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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