated by anion exchange chromatography (Seuwen *et al.*, 1990). Results are expressed as the ratio of inositol phosphates / inositol phosphates + inositol counts.

L-arginine transport – Transport was assayed in whole cells using L-[2,3,4,5^{3}H] arginine monohydrochloride as reported [15].

RESULTS

As a prototype of an active ACE inhibitor we used enalaprilat and initially measured the increase in $[Ca^{2+}]_i$ in IMR-90 and BPAE cells, which express B_1 and B_2 receptors [14]. Subsequently, we also measured NO release from BPAE cells using a porphyrinic electrode in real-time (Fig. 2). The receptors were activated with either the peptide agonists desArg^{10}-kallidin and bradykinin or ACE inhibitor enalaprilat. Bradykinin (10 nM) and des-Arg^{10}-kallidin (10 nM) released NO in distinctly different patterns. Bradykinin, a B_2 receptor ligand, released NO in a brief burst, while desArg^{10}-kallidin, agonist of the B_1 receptor, brought about a more prolonged and substantially higher liberation of NO than after B_2 receptor stimulation (Fig 2A). Enalaprilat (10 nM), in the absence of a peptide agonist, significantly increased NO, similarly to desArg^{10}-kallidin (Fig.2A). The response to enalaprilat was completely inhibited by the B_1 antagonist desArg^{10}-Leu^9-kallidin (Fig.2C).

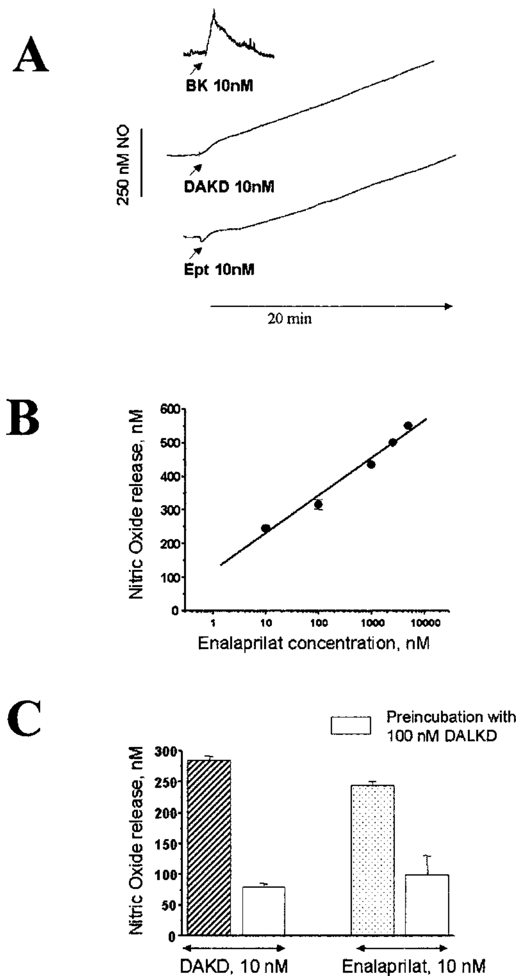

Figure 2. Stimulation of NO generation in endothelial cells by B1 agonists. NO production was measured in BPAE cells using a porphyrinic microsensor in real time. A, the addition of enalaprilat (Ept) or des-Arg10-kallidin (DAKD) (denoted by the arrows) caused an immediate generation of NO, which continued to increase over 20 min. In contrast, BK stimulated a transient increase in NO, which returned to baseline by about 5 min. B, the dose-response curve for enalaprilat is shown. BPAE cells were stimulated with increasing concentrations of enalaprilat and the NO concentration generated at 20 min taken as a measure of the response. Results represent the mean values of five (10 and 100 nM) or two (μM concentration points) independent experiments. C, the B1 receptor blocker inhibits enalaprilat stimulation of NO production. Des-Arg10-kallidin and enalaprilat were added to cells pretreated for 2 min with or without the B1 receptor antagonist (DALKD) as indicated, and the NO concentration generated at 20 min was taken as a measure of the response. Shown are the mean values from four or more independent experiments. Reproduced with permission from ref.[14].

To explain the mode of activation of B_1 receptor by ACE inhibitors we performed another set of experiments, where we used various cell lines and transfected them to express the human B_1 receptor [14]. These experiments further indicated that ACE inhibitors directly activate the B_1 receptor, even in the absence of ACE, behaving essentially as receptor agonists. To clarify how they activate the B_1 receptors, we compared the amino acid sequence of ACE with the bradykinin B_1 and B_2 receptors [16, 17]. Although there is little overall homology, the human B_1 receptor contains in its second extracellular loop (residues 195-199) an HEAWH sequence (Fig. 2), which is absent in the B_2 receptor. This is similar to the sequence in the active centers of the two domains of ACE (HEMGH) [11] and matches the HEXXH zinc-binding consensus sequence [18] in other zinc metalloproteases. ACE inhibitors combine with the active centers of ACE via the Zn^{2+} cofactor with their SH or COO groups. If ACE inhibitors bind in a similar fashion to the B_1 receptor, then mutation in the HEXXH motif should eliminate inhibitor binding [19]. We constructed a point mutation (H195A) at the putative Zn-binding site (HEAWH→AEAWH). The [H195A]B_1 receptor mutant was transiently expressed in HEK 293 cells. In HEK/[H195A]B_1 cells, des-Arg^{10}-kallidin activated the receptor, whereas enalaprilat was inactive (Fig.3). Consequently, the HEAWH sequence in the second extracellular loop is essential for the direct activation of the B_1 receptors by enalaprilat but not for desArg^{10}-kallidin, which acts on other epitopes in the third extracellular loop [20, 21].

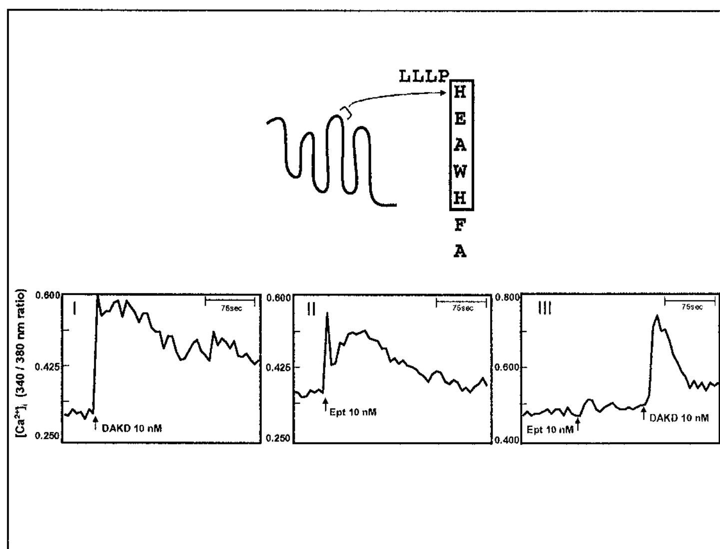

Figure 3. Site of the activation of the B1 receptor by enalaprilat. A, a schematic of the structure of the human B1 receptor is shown. The HEAWH motif in the second extracellular loop (residues 195-199), the proposed binding site for enalaprilat, is enlarged. This putative zinc-binding sequence (HEAWH) is well conserved in B1 receptors across species. B, tracings from single HEK cells expressing wild type B1 (panels I and II) or the [H195A]B1 mutant (panel III) are shown. The cells were stimulated with des-Arg10-kallidin (DAKD) and enalaprilat (Ept). Notice that the H195A mutation of the B1 receptor in the Zn-binding region abolished only the effect of enalaprilat, whereas des-Arg10-kallidin remained active. Results are representative of five independent experiments. Reproduced with permission from ref. [14].

To confirm our findings obtained with the [H195A]B_1 mutant, we used a synthetic undecapeptide (LLPHEAWHFAR) corresponding to residues 192-202 of the B_1 receptor, which contains the putative ACE inhibitor binding site [14]. This undecapeptide blocked the effect of ACE inhibitor specifically and completely, whereas des-Arg^{10}-kallidin's activity was not affected [14].

We next sought to investigate how the activation of B_1 receptors releases NO from endothelial cells. To this end we used two types of cells: BPAE cells, which constitutively express the B_1 receptor and HLMVE cells where we induced the receptor with inflammatory cytokines, IL-1β and IFN- γ. To determine the contributions of different NO synthase (NOS) isoforms to NO production, we used inhibitors relatively specific for endothelial NOS (eNOS) or inducible NOS (iNOS). 1400W, iNOS inhibitor, supressed the majority of NO responses elicited both by desArg^{10}-kallidin or enalaprilat, when tested in cytokine-

stimulated HLMVE cells (Fig. 4A). In these cells, both agonists release NO via iNOS, in a similar, calcium-independent manner [15] , by increasing the uptake of NOS substrate, arginine (Fig. 4B).

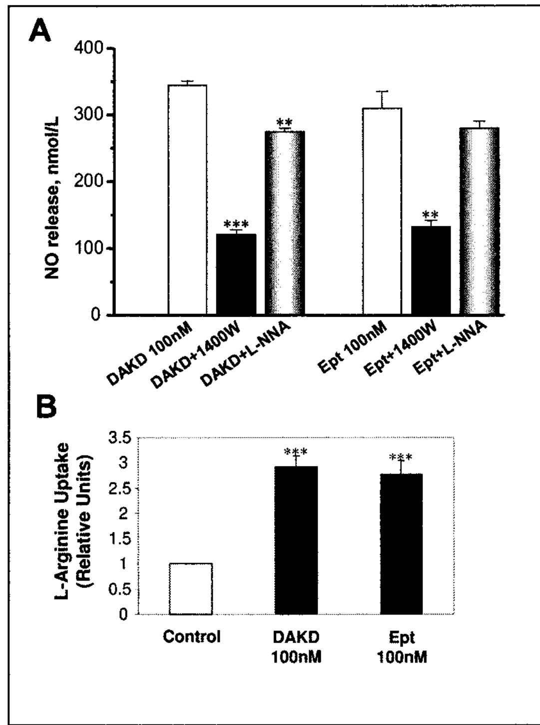

Figure 4. B1 agonist peptide and ACE inhibitor release NO mainly via iNOS and increase arginine uptake in cytokine-stimulated HLMVE cells.

A. ***NO Release.*** *Cells were preincubated (15 min, 37°) with selective eNOS (2 μM Nω-nitro-L-arginine (L-NNA)) or iNOS inhibitor (2 μM 1400W) and NO production in response to B1 agonists was measured with a porphyrinic electrode in real-time. Shown are mean values obtained with 100 nM desArg10-kallidin (DAKD) or 100 nM enalaprilat (Ept) in the absence and presence of eNOS and iNOS inhibitors (n=4).*
, significantly different from control (p<0.05); *, significantly different from control (p<0.001)*
Reproduced with permission (ref. 15, Fig. 6).

B. ***L-arginine uptake.*** *HLMVE cells were stimulated for 1 minute with 100 nM desArg10-kallidin (DAKD) or 100 nM enalaprilat (Ept). The abscissa shows the uptake of arginine relative to control. The results represent the means ±SE from 9 independent experiments (done in triplicate). ***, significantly different from the control (p<0.001).*
Reproduced with permission (ref.15, Fig. 7).

In contrast, in BPAE cells, desArg^{10}-kallidin and enalaprilat activate NO synthesis by different mechanisms. DesArg^{10}-kallidin releases NO via eNOS activation, dependent on intracellular calcium elevation (not shown;[15]). Enalaprilat activates mainly eNOS but to some degree iNOS to release NO (Fig.5). Consistent with these findings, the intracellular calcium chelator, BAPTA, had less effect on the response to enalaprilat compared to that obtained with desArg^{10}-kallidin (not shown;[15]).

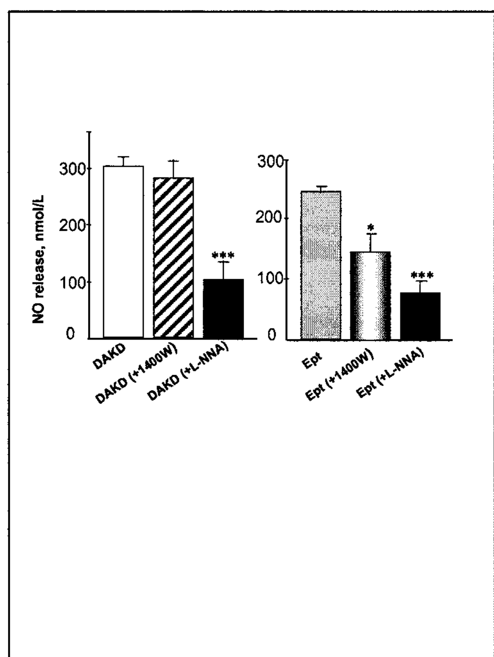

Figure 5. ***NO release from BPAE cells.*** *BPAE cells were preincubated with L-NNA or 1400W and NO production in response to B1 agonists was measured with a porphyrinic electrode. Mean values (±SE) obtained with 100 nM desArg10-kallidin (DAKD) or 100 nM enalaprilat (ept) in the absence and presence of eNOS and iNOS inhibitors (n=4-5).*
, significantly different from control ($p<0.05$); *, significantly different from control ($p<0.001$)*
Reproduced with permission from ref. [15].

In BPAE cells, B_1 receptor ligands desArg^{10}-kallidin and enalaprilat also raised intracellular calcium by different mechanisms [15] (Fig.6). Enalaprilat activates the B_1 receptor via a Gs-protein dependent pathway and it stimulates the influx of external calcium, independent of inositol 1,4,5-tris-phosphate (IP3) generation. In contrast, desArg^{10}-kallidin acts through Gq-protein to first release internal calcium by IP3 liberation and then subsequently stimulates calcium entry. In addition, protein kinase C inhibitors (calphostin C and chelerythrine) blocked the elevation of $[Ca^{2+}]_i$ triggered only by ACE inhibitors but not that stimulated by desArg^{10}-kallidin [15].

DISCUSSION

Numerous studies demonstrate the effectiveness of ACE inhibitors in cardiac and renal patients. For instance, trials involving more than 9000 patients showed that ACE inhibitor substantially lowered the rates of death, heart attack, stroke, heart failure and complications related to diabetes mellitus [22, 23].

We investigated the cellular and molecular mode of action of these drugs in order to gain better understanding of their actions. We found, using several types of cultured cells, that ACE inhibitors, in low, therapeutically relevant concentrations, directly activate the bradykinin B_1 receptor in the absence of endogenous kinins or ACE [14, 24]. This activity differs from the potentiation of bradykinin actions on its B_2 receptor by ACE inhibitors, previously decribed by our group [6, 7], which is indirect and requires the expression of both the B_2 receptor and ACE. Human B_1 and ACE, although lacking overall similarities, share a common structural element- a zinc binding motif. This sequence, represented by HEAWH residues in the B_1 receptor, is the likely site of attachment of ACE inhibitors. This hypothesis was confirmed in experiments where we found that point mutation of the zinc binding motif of B_1 receptor or the pretreatment of cells with the undecapeptide representing this Zn-binding site blocked the effect of ACE inhibitors but not that of the peptide ligand, desArg^{10}-kallidin [14].

Activation of B_1 receptors by either the peptide ligand or ACE inhibitor releases NO [12, 14, 15, 25-28]. B_1-mediated NO release is sustained and the time course differs from that caused by B_2 receptor activation, which produces a shorter, transient response [12, 14]. We investigated the mechanism of NO release triggered by the activation of either a constitutively expressed or induced B_1 receptor. To this end we used two types of endothelial cells, BPAE and cytokine-stimulated HLMVE, as suitable models for the native, constitutive [14, 29] or inducible expression of B_1 receptors [12]. We hypothesized that different NOS isoforms may be involved under native or cytokine-treated conditions, since interleukin-1β stimulates the expression of iNOS [30-32] and B_1 receptor ligands, desArg^{10}-kallidin and enalaprilat may stimulate distinct signaling pathways [14].

In BPAE cells we found that the mode of NO release by ACE inhibitor and peptide ligand differs [15]. The synthesis of NO by desArg^{10}-kallidin largely depends on the elevation of $[Ca^{2+}]_i$ and eNOS activation. In contrast, enalaprilat releases NO largely independent of $[Ca^{2+}]_i$ via eNOS and iNOS activation. Although both B_1 receptor ligands raise $[Ca^{2+}]_i$, they do so through stimulation of dissimilar

signaling pathways. ACE inhibitor enalaprilat enhances entry of extracellular calcium, while desArg^{10}-kallidin first releases internal calcium via IP3 liberation and then subsequently augments calcium influx. This is mediated by differential coupling to G proteins. DesArg^{10}-kallidin acts through Gq-protein to activate phospholipase Cβ, generate IP3 and raise cytosolic calcium, whereas enalaprilat stimulates calcium entry through a Gs- and PKC dependent pathway (Fig.6). PKC may phosphorylate and upregulate calcium channels by opposing Gβγ-induced inhibition [33].

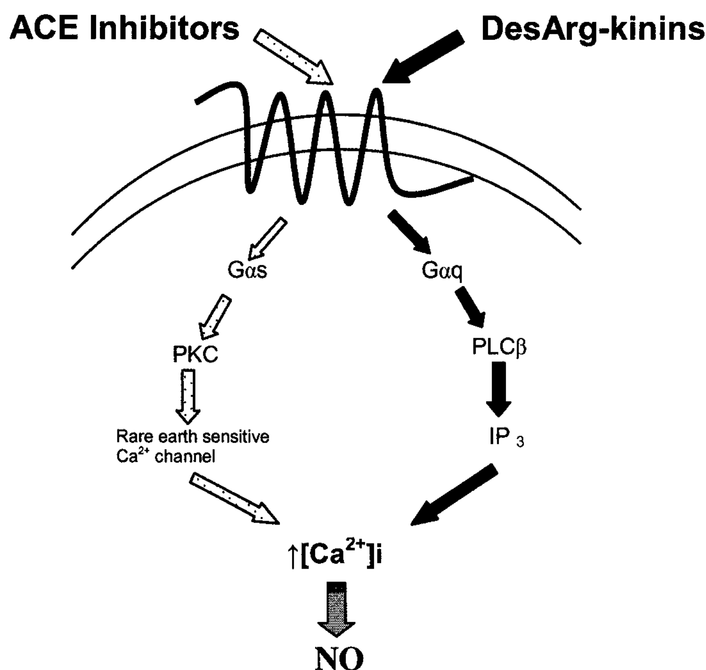

Figure 6. Scheme to illustrate how the peptide desArg10-kallidin and ACE inhibitor enalaprilat activate the B1 receptor to stimulate elevation of [Ca2+]i and NO release through different pathways in BPAE cells.

HLMVE cells, exposed to inflammatory cytokines IL-1β and IFN-γ, were used to mimic pathological conditions, which induce the B_1 receptor expression *in vivo* [9, 24]. In these cells, both desArg^{10}-kallidin and ACE inhibitor release NO mainly via iNOS stimulation, independent of $[Ca^{2+}]_i$ elevation. Both of the B_1 ligands employed enhanced the uptake of extracellular arginine, which is necessary for iNOS, but not

eNOS activity. These results indicate that although the activation of both constitutively expressed and cytokine-induced B_1 receptor causes a prolonged NO release, the mechanisms of these processes may be different. The signaling in cells constitutively expressing B_1 receptors or in cells stimulated with cytokines to induce B_1 receptors may be predominantly linked to eNOS and iNOS activity respectively.

Upregulation and activation of iNOS to produce high levels of NO can be deleterious, but several reports also document the benefits of its expression. For instance, iNOS protects against oxidative damage [34, 35], inhibits platelet adhesion [36] or contributes to prolonged protective effects of ischemic preconditioning [37]. NO can also blunt the inflammatory response by inhibition of NF-κB activation, increasing the expression, nuclear translocation and stabilization of its inhibitory protein IκB [38, 39].

Augmenting NO release by direct activation of B_1 receptors may play a significant role in the effectiveness of ACE inhibitors, since a deficiency of NO contributes to a variety of cardiovascular diseases [40-42]. B_1 receptors, induced during ischemia are cardioprotective [43]. Activation of B_1 receptors also contributes to the protective effects of ischemic preconditioning in coronary arteries [44]. Heart failure is accompanied by increased production of cytokines [45, 46], which, in turn, stimulate B_1 receptor expression. The B_1 receptors are induced after myocardial infarction and they may contribute to wound healing and tissue repair [47].

In summary, ACE inhibitors directly activate B_1 receptor, in the absence of ACE and endogenous kinins and this results in a prolonged NO release [14, 15]. B_1 receptors contribute to the effects of ACE inhibitors when tested in cultured cells [14, 15], laboratory animals [48-50] or heart failure patients [51]. Some of the beneficial effects of ACE inhibitor therapy can be due to direct activation of the peptide B_1 receptor, even in cells lacking ACE expression, especially in cardiac functions.

Acknowledgements

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Chapter 14
REMODELING IN HYPERTENSIVE HEART DISEASE: ROLE OF THE RENIN-ANGIOTENSIN-ALDOSTERONE SYSTEM

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INTRODUCTION

The classical criterion in defining hypertensive heart disease (HHD) is a greater than normal left ventricular mass (i.e., left ventricular hypertrophy) in the absence of a cause other than arterial hypertension. However, it is now accepted that besides this quantitative trait, changes in the composition of myocardial tissue (i.e., remodeling) develop in the hypertensive heart [1]. Whereas left ventricular hypertrophy provides the physiological response of the cardiomyocyte compartment to pressure overload in an attempt to normalize systolic wall stress, myocardial remodeling is mostly the consequence of a number of neurohormonal- and cytokine-mediated pathological processes occurring both in the cardiomyocyte and the noncardiomyocyte compartments of the whole heart. The clinical relevance of this distinction is that remodeling may be involved in both the development of heart failure and the increased cardiovascular risk of patients with hypertensive heart disease (HHD) [2]. Some of these processes are related to the exaggerated loss of cardiomyocytes due to apoptosis and the excessive accumulation of collagen type I and III fibers in the myocardium. This review focuses on the role of angiotensin II (ANG II)- and aldosterone (ALDO)-triggered pathways involved in such processes.

RELEVANCE OF MYOCARDIAL REMODELING IN HYPERTENSIVE HEART DISEASE

Clinical evidence

Besides a large body of experimental data there is now evidence from clinical studies showing that both fibrosis and apoptosis are present in the human hypertensive myocardium (Figure 1).

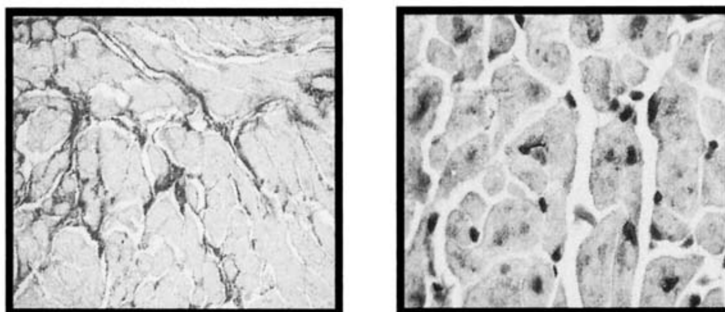

Figure 1. Endomyocardial tissue from one patient with hypertensive heart disease. Left panel, section was stained with Picrosirius Red, and the interstitial and perivascular collagen tissue was identified in red (x200). Right panel, section was immunostained with an antibody anticaspase-3 (active form), and the caspase-3 positive cells were identified in brown (x400).

Evidence of fibrosis

The available evidence indicates that myocardial fibrosis due to an exaggerate accumulation of fibrillar collagen types I and III within the myocardial interstitium and surrounding intramural coronary arteries and arterioles is one of the key pathologic features of myocardial remodeling in human HHD. In fact, a number of studies performed in postmortem human hearts [3-5] and endomyocardial human biopsies [6-9] have shown that myocardial collagen volume fraction, a morphometric measure of the amount of tissue collagen, is constantly increased in patients with HHD compared with normotensive controls. In addition, we have shown recently that collagen volume fraction further increases in patients with HHD who develop heart failure [10]. Interestingly, most of the collagen tissue accumulated in the failing hypertensive heart corresponds to collagen type I fibers.

Evidence of apoptosis

Cardiomyocyte apoptosis has been shown to be abnormally

stimulated in patients with HHD, no angiographic evidence of coronary artery disease and normal cardiac function [11,12]. In addition, recent findings from our laboratory indicate that cardiomyocyte apoptosis is increased in patients with HHD and heart failure compared with patients with HHD and normal cardiac function [13]. In fact, i ncreased cardiomyocyte apoptotic index and active caspase-3 expression was found in hypertensive failing hearts compared with hypertensive hypertrophied hearts and normotensive hearts in this study.

Clinical consequences

The available evidence reviewed above suggests that myocardial fibrosis and cardiomyocyte apoptosis precede the impairment in ventricular function and its exacerbation accompanies the development of heart failure in patients with HHD (Figures 2 and 3). Several mechanisms may link myocardial remodeling with heart failure in these patients.

Fibrosis and heart failure

Initially, fibrosis of the myocardial interstitium compromises the rate of relaxation, diastolic suction and passive stiffness, contributing to impaired diastolic function [14]. In accordance with this, we have shown recently that an association exists between myocardial collagen content and left ventricular chamber stiffness in patients with HHD [15]. A continued accumulation of fibrous tissue further impairs diastolic filling and now compromises transduction of cardiomyocyte contraction into myocardial force development, thus impairing systolic performance [16]. In support of this possibility we found that an association exists between the increase of myocardial accumulation of collagen type I fibers and the deterioration of ejection fraction in hypertensives. Furthemore, we found that the values of collagen volume fraction were higher in heart failure hypertensives with the most severe functional impairment (e.g., depressed ejection fraction) and with the most severe New York Heart Association class (e.g., class IV) [10]. In addition, myocardial fibrosis is one the structural substrates for two alterations playing a major role in the progression of heart failure, arrhythmogenicity and diminution of coronary reserve [17].

Apoptosis and heart failure

It has been hypothesized that cardiomyocyte apoptosis is one of the mechanisms involved in the loss of contractile mass that facilitates the transition to heart failure in HHD [18,19]. In support of this possibility are

recent findings from our group showing that increased cardiomyocyte apoptosis is associated with diminished cardiomyocyte density and reduced ejection fraction in patients with HHD and heart failure [13]. In addition, impaired contractile function may reflect not only a decrease in the number of viable, fully functional cardiomyocytes, but also a decline in the function of viable cardiomyocytes, or a combination of these mechanisms. It has been reported recently that caspase-3 cleaves cardiomyocyte myofibrillar proteins, resulting in depression of cardiomyocyte contractile function [20]. In addition, Laugwitz et al.[21] have demonstrated that blockade of caspase-3 activation improves contractility in the failing myocardium. This possibility is of interest, taking into account that overexpression of the active form of caspase-3 has been reported in the myocardium of patients with HHD [13].

ROLE OF ANG II AND ALDO IN HYPERTENSIVE MYOCARDIAL FIBROSIS

It has been proposed, that the excess of myocardial collagen seen in HHD is mainly due to the uncoupling between increased synthesis and unchanged or decreased degradation of collagen type I fibers [22]. Hemodynamic loading, ischemia, hormones, and growth factors may be involved in such an uncoupling [22].

Role of ANG II

Increasing evidence strongly indicates that ANG II exerts multiple profibrotic effects within the heart including induction of fibroblast hyperplasia, activation of collagen biosynthetic pathways and inhibition of collagen degradative pathways [23]. In addition, available data indicates that these effects may result from either the direct action of ANG II or a synergistic cooperation between this peptide and other profibrotic factors (e.g., transforming growth factor-β and endothelin-1) [23].

In this context, various lines of evidence support a role for ANG II as a critical candidate factor to induce myocardial fibrosis in HHD (Figure 2). Endogenous elevations in circulating ANG II that accompany unilateral renal artery stenosis [24] or the infusion of exogenous ANG II [25] are associated with increased blood pressure and fibrosis. Two observations suggest that the ability of ANG II to induce cardiac fibrosis in these models is independent of its hypertensive action. First, fibrosis in the renal artery stenosis model develops in both low-pressure right and left atria and right ventricle and high-pressure left ventricle [26]. Second, cardiac fibrosis in the ANG II infusion model can be prevented by either angiotensin converting enzyme (ACE) inhibitors or angiotensin type 1 (AT_1) receptor antagonists,

but not by hydralazine or prazosin, despite a similar antihypertensive efficacy of these compounds [27,28].

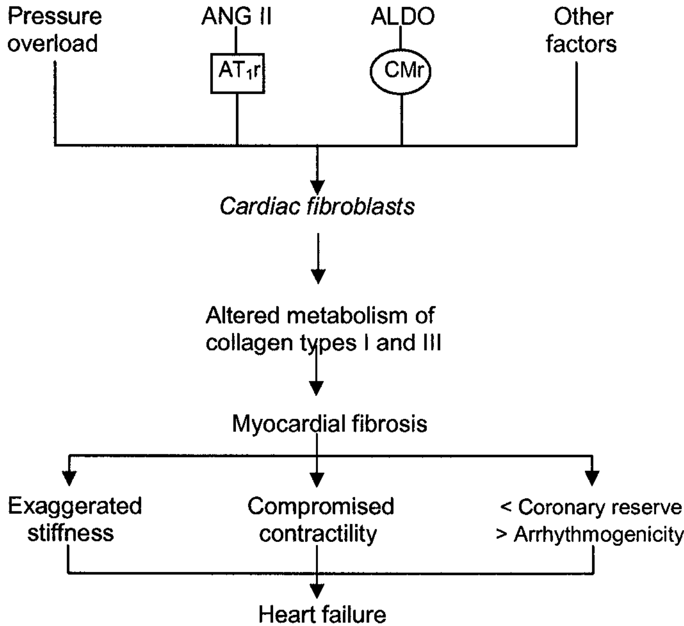

Figure 2. Involvement of angiotensin II (ANG II) and aldosterone (ALDO) in myocardial fibrosis and heart failure in hypertensive heart disease. (AT_1r, angiotensin II type 1 receptor; CMr, cytosolic minerolocorticoid receptor).

The hypertensive Ren2 rat provides a well-established model of ANG II-dependent cardiac hypertrophy [29]. Several studies have revealed that interstitial and perivascular fibrosis, along with extensive collagen types I and III deposition are present in Ren2 [30-32]. Increased cardiac renin and ANG II levels have been described in this transgenic rat model [29]. In addition, cardiac lesions are very sensitive to ACE inhibition and AT_1 receptor antagonism in Ren2 rats [33]. As a result, the development of hypertrophy and fibrosis in the heart of these animals has been attributed, at least partially, to a local activation of the cardiac renin-angiotensin system.

In rats with spontaneous hypertension (SHR) and left ventricular hypertrophy, myocardial fibrosis has been shown to regress by treatment with the ACE inhibitor lisinopril [34]. This effect occurred independently of the drug's antihypertensive effect. On the other hand, it has been found that chronic AT_1 receptor antagonism with losartan resulted in reversal of

fibrosis, inhibition of the post-transcriptional synthesis of procollagen type I, inhibition of tissue inhibitor of metalloproteinase-1 expression and stimulation of collagenase activity in the left ventricle of adult SHR [35,36]. Analysis of the individual data showed that the intensity of these myocardial changes was independent of the antihypertensive efficacy of the drug [35,36].

The fibrogenic role of ANG II in humans has been investigated in 3 recent prospective trials of limited size using biopsy-proven myocardial fibrosis in patients with HHD. Schwartzkopff et al. [7] studied 14 patients before and after 1 year of treatment with the ACE inhibitor perindopril. Structural analysis revealed diminution of perivascular and interstitial fibrosis with treatment. The regression of fibrosis on ACE inhibitor treatment was observed in the non-pressure-overloaded right ventricle, indicating that the antifibrotic effect was not accounted for by left ventricular pressure reduction alone. Brilla et al. [8] randomized 35 previously treated patients with controlled blood pressure to receive either the ACE inhibitor lisinopril or the diuretic hydrochlorothiazide for 6 months. Only patients randomized to lisinopril had a significant reduction in myocardial fibrosis. Blood pressure reduction was similar in patients treated with either lisinopril or hydrochlorothiazide. Finally, López et al. [37] studied 37 treated patients with uncontrolled blood pressure. After randomization, 21 patients were assigned to the AT_1 receptor antagonist losartan and 16 to the calcium channel blocker amlodipine for 12 months. Whereas myocardial fibrosis decreased significantly in losartan-treated patients, this parameter remained unchanged in amlodipine-treated patients. A similar reduction of blood pressure in losartan-treated patients than in amlodipine-treated patients was reported in this study. Collectively, these observations support the concept that in addition to pressure overload, ANG II induces myocardial fibrosis in essential hypertension.

Role of ALDO

Experimental studies have demonstrated the central role of ALDO in promoting cardiac fibrosis, probably through a direct action on the heart mediated by cardiac mineralocorticoid receptors [38-40] (Figure 2). In fact, ALDO has been shown to stimulate collagen synthesis through the mineralocorticoid receptor in isolated cardiac fibroblasts [41]. On the other hand, in experimental studies on rats with renovascular hypertension, hyperaldosteronism, or spontaneous hypertension, the ALDO antagonist spironolactone was able to prevent or reverse the development of myocardial fibrosis even though the drug did not normalize blood pressure and did not prevent left ventricular hypertrophy [26,42-45]. Thus, an increase in this mineralocorticoid may be a mechanism for ANG II-induced cardiac fibrosis in some forms of arterial hypertension.

Interestingly, an increase in the density of AT_1 receptors has been observed in the heart of ALDO-salt treated rats [46]. In addition, the AT_1 receptor antagonist losartan prevents fibrosis and upregulation of collagen types I and III mRNAs in the heart of ALDO-salt treated rats [47]. Taken together, these findings support the hypothesis that one mechanism by which ALDO induces cardiac fibrosis involves ANG II acting through AT_1 receptors. Since the production of ALDO is activated in the hypertrophied left ventricle of SHR [48] and hypertensive patients [49], it is thus possible that this horm one contributes to ANG II-mediated myocardial fibrosis in primary hypertension.

The potential clinical relevance of these interactions is given by several observations. In essential hypertension, a low dose of the ALDO antagonist canrenone added to antihypertensive treatment has been shown to significantly improve left ventricular diastolic function [50]. This improvement, not accounted for by changes in blood pressure and left ventricular mass, can be therefore ascribed to a direct action of the drug on the myocardium. This is further supported by recent studies showing that chronic administration of either spironolactone [51-55] or potassium canrenoate [56] is associated with a reduction in the circulating levels of markers of collagen turnover in patients with different cardiac diseases that evolve with myocardial fibrosis.

ROLE OF ANG II AND ALDO IN HYPERTENSIVE CARDIOMYOCYTE APOPTOSIS

Cardiomyocyte apoptosis has been proposed to occur as a result of an imbalance between the factors that induce or block apoptosis [57]. Thus, arterial hypertension may represent a condition in which inducers of cardiomyocyte apoptosis predominate over suppressors of cardiomyocyte apoptosis [58]. It is now recognized that besides the mechanic factor secondary to hemodynamic overload, local humoral factors may induce cardiomyocyte apoptosis in HHD.

Role of ANG II

Several arguments suggest that ANG II may be one of the humoral factors potentially involved in cardiomyocyte apoptosis in HHD (Figure 3). First, cardiomyocyte apoptosis increases in ANG II-infused hypertensive Sprague-Dawley rats and blockade of the AT_1 receptor with losartan prevents this effect despite the persistence of increased blood pressure [59]. Second, an association has been found between enhanced cardiomyocyte apoptosis

and exaggerated ACE activity in the hypertrophied left ventricle of SHR [60]. Finally, chronic treatment with losartan at doses that do not normalize blood pressure is associated with reduction of cardiomyocyte apoptosis in both SHR [61] and patients with HHD [11].

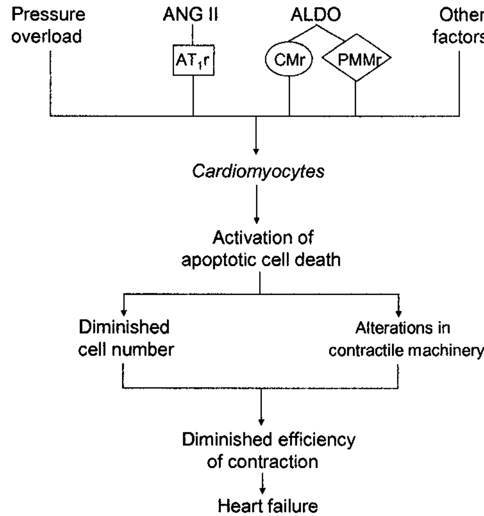

Figure 3. Involvement of angiotensin II (ANG II) and aldosterone (ALDO) in cardiomyocyte apoptosis and heart failure in hypertensive heart disease. (AT_1r, angiotensin II type 1 receptor; CMr, cytosolic minerolocorticoid receptor; PMMr, plasma membrane mineralocorticoid receptor).

In vitro studies have shown that ANG II binding to AT_1 receptors triggers apoptosis by a mechanism involving stimulation of p38 MAP kinase activity, activation of p53 protein and subsequent decrease of the Bcl-2-to-Bax protein ratio, activation of caspase-3, stimulation of calcium-dependent DNase I, and internucleosomal DNA fragmentation [62-66]. Although ANG II has been shown to induce apoptosis in other cardiovascular cells through stimulation of the AT_2 receptor [67], recent findings suggest that it is unlikely that this receptor is a strong signal to induce cardiomyocyte apoptosis in vivo. In fact, apoptosis is not increased in the heart of transgenic mice overexpressing AT_2 receptors in the myocardium [68]. In addition, Diep et al. have reported that blockade of AT_1 receptors with losartan is accompanied by normalization of cardiac apoptosis in rats with ANG II-induced hypertension that exhibit increased expression of AT_2 receptors in the heart [59].

Role of ALDO

Besides its profibrotic actions, ALDO may also exert proapoptotic actions in the heart (Figure 3). Recently, De Angelis et al. [69] have reported that ALDO induces ventricular cardiomyocyte apoptosis in vivo and in cultured cells. The effect of aldosterone seems to be mediated by mineralocorticoid receptors, since it is abolished by spironolactone in primary culture of these cells. More recently, Mano et al. [70] have reported that ALDO directly induces cardiomyocyte apoptosis by activating its phospholipase C-coupled membrane receptor-mediated mitochondrial apoptosis signalling associated with the calcineurin-Bad pathway.

Interestingly, cardiomyocyte apoptosis induced by infusion of ANG II is reduced by 50% in hearts of rats pre-treated with spironolactone, suggesting that the pro-apoptotic effect of the peptide could be due, at least in part, to ALDO [69].

CONCLUSIONS

ANG II has endocrine, autocrine and paracrine properties that influence the behaviour of cardiac cells and matrix via AT_1 receptor binding. Thus, various paradigms have been suggested, including ANG II-triggered apoptosis of cardiomyocytes and ANG II-mediated upregulation of collagen types I and III formation and deposition in HHD. On the other hand, a growing body of evidence deals with the potential role of ALDO, either local or systemic, in inducing cardiac fibrosis and apoptosis. Thus, aldosterone might also mediate the profibrotic and proapoptotic actions of ANG II. To reduce the risk of heart failure that accompanies HHD, its adverse structural remodeling must be targeted for pharmacological intervention. Available experimental and clinical data suggest that agents interfering with either ACE, the AT_1 receptor, or the mineralocorticoid receptor may provide such a cardioprotective effect.

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Chapter 15
CARDIAC ALDOSTERONE PRODUCTION: A VASCULAR PROBLEM?

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ABSTRACT

Elevated circulating aldosterone level is associated with impaired cardiovascular function. As shown by RALES and EPHESUS clinical studies, aldosterone antagonists decrease total and cardiovascular mortality in heart failure and post-MI with left ventricular dysfunction. Aldosterone induces cardiac fibrosis in both experimental models and clinical conditions. Nevertheless, the pathophysiology of aldosterone-induced cardiac alterations remains in large part unclear. To resolve this issue we have explored the possibility that the heart may produce aldosterone. We found that there is in fact a myocardial production of the hormone which is regulated by the same physiological stimuli than in the adrenals. This local aldosterone production is increased in post-MI and is in part responsible for cardiac fibrosis. Then, transgenic mice that overexpress the terminal enzyme of aldosterone biosynthesis, aldosterone-synthase, AS, in the heart have been raised by gene targeting with the alpha-myosin heavy chain promoter. Aldosterone concentration was enhanced 1.7-fold in transgenic hearts with no evidences of either structural or functional myocardial alterations. In contrast, male transgenic mice displayed a major endothelium-independent alteration of the coronary vasodilatation. To conclude, a moderately increased local concentration in aldosterone results in a pronounced coronary dysfunction without other cardiac alterations, which is a newly identified effect of aldosterone. This aldosterone-induced decrease of coronary reserve may be a new risk factor for cardiovascular diseases.

Heart failure, the new landscape

During millions of years the main cause of heart failure was endocarditis and rheumatic disease (the Bouillaud disease), and the average lifespan was around 45 years. Rather recently, in our countries infections and denutrition were controlled, the lifespan now reaches approximately 75 years and heart failure has different origins. These include essential arterial hypertension, the clinical consequences of atherosclerosis, diabetes, obesity which all were favoured by senescence. Such an evolution of epidemiology was restricted to certain areas of the world and, as shown in a big worldwide epidemiologic study, depends upon the socioeconomic level that has been reached by each country (1).

In 2004, in our countries, heart failure results from two groups of factors: (i) mechanical overload, which induces an adaptational process through mechanosensation and mechanotransduction, and is an ancient evolutionary process already utilised in the past to adapt the heart to valvular diseases; (ii) recently, at least three different additional factors were involved which all participate in the remodelling process, including the multiple forms of cell deaths and fibrosis which both are multifactorial, and the trophic consequences of the neurohormone reaction (2) (Figure 1). Fibrosis itself became a major target for pharmaceutical research, and several trials have evidenced a reversibility of cardiac remodeling and myocardial fibrosis (3), including trials using anti aldosterone (4, 5, 6).

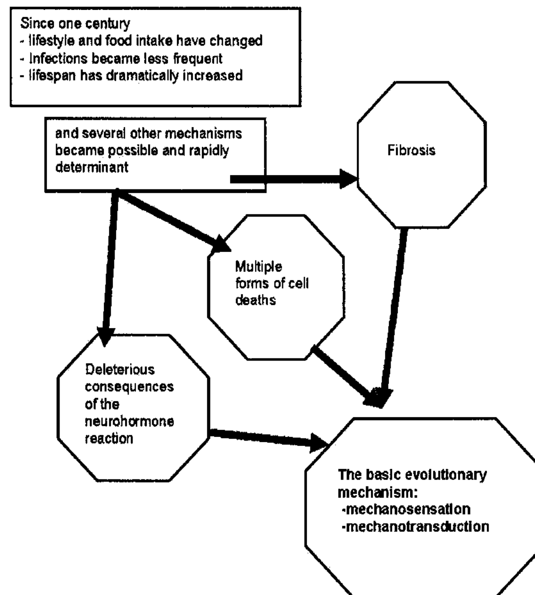

Figure 1. The new landscape of heart failure

Aldosterone-induced myocardial fibrosis

Both clinical and experimental studies have convincingly evidenced a deleterious effect of aldosterone. C. Brilla and K. Weber were the first to show, using an experimental aldo-salt model of hypertension that aldosterone was able to induce myocardial fibrosis in both the overloaded (left) and the non overloaded (right) ventricles (7). This was confirmed by our group, and other laboratories (8, 9). Nevertheless, such an experimental setting showed that aldosterone is deleterious for the heart, but only at very high hormonal concentrations. On the other hand, clinical trials provide arguments in favor of a cardiotoxicity of the hormone, but these arguments were quite indirect. Two trials have indeed shown a spectacular beneficial effect of two aldosterone antagonists, spironolactone and eplerenone, at subhypotensive doses in heart failure (4, 5). However, the mechanisms of aldosterone-induced myocardial fibrosis in clinical conditions still remain unclear mainly because in clinical conditions, the augmentation of the plasma levels of aldosterone was too low to account for the fibrogenic process.

In fact, as initially shown in our laboratory the myocardium itself is an endocrine which is capable to synthesize the hormone. The tissular aldosterone concentration which have been measured in normal rat heart is approximately 15 times higher than the plasma concentration of the hormone (1 nM) and rose up to 50 times after a myocardial infarction (10, 11). Here also, experimental findings were confirmed in clinical settings. In humans, the activation of several steroidogenic genes including the aldosterone-synthase (AS) has been detected in the failing heart (12-15), and the myocardial aldosterone production, as measured by the arteriovenous differences in the hormone, was significantly enhanced in the failing heart (15).

A pericoronary inflammatory reaction with macrophages, inflammatory markers, and necrosis is a very early step of cardiac damage in the aldosterone-salt model (9, 16, 17). It has been suggested that, at least in this model, the coronary arteries may be a target for aldosterone in the heart, and that vascular damages may be one of the primary events in aldosterone-induced fibrogenesis. Nevertheless, it remains to know whether the local production of aldosterone (which may strongly enhance the myocardial aldosterone concentration) is capable to produce an inflammatory perivascular reaction and then fibrosis.

Transgenic strain of mice with cardiac targeted overexpression of aldosynthase

To resolve this issue, we have raise a transgenic strain of mouse in which aldosterone was specifically increased in the heart (18, 19). The expression of a transgene containing the 1500 bp coding sequence of

aldosynthase has been driven by the mouse alpha-MHC gene promoter. Two independent founder lines were established in FVB mice with a 100-fold increase in aldosynthase mRNA. The aldosterone concentration is increased by 1.7 fold in the right and left ventricles, and in the atria, whereas it is unchanged in the plasma. Post-natal development and basic phenotype parameters are similar in the two lines. The study was performed only in male animals.

Cardiac structure and function in male transgenic mice

Male transgenic mice have normal blood pressure, heart rate, ejection fraction, velocity of shortening, relaxation time, and E/Ea ratio at echocardiography (Table 1). In addition, the cardiac capillaries density is unchangeed in transgenic mice compared to wild-type (WT) ones. Histologic examination of ventricles shows no alteration of ventricular tissue and of coronary vessels, including the perivascular area, in transgenic mice. The levels of ventricular AT1 and AT2 receptors and the collagen volume fraction are also unchanged in 3 month-old male transgenic mice. Using real–time PCR, we find no difference in the levels of expression of mRNAs coding the endothelin converting enzyme, Pre-Pro-endothelin and the endothelin-1 type a receptor in transgenic mice ventricles as compared to WT.

Echocardiographic evaluation has been performed in separate groups of 15 and 27 week-old mice. The left ventricle of transgenic mice is neither dilated nor hypertrophied, and systolic and diastolic echocardiographic parameters are similar in transgenic and WT mice (Table 1). At 36 weeks, LV function is also normal (not shown). The aldosynthase overexpression does not alter the L-type calcium current $I_{Ca,L}$ of isolated cardiac myocytes, both under basal conditions and under maximal beta-stimulation (isoproterenol 10^{-7} M), or the time-dependent and -independent components of the transient outward potassium channel current, I_{to1} and Isus. T-type calcium current is absent in both groups.

Using the isolated perfused heart, the left ventricular function in unstimulated WT and transgenic hearts is the same and the response to calcium of the transgenic hearts is not modified.

Table 1. Phenotype of male Wild Type (WT) and Transgenic (TG) mice (from 19)

	WT		**TG**	
	15 wk	**27 wk**	**15 wk**	**27 wk**
Anatomical data				
Body weight, g	25.5±0.5		26.2±0.5	
Ventricles weight, mg	101.1±3.1		109.4±3.6	
VW/BW, mg/g	3.97±0.11		4.17±0.09	
Adrenal glands weight, mg	2.47±0.11		2.36±0.06	
Physiological data				
Systolic pressure, mmHg	151±13		152±15	
Diastolic pressure, mmHg	110±10		109±12	
Heart rate, bpm	545±17	530±15	518±15	516±13
Quantitative morphometry				
Capillaries density, n/mm2	39±0.70		40±1.02	
Myocytes density, n/mm2	22±0.83		20±0.90	
Capillaries/myocytes ratio	1. 80±0.07		1.99±0.07	
Total collagen volume fraction, %	0.40±0.08		0.42±0.09	
Echocardiography				
Ejection Fraction, %	76±3	86±3	78±3	82±3
VcFc, circ/s	2.42±0.15	2.98±0.2	2.53±0.18	2.73±0.14
IVRT, ms	12.4±0.5	15.0±0.91	13.6±0.67	18.0±1.6
E/Ea	23.9±1.53	19.8±1.31	26±1.78	24.1±1.16
Hormones, nM/L				
Aldosterone				
Plasma	0.40±0.09		0.60±0.22	
Ventricles	***9.8±1.6***		***16.8±2.1****	
Adrenals	128±38		158±11	
Corticosterone				
Plasma	422±90		551±320	
Ventricles	111±22		99±12	
Adrenals	12,698 ±3,799		12,917 ±2,380	

* $p<0.02$
VcFc: Velocity of circumferential Fibers shortening corrected for heart rate - IVRT: IsoVolumic Relaxation Time - E: maximal velocity of E wave of transmitral blood flow - Ea: maximal velocity of E wave of the mitral annulus tissue Doppler.

Coronary function

In fact, the major defect that is observed in transgenic animals concerns the coronary function. Changes in coronary perfusion pressure have been studied on an isolated perfused heart set-up. (i) The coronary flow was reduced by 55 % in transgenic hearts. (ii) Infusion of acetylcholine in the perfusion line induced a decrease in coronary resistance, which is expressed in

fig 2 as a percent change in coronary perfusion pressure (CPP) relative to baseline values in WT mice. The vasodilatory response was observed at 10^{-6} M acetylcholine and was dose-dependent in controls. In contrast, the vasodilatory response of acetylcholine were almost abolished in transgenic mice (Fig. 2). In addition, the coronary responses to bradykinin were also decreased by 60% in transgenic animals. (iii) Nevertheless, the maximal response to sodium nitroprusside was also decreased in transgenic hearts, which finally indicates that the defect was endothelium-independent.

Altered CoronaryFunction in male AS+ mice

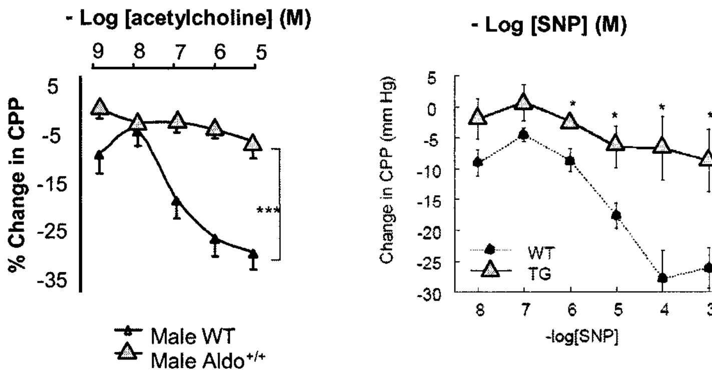

Figure 2. Coronary function in transgenic mice overexpressing aldosynthase in the heart. Isolated coronary perfused hearts at constant flow. CPP: coronary perfusion pressure. SNP: sodium nitroprusside [from 19]

The vessel, a major target for aldosterone

The major effect of the transgenic myocardial overexpression of aldosynthase in males is that a slight (as in Table 1) increase in the aldosterone concentration which had been targeted in the myocardium is able to induce a major coronary vascular dysfunction without altering cardiac structure and function.

This finding may explain how patients who suffer from heart failure, and as such activate their own myocardial production of aldosterone, can undergo vascular damages even with a weak elevation of their plasma level of the hormone. Observations from our transgenic mice suggest that the weak increase of aldosterone tissular concentrations is unsufficient to induce inflammation, necrosis and finally fibrosis. The situation, however, may be

quite different in pathological situations where not only aldosterone but also other hormones, such as angiotensin II, are increased.

In transgenic hearts, the aldosynthase mRNA is increased by a factor of 100, the corresponding protein is enhanced by a factor of 4, and the final product aldosterone is augmented by only 1.7-fold. Such a modest increase is observed in the whole homogenate which is likely to underestimate the in vivo situation. Several studies have demonstrated an increased production of cardiac aldosterone after myocardial infarction in the rat (11), but also in the failing human heart (14,15, review in 20). Our results show that a slight elevation of aldosterone synthesis is able to seriously impair coronary reserve and suggest that a long term impregnation with slightly elevated aldosterone may be determinant for the coronary function. Thus, the treshold for a deleterious concentration of aldosterone in cardiac tissue appears to be lower than what was previously thought.

The marked alteration of the coronary vascular responsiveness includes a decrease in the basal coronary flow together with an endothelium independent (and possibly partly endothelium-dependent) dysfunction with a normal contraction, relaxation and normal ionic currents in cardiomyocytes. The calcium current which is a target for aldosterone is unchanged (21). Several papers support the view that the vessels are a target for aldosterone, including the work of Farquharson (22) who evidenced an improved endothelium-dependent arterial function under aldosterone-inhibitor in heart failure. It has been suggested by others that the deleterious effects of aldosterone may be nitric oxide-mediated through the superoxide anion formation (23). This finding extends previous observations made in our group and obtained from the rat aldosterone-salt model, in which fibrinoid necrosis and the presence of inflammatory cells surrounding coronary arteries have been evidenced (24), and confirms the findings from other groups who have shown a pericoronary inflammatory reaction that appears early in the aldosterone-salt-treated rats (9, 16, 17).

To conclude (Fig 3), the present study provides the first evidence that locally produced aldosterone alters coronary vascular function even at low dose. Thus, this is a newly identified risk factor that may play a role in chronic cardiovascular diseases.

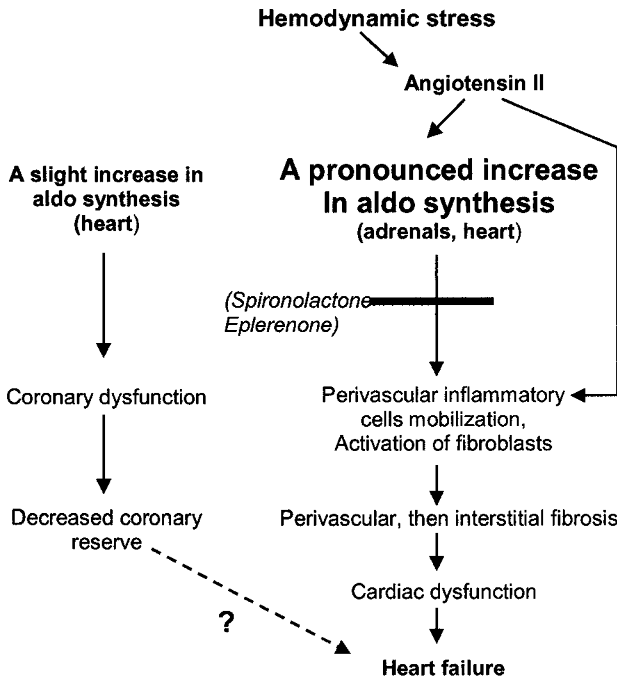

Fig 3. Myocardial consequences of aldosterone production - A working hypothesis. (from refs 9, 11, 16, 17, 19).

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Chapter 16
CELLULAR OXIDATIVE STRESS, AGING, AND THE LOCAL RAS

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AGING, MITOCHONDRIA, AND OXIDATIVE STRESS

Aging is the accumulation of the more or less random diverse deleterious changes that occur over time. Some changes are inheritable, while the majority increases the chance of disease and death with advancing age. Together, these aging changes ensure evolution. Some of them, which are shared by all species (1), are progressive, structural modifications that are generally associated with the gradual loss of organ function (2, 3).

In mammals, degenerative processes such as atherosclerosis, senile plaques in the brain, and the replacement of functional parenchyma by fibroconnective tissue in a variety of organs are considered manifestations of aging (4). Ultrastructurally, lipofuscin pigment and a reduction in the number of cellular organelles such as mitochondria are common in aged animals (5-8).

Aging and the degenerative changes that simultaneously cause it and characterize it, is a complex process and certainly multifactor. Postulated mechanisms include telomere shortening (9), oxidative damage to critical macromolecules by reactive oxygen species (ROS) (1, 10), nonenzymatic glycation of long-lived proteins, altered gene expression patterns (11), and damage to DNA leading to genomic instability (nuclear and mitochondrial DNA) (12-13).

THEORIES OF AGING: FREE RADICAL THEORY

Many theories (14, 15) have been advanced to account for the aging process. No theory is completely accepted, but the Free Radical Theory of Aging (15-17) has been widely examined and has gained substantial support, mostly because of molecular and cellular evidence (18). It, and the simultaneous discovery of the important, ubiquitous involvement of free radicals in endogenous metabolic reactions, arose in 1954 (16, 17). The theory is based on the premise that a single common modality, modifiable by genetic and environmental factors, is responsible for the aging and death of all living things.

According to this theory, the common aging process is primarily a consequence of free radical damage to critical biological molecules including DNA, fats, and proteins. These reactions, however initiated, are responsible for the progressive deterioration of biological systems over time. Extended in 1972 (19) and again in 1983 (20), the theory now also suggests that:

1. Most free radicals are generated by the mitochondria at an increasing rate with age, and
2. The lifespan is determined primarily by the rate of free radical damage to the mitochondria; primarily mediated by the superoxide radical. Subsequently, cellular death occurs when the accumulation of free radical insults, primarily in the mitochondria, reach a critical level where the cell can no longer survive. Collectively, the free radical reactions initiated by the mitochondria constitute the inherent aging process, but damage to membranes in general and more specifically, to genomic DNA may play an important secondary role.

MITOCHONDRIA IS A PERMANENT SOURCE OF ROS

In the past decade, several investigators have established that the respiratory function of mitochondria declines with age (21-23). In addition, it has been shown that the impairment of the electron transport chain —elicited by respiratory inhibitors, mtDNA mutation, or gene knockout— results in the enhanced production of mitochondrial ROS due to the incomplete reduction of oxygen (24-26). Thus, it is reasonable to conjecture that the aging tissues will exhibit defective respiratory function and mtDNA mutations, which will further expose them to the higher oxidative stress elicited by the enhanced production of

mitochondrial ROS.

AGING, MITOCHONDRIA, AND RENIN-ANGIOTENSIN SYSTEM

Aging is frequently associated with a reduction in the number of mitochondria in certain organs (27, 28). Such a reduction may be a consequence of the oxidation of mitochondrial components; in this respect, oxidative changes within the mitochondrial genome may lead to a progressive loss in the capacity to regenerate these organelles (29).

In mice, chronic long-term treatment with enalapril was shown to attenuate age-related structural and functional changes in several organs and to prevent a decrease in the number of mitochondria in the heart and liver (30). These results suggest that ACE inhibitors (ACEi) might have reduced age-related oxidative damage to mitochondria.

In the following figure (Figure 1), we show 3 photographs taken of animals in our laboratory. The first one shows two CF1 male mice, one from the control group and one from the enalapril-treated group. Both animals are the same age. The differences in their aspects are evident and correspond with the structural and functional changes seen in their organs (30). The other two photographs show similar results found in 24 month-old Wistar rats (31).

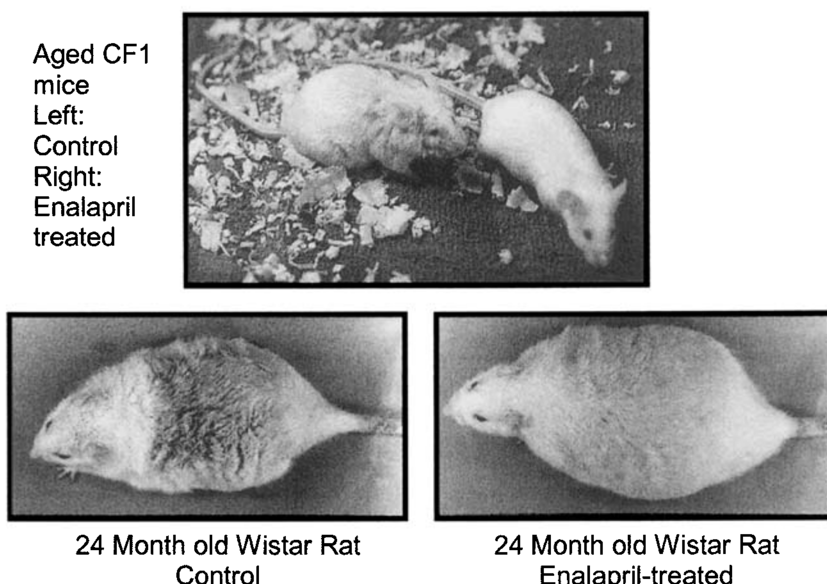

Figure 1: Aged CF1 mice and Wistar rats.

Tissues obtained from aged animals show not only a reduced number of mitochondria, but they also display changes in mitochondrial structure such as swelling, shortening of the cristae, and matrix vacuolization (27, 28, 32). These changes were associated with an increased generation of superoxide anion and hydrogen peroxide, as well as with a decline in energy production (33-35).

OXIDATIVE STRESS ON TISSUES

Enhanced Oxidative Stress and Damage in Aging Tissues

As mice and Wistar rats age, we have observed in our studies a decrease in the number of mitochondria in renal, myocardial, and hepatic tissues. An increase in oxidative stress tissue, as well as a decrease in glutathione, SOD, and other antioxidant defenses was also observed. Likewise, the tissues showed an increase in collagen and TGF-β1, a decrease in the numbers of capillaries, and other signs of aging (30, 36, 37).

Angiotensin II and oxidant stress

Ang-II stimulates superoxide production via AT_1receptors by activating NAD(P)H oxidase localized in endothelial cells, vascular smooth muscle cells, and fibroblasts of the vascular wall (38). In endothelial cells, angiotensin II also activates eNOS to release nitric oxide (NO) (39). Superoxide can react with NO, generating peroxynitrite, which is a strong oxidant. Consequently, Ang-II, by stimulating superoxide production, reduces local NO tissue content and generates species capable of oxidizing nucleic acids, lipids, and proteins (40). NAD(P)H is probably not the only source of oxidants stimulated by angiotensin II. Under certain physiopathological conditions, eNOS can produce superoxide (41) that results from the oxidative destruction of tetrahydrobiopterine, a critical cofactor for eNOS function (42). It has been suggested that any condition that increases superoxide production in the endothelium may lead to the production of important amounts of superoxide from uncoupled eNOS (43). Several studies indicate that clinical conditions that display increased Ang-II levels also exhibit increased superoxide production (44, 45) in concurrence with a reduction of NO bioactivity (46) and an elevation of oxidation products (47, 48). The physiological consequences of reactive oxygen and nitrogen species

(RONS) formation by angiotensin II include the induction of vascular inflammatory responses (49), the growth of vascular smooth muscle cells (50), the impairment of endothelium-dependent relaxation (51), and cardiac hypertrophy (52). In this setting, both ACEi and Angiotensin Receptor Blockers (ARB), by attenuating Ang-II formation or preventing Ang-II from binding to its respective receptor, can decrease superoxide generation and increase NO bioavailability, thus reducing oxidant stress. Based on these concepts, it was postulated that ACEi might act as "magic bullets" against oxidant stress (53). This may explain some of the beneficial effects of ACEi that cannot be ascribed to their action on blood pressure. ARB, being a newer class of drug, have not been so widely tested as ACEi, but evidence gathered up to the present time suggests that they too would, in certain ways, limit superoxide production and so modulate RONS generation (54, 55).

The value of renin-angiotensin system in aging

In the field of life extension, the current "gold standard" for screening interventions to retard the aging process presents some major obstacles: the assay requires approximately 4 years to complete and provides imprecise data on the rate of aging in individual organ systems (e.g., did the candidate mimetic retard aging in the heart?).

Our previous work pinpoints angiotensin converting enzyme inhibitors (ACEi) as highly promising candidates to increase lifespan.

Renin-Angiotensin System in Aging

The renin-angiotensin system (RAS) plays an important role in cardiovascular homeostasis and in the embryologic development of the kidney and other organs in mammals, as well as in different vertebrates (57-59).

Molecular probes have shown that the majority of the body's organs contain mRNA, which codifies angiotensinogen (60).

Recent studies showed that inhibition of the RAS, with either ACEi or ARB, could attenuate the effects of aging in various tissues (62-64). Based on these results, and in the context of mitochondria being involved in the aging process, we hypothesized that long-term treatment with ACEi (enalapril) or ARB (losartan) might attenuate the structural and functional changes that occur in mitochondria upon aging. When compared to kidney mitochondria isolated from untreated 22-month old

rats, mitochondria isolated from 22-month old rats that had been treated for 8 months with enalapril or losartan showed an improved capacity for energy production, a lower generation of hydrogen peroxide, a higher activity of mitochondrial nitric oxide synthase, and a higher content of uncoupling protein-2 (35). Electron microscopy of proximal tubular epithelial cells from enalapril- or losartan-treated rats showed a higher number of mitochondria, a better definition of mitochondrial cristae, and lower numbers of osmiophilic bodies (probably derived from lipid oxidation), as compared to cells from untreated rats. Furthermore, both treatments prevented the alteration of mitochondrial distribution inside proximal tubular cells that was observed in untreated rats. In addition, both treatments attenuated glutathione oxidation in renal tissue. These results suggest that enalapril and losartan may protect against the effects of aging by attenuating oxidative damage to mitochondrial components (35).

Many of the accepted mechanisms for development of renal lesions are associated with the hyperfiltration phenomenon among them is glomerulosclerosis due to aging.

The progressive development of glomerulosclerosis (GS) is a well-known phenomenon of the aging kidney that affects a variety of mammalian species. It is also known that in both rats and CF1 mice, the development of glomerulosclerosis is a biological phenomenon of aging (65) that also occurs in human (66). The prevalence of sclerosed over normal glomeruli found in human kidneys after the age of 70 is 10-20% higher (66).

When compared to the ACEi treated animals, our control animals showed a significant decrease in the number of cortical glomeruli. This suggests that ACEi prevented the loss of glomeruli associated with the normal aging process in CF1 mice.

In those models in which the systemic blood pressure is not increased, ACEi are still protective. This suggests that the effect doesn't depend upon their antihypertensive action. In addition, doses that do not modify systemic arterial pressure still retard the appearance of sclerosis (67-70). In summary, these experiments demonstrate that the daily administration of ACEi in CF1 mice from the time of weaning significantly retards the progressive development of mesangial expansion and focal or diffuse glomerulosclerosis seen in untreated mice.

Based on these findings (71), and based on the model of the CF1 aging mouse, we decided to investigate the effects of ACEi on cellular and tissue function and structure in a variety of organs during the aging process.

The most important results in these trials were found in animals

receiving ACEi and were not dose related. They include (30):

A. *Increased survival*: The increased survival in normal rodents, as described in the literature, is related to dietary restriction (72). Moreover, according to the literature, hypoproteic diets in rats decrease the numbers of Angiotensin II receptors (73).

B. *Lower cardiac weight, lower myocardial and glomerular sclerosis, and lower middle vascular layer weight* (71, 74): Aging can be considered as a physiologic situation leading to cardiovascular alterations structurally and functionally similar to those observed in diseases such as diabetes and arterial hypertension, among others. The increase of cardiac weight is a common finding in the aged (75). In our model, treated animals showed a significantly lower cardiac weight compared to control animals. This was correlated with an important decrease of myocardial sclerosis. A review by Weber *et al.* (76) highlights the role of AngII and aldosterone in the development of sclerosis in the cardiac muscle.

   Several mechanisms have been proposed to explain the glomerulosclerosis. Some tests carried out with micropuncture and morphometric techniques indicate that the main pathogenic factor of the GS is the increase of glomerular pressure, which injures the epithelial, endothelial, and mesangial cells (77). ACEi decrease glomerular pressure by reducing the resistance of the glomerular efferent arteriole (78). Administration of these drugs reduces the development of GS in various experimental models (61, 79, 80), as in the model we studied.

C. *Maintainance of the number of mitochondria in heart and liver cells*: As described previously, aging is accompanied by a decrease in the number of mitochondria. Ultrastructurally, in our study, we found a significant difference in the number of mitochondria in heart and liver cells. The maintenance of mitochondrial number seen in the heart muscle cells of enalapril-treated animals was associated with less sclerosis and increased survival. The same results were obtained in the hepatocytes as they maintained the number of mitochondria. This suggests that the effect of ACEi is not restricted to the cardiovascular system.

   A possible hypothesis to explain the survival of the number of mitochondria is the role of the free radicals in

their injury; current evidence that free radicals participate in the pathogenesis of progressive cell injury comes predominantly from indirect studies (81, 82). Quite likely, the ACEi act as antioxidants and scavengers by inhibiting the role of angiotensin in promoting free radicals. Animals treated with ACEi were shown to maintain a consistent number of mitochondria. Accordingly, these animals lived longer and displayed a number of advantages: less sclerosis of the kidneys, heart, and liver as well as less apoptosis in these tissues. Taken together, these facts suggest a connection between ACEi and the formation of free radicals, which are the foremost mediators of age-related pathology.

In our model of aged mice, the effect of enalapril suggests multiple interconnections having to do with the improvement of antioxidant enzyme activity, mitochondrial number, myocardiocyte replicative capacity, apoptosis, and myocardial fibrosis (56).

These results present new questions: 1. is the protective effect on organ damage related to lower blood pressure?; 2. could this effect be detected in other species? In response to the first question, we decided to repeat our studies by giving propranolol, nifedipine, hydrochlorothiazyde, and enalapril to different groups of CF1 mice. The results revealed that even though these drugs induced similar hypotensive effects, only enalapril-treated animals experienced protection from organ damage and a prolonged lifespan (Figure 2) (84).

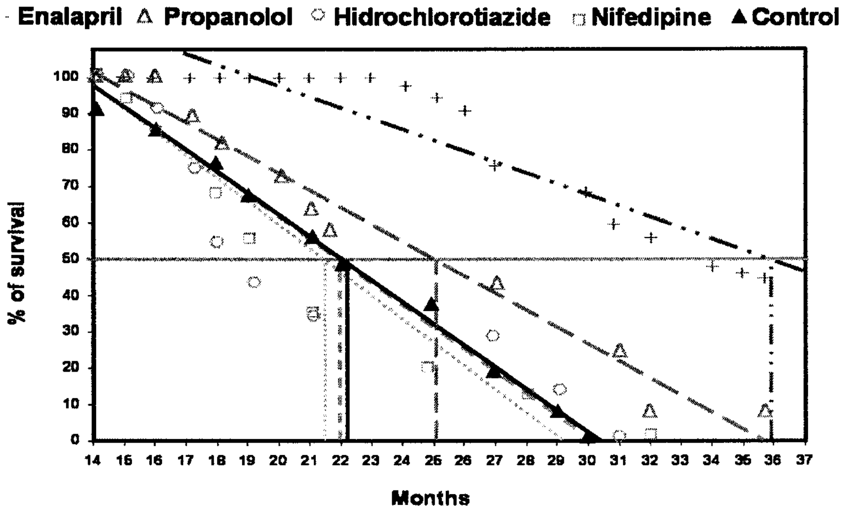

Figure 2: Survival Tendencies and L50

To answer the second question, we repeated these studies in Wistar rats and found results similar to those obtained in the CF1 mice (85, 86).

Effects of aging on the kidney

To further define the role of the renin-angiotensin system in aging, we administered enalapril (10 mg/kg/day) and the angiotensin receptor antagonist losartan (30 mg/kg/day) to Wistar rats from the time of weaning. After 7 months of treatment, both losartan and enalapril significantly decreased tubular atrophy as well as glomerular and interstitial fibrosis (31, 87).

The protective effects of losartan and enalapril on the kidneys of the animals at 18 and 22 months was also analyzed. Reduced kidney damage was definitively shown,; both treated groups presented lower glomerular and tubulointerstitial fibrosis, monocytic or macrophage infiltrates, and tubular atrophy than control animals (88). The differences in the proximal tubule cells at 18 months between control and treated groups (analyzed by electron microscopy) were extreme; higher brush border size, number of mitochondria, and conservation of mitochondrial ultrastructure and function stood out in the treated animals, and were similar to those found in younger rats (35). Because of that, we repeated the experiments but modified the treatment protocol to start when the animals reached 12 months of age; the results were evaluated 6 months later (89). In this experiment, we also found that either enalapril or losartan could produce protective renal effects. These data support the hypothesis that the RAS plays a considerable role in the natural aging of the kidney.

Effects of aging on the heart

Cardiovascular alterations due to aging are similar to those attributable to high blood pressure overload with respect to the biochemical, mechanical, and electrophysiological properties of the heart and blood vessels (90, 91). In rats, both hypertension and age produce an increase in left-ventricular weight and myocardial fibrosis (92). Alternately, the structure and function of the arterial wall also show evidence of aging attributable to hypertension; this mainly being an enhanced stiffness (93), which is also associated with smooth-muscle-cell hypertrophy and collagen accumulation (94). Endothelial and

smooth-muscle dysfunction along with aging and hypertension have also been described in rat aortas (95).

Angiotensin II has been shown to affect the structure of the cardiovascular system. It can modify cardiac and vascular structure (96), probably through hemodynamic effects, but also through its growth-promoting properties (97). It is not surprising, therefore, that ACEi and angiotensin receptor antagonists can prevent vascular hypertrophy in a variety of experimental models (97-99), as well as protect against the structural changes induced in the vasculature by hypertension (100). The protective actions of ACEi seem to be independent of arterial pressure, because they are similar in normotensive and hypertensive animals (100).

Blockade of the RAS can prevent left-ventricular hypertrophy and myocardiosclerosis in animals as well as in humans (74, 92, 101-103). Supporting these reports, our recent data in Wistar rats indicate that chronic treatment (6 or 18 months) with enalapril or losartan (10 mg/Kg/day) can attenuate several age-related cardiovascular changes (76, 104, 105). The protective effects of these compounds include the reduction of heart weight and myocardiosclerosis in 6-month-old rats (104). Moreover, morphometric and biochemical analyses of heart tissue revealed a reduction of DNA, collagen concentration, and the percentage of fibrosis in rats treated with either agent.

Mechanisms by which ACEi and ARB may alter the aging process

It seems very likely that ACEi and ARB exert their effects by blocking free radical formation and other adverse reactions associated with angiotensin.

Experimental data from our laboratory show that 7 months of RAS inhibition treatment can increase superoxide dismutase activity and total glutathione content in several rat tissues. This enhancement of antioxidant defenses is associated with a decrease in lipid oxidation relative to untreated controls (83).

With the objective of elucidating possible mechanisms, we decided to analyze the function of the mitochondria in the kidney by comparing control animals with animals treated with enalapril or losartan. The results can be found in Table 1 (106).

Table 1: Enalapril and losartan attenuate renal mitochondrial changes in aging rats. Values from treated 22-month old rats and 4-month-old untreated rats[a]

	Old	Enalapril	Losartan	Young
State 3/State 4	3.04±0.68	6.13±1.25*	5.37±1.08*	5.62±0.61*
ADP/O	1.64±0.15	2.50±0.31*	3.14±0.26*	2.83±0.28*
H_2O_2 (nmol/min^{-1}/mg protein^{-1})	10.1±1.0	7.17±1.61*	7.10±1.32*	3.39±0.68#
NOS (nmol NO/min^{-1}/mg protein^{-1})	0.72±0.06	0.98±0.05*	1.18±0.07*	0.98±0.04*
UCP2 (arbitrary units)	0.38±0.01	1.25±0.10*	1.77±0.51*	1.63±0.20*
Mn-SOD (units/mg protein)	71.0±0.1	47.2±0.1*	49.0±0.22*	41.8±0.18*

[a]Values are mean ± SE of 8 animals. *P< 0.05 vs. Old; #P < 0.01 vs. Old, Enalapril, Losartan

We studied whether chronic enalapril or losartan treatments can prevent the decline of the respiratory control ratio and /or the increase of hydrogen peroxide production that occur in mitochondria upon aging. These results indicate that the inhibition of the renin-angiotensin system could upregulate kidney mitochondrial respiration, and ameliorate certain functional and structural changes that occur with age (35).

Our working hypothesis is that AngII inhibition fosters changes in the mechanism of oxidative stress, especially at the mitochondrial level. With ample evidence that Ang II is intimately associated with free radical mayhem, this hypothesis is rational. In this regard, all the effects of ACEi and ARB in preventing diminution in mitochondrial damage and number and DNA oxidation are readily understandable. It follows that these effects most certainly play a role in slowing the aging process in both the kidney and heart. Moreover, a lower rate of oxidative stress could be the key to the reduction of the inflammatory process, cytokines, and growth factor production. Diminished fibrosis and the protection of kidney function and other tissues is another logical consequence.

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