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NEW PHYSIOLOGICAL CONCEPTS OF THE RENIN ANGIOTENSIN SYSTEM FROM THE INVESTIGATION OF PRECURSORS AND PRODUCTS OF ANGIOTENSIN I METABOLISM:

The Novartis Award Lecture

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Introduction

Research on the regulatory actions of the renin angiotensin system (RAS) continues to provide a wealth of information on how cells maintain their internal homeostatic environment, regulate metabolic processes, and adapt or contribute to disease. Not that long ago, the active product of the system, angiotensin II (Ang II), was considered the single critical hormone product of an endocrine system involved in regulating blood volume and vascular tone. A revised concept emerged following the demonstration that renin and angiotensinogen are present in tissues. These findings suggested that the RAS is comprised of a dual, independently regulated, blood-borne and tissue systems. Today, a broader and complex system is being revealed by advanced genetic and molecular tools, as well as the outcome of clinical studies using medications selective for one or more of the proteins contributing to the generation of angiotensin peptides.

Recognition that the RAS contains both a pressor and depressor arm in exerting regulatory functions on vascular tone and cellular signaling paved the way for the generation of an alternate hypothesis as to how an imbalance of their function contributes to cardiovascular disease.(1) This review summarizes the data supporting the hypothesis of a counter regulatory arm that within the RAS opposes the actions of Ang II. We build upon these earlier discoveries to provide a new insight into an additional pathway in which an extended form of angiotensin I (Ang I), proangiotensin 12 [Ang-(1–12)], may be an alternate substrate for the production of the biological active angiotensins. A comprehensive evaluation of this topic cannot be achieved within the assigned space; therefore, only the key elements of the topic will be addressed, asking for indulgence in not providing a detailed listing of all published studies.

Angiotensin-(1–7): The Paradigm Shift

The period from approximately 1970 to 1980 represented the beginning of a shift in the concept of how the RAS was involved in cardiovascular pathology. Renewed enthusiasm for its study was stimulated by the demonstration that the administration of the angiotensin converting enzyme (ACE) teprotide had a dramatic effect in reducing the blood pressure of hypertensive subjects. (2) These results prompted many laboratories to undertake newer approaches to the investigation of the physiology of Ang II, isolate its receptor, and undertake the eventual synthesis of orally active Ang II receptor antagonists. (3)

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Conflict of Interest

There are no conflicts of interest.

The progress made throughout these exciting discoveries, nevertheless continued to posit Ang II as the biologically relevant product of the biochemical cascade that initiated by renin culminated in the production of Ang II. Alternate processes were assumed to have no major relevance in terms of biological function. The publication of the first description that the N-terminal derived heptapeptide, angiotensin-(1-7) [Ang-(1-7)], stimulated the release of vasopressin from rat hypothalamic explants (4) would over time decisively alter the former view.

Although initial studies found that Ang-(1-7) acted as a vasodilator, (5) further research showed that this effect could be best demonstrated in isolated vessels, (6;7) in animals in which the baroreceptors are eliminated, (5) or in conditions in which endogenous levels of Ang II are increased by maneuvers such as sodium depletion (8;9) or renovascular hypertension. (10) These findings underscored the concept that Ang-(1-7) acts as a paracrine hormone when formed in close proximity to the vascular smooth muscle or that its actions depend upon a change in the signaling effector mechanisms associated with increased expression or activity of AT₁ receptors. This is not an unreasonable possibility since it has been documented that the antihypertensive action of ACE inhibitors and Ang II receptor antagonists is amplified by concomitant use of thiazide diuretics.

Over the following decades, research would demonstrate that Ang-(1-7) contributes to the cardio-renal control of blood pressure via actions that within the heart, kidney, and the blood vessels opposed the activity of Ang II. (11-13) Ang-(1-7) was shown to reverse the hypertrophic and profibrotic effects of Ang II in the heart and the vasculature, (14-17) oppose Ang II-mediated cardiac arrhythmogenic activity, (18) possess antiatherogenic and antithrombotic actions, (19-24) inhibit oxidative stress and the generation of radical oxygen species, (25;26), and modulate hematopoietic function. (27;28)

Identification of the mas receptor as the conveyor for the cellular signaling responsible for Ang-(1-7) actions (29) and the demonstration that genetic deletion of this receptor abrogates the protective actions of the heptapeptide (30-36) has affirmed its participation in the regulation of cardiovascular function. Second messenger mechanisms responsible for the cellular response mediated by the binding of Ang-(1-7) to the mas receptor include inhibition of the mitogen activated protein (MAP) kinase pathway, stimulation of cellular phosphatases, inhibition of cyclooxygenase 2 (COX2) and facilitation of nitric oxide release. (28;37-44)

ACE2 and Ang-(1-7)

The pace of research on the counter lever role of Ang-(1-7) on Ang II expanded with the identification of an ACE homologue, ACE2, that cleaved Ang II into Ang-(1-7). (45;46) As reviewed elsewhere,(47-50) ACE2 differs from ACE in acting as a mono-carboxypeptidase to cleave the Pro⁷-Phe⁸ bond of Ang II to form Ang-(1-7) and not been inhibitable by exposure to ACE inhibitors. A stepping stone in the evolving understanding of the role of ACE2 in cardiac function followed the demonstration that deletion of the ACE2 gene is accompanied by severe cardiac contractility defects and that ACE2 mRNA protein and expression were reduced in three different models of experimental hypertension. (51) Selective overexpression of cardiac ACE2 in rats is associated with reversal of cardiac hypertrophy (52-54) and progression of atherosclerosis (55) while chemical inhibition of ACE2 worsened the progression of renovascular hypertension. (56) While ACE2 has been shown to convert Ang II into Ang-(1-7) in both animals (57) and humans, (58;59) further work is necessary to affirm whether the predominant effects of ACE2 inhibition are primarily due to prevention of Ang II metabolism. Nevertheless, this hypothesis is in keeping with the findings of reduced ACE2 expression or activity in experimental models of hypertension, (51;56;60-64) human prehypertension, (65) heart failure, (65-68) renal disease and type 2 diabetes. (69-72)

Following the first demonstration that blockade of AT₁ receptors in rats with myocardial infarction were associated with upregulation of cardiac ACE2 mRNA (73), newer studies suggest that ACE2 gene transcription is negatively regulated by Ang II while Ang-(1–7) counteracts the inhibitory effect of Ang II on ACE2 gene expression. (74–76)

Altogether, the proposal that Ang-(1–7) opposes the actions of Ang II became the underpinning for the recognition that the RAS is biochemically constituted by alternate enzymatic pathways leading to the generation of separate peptides acting at receptors of which the Ang II AT₁ receptor subtype is only a part of the system. In accepting a more complex view of the system and its function in the regulation of blood pressure and vascular structure, the concept that the arm of the RAS comprised by the *ACE2/Ang-(1–7)/mas receptor axis* counterbalances the activity of the other arm (*ACE/Ang II/AT₁ receptor axis*) has gained acceptance. The new knowledge is stimulating further research into its possible role as, at the very least, a permissive contributor of the cardio-renal remodeling accompanying the pathogenesis of hypertensive vascular disease and target-organ damage.

Proangiotensin 12 (a.k.a., Angiotensin-(1–12))

As the science dissecting the contribution of the RAS to cellular function continues to evolve, a study from Japan challenged in 2006 the idea that renin-dependent hydrolysis of angiotensinogen (Aogen) constitutes the rate-limiting step for the formation of angiotensin peptides. (77) Their observations opened a new window toward exploring how angiotensin peptides may be formed within the interior milieu of cells or their immediate surrounding environment. The isolation of a new Aogen-derived peptide by Nagata et al. (77) from the rat's small intestine contains a shorter form of the synthetic tetradecapeptide synthesized by Skeggs et al. (78) in 1958. The Aogen-derived substrate was termed by them proangiotensin-12 based on its role as an Ang II precursor. In following the terminology approved by the Nomenclature Committee of the Council for High Blood Pressure Research in 1991, (79) we will use the term angiotensin-(1–12) [Ang-(1–12)] throughout this review, as it best follows the appropriate convention in defining the amino acid sequence of the polypeptide within the family of angiotensins (Table 1). Critically important, their study showed the endogenous presence of the peptide in Wistar rats and its ability to serve as a substrate for the *in vitro* and *in vivo* generation of Ang II, a finding that strongly differentiates Ang-(1–12) from the tetradecapeptide isolated previously. As illustrated in Figure 1, Ang-(1–12) levels in the small intestine, liver, lungs, adrenal gland, heart, brain, and pancreas are higher than corresponding concentrations of Ang I. The ability of Ang-(1–12) to act as an endogenous substrate for Ang II production followed the observation that Ang-(1–12) vasoconstrictor effects were prevented by prior blockade with either captopril or the AT₁ receptor antagonist candesartan in both the isolated aorta and the systemic circulation. (77) Their findings attracted the interest of our laboratory because this was the first time that an extended form of Ang I had been shown to exist endogenously.

Intrigued by the potential importance of Ang-(1–12) as an alternate substrate for the formation of angiotensin peptides, we explored the location of Ang-(1–12) in cardiac and renal tissues of both Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats. Polyclonal antibodies targeting the specific sequence of rat Ang-(1–12) revealed intense and selective staining of cardiac myocytes and renal tubular cells of both strains (Figure 2). The patchy staining observed in cardiac myocytes of the left ventricle of WKY rats was markedly altered in the SHR, since almost all the cardiac tissue displayed intense staining. (80) To more precisely determine whether the visual display of increased Ang-(1–12) immunoreactive staining reflected a greater endogenous expression of the peptide, we measured Ang-(1–12) tissue concentrations by radioimmunoassay. These experiments showed that the endogenous content of Ang-(1–12) in the cardiac tissue of SHR was 47% higher than those found in WKY. (80)

Rat kidney also displayed selective expression of Ang-(1–12) in the proximal, distal, and collecting renal tubules within the deep cortical and outer medullary zones in both strains (Figure 2); however, Ang-(1–12) concentrations in renal tissue were slightly reduced in SHR. (80)

Differential tissue expression of Ang-(1–12) led to a second study focusing on whether renin accounted for the cleavage of Ang-(1–12) into Ang II, Ang-(1–7), or both in isolated perfused hearts from three normotensive and two hypertensive rat strains. (81) As illustrated in Figure 3, Ang-(1–12) caused the rapid appearance of both Ang I and Ang II in the perfusate of WKY and SHR, with highest levels occurring between 30 and 60 min of recirculation (Figure 3). Although renin was present both in the cardiac tissue and in the effluent of isolated perfused WKY and SHR hearts (Figure 3), addition of a selective rat renin inhibitor (WFML-1) did not prevent conversion of Ang-(1–12) into angiotensin peptides. (81) These data thus excluded renin from acting on Ang-(1–12). Recent studies in anephric rats are in agreement with this interpretation. (82) In the anephric state the loss of renal-derived renin resulted in the expected fall in the circulating concentrations of Ang I and Ang II to barely detectable levels while it had only a small reducing effect on plasma Ang-(1–12) levels (Figure 4). In contrast, parallel measures of the cardiac content of angiotensin peptides documented a 91% increase in cardiac Ang-(1–12) tissue concentrations 48 h after bilateral nephrectomy. (82) Furthermore, absence of renin transcripts in cardiac tissue of both sham and nephrectomized rats suggests that both the processing of Aogen into Ang-(1–12) and its secondary conversion to Ang I occurs via a non-renin pathway. Therefore, the biochemical processes associated with the expression and cleavage of Ang-(1–12) through a non-renin pathway suggest an additional level of complexity within the enzymatic pathways accounting for the expression of biologically active peptides.

Insights into the enzymatic pathway (s) responsible for the conversion of Ang-(1–12) into Ang II have been recently expanded by studies of Prosser et al. (83) in the isolated heart of Sprague Dawley rats. In this preparation, *ex-vivo* and *in-vivo* conversion of Ang-(1–12) into Ang II was prevented by the administration of chymostatin using combined high performance liquid chromatography and tandem mass spectrometry analysis (83). Urata et al. (84) first implicated a functional role for chymase, a member of the serine protease family, as a tissue enzyme involved in the conversion of Ang I into Ang II. The location of chymase in secretory granules and its ubiquitous existence in mast, vascular endothelial and mesenchymal cells provides a mechanism for the intracellular formation of angiotensin peptides and their functioning as intracrine and paracrine regulators. (85) A potential limitation in terms of ascribing a primary role for chymase in the metabolism of Ang-(1–12) is the previous report by Nagata et al. (77) who found that captopril abolished the constrictor response of aortic strips to Ang-(1–12) exposure, as well as preventing the pressor effect of intravenous Ang-(1–12). Differences in the findings between both studies may have been influenced by employed methodology and the use by Prosser et al. (83) of human recombinant chymase. In this connection, rat, but not human chymase cleaves the Tyr⁴-Ile⁵ bond of Ang II. (86) In addition, tissue damage and edema associated with saline perfusion of the preparation may have increased cell permeability exposing the peptide to intracellular chymase. (83)

The tantalizing evidence for the existence of an alternate renin-independent substrate for extra- or intracellular processing of angiotensin peptides awaits further studies as to whether its biological activity is expressed through the formation of Ang II or may act independently of its processing into the known biological peptides of the renin angiotensin system. Further studies should be undertaken to unravel the potential importance of Ang-(1–12) as an alternate substrate for the formation of angiotensin peptides as well as determining the enzyme(s) accounting for the cleavage of Ang-(1–12) from Aogen and its subsequent conversion into Ang II, Ang-(1–7) or both. As research delves further into the potential role of Ang-(1–12) as a source of tissues angiotensins formation the question of whether increased formation of Ang-

(1–12) may contribute to pathology comes to the forefront. A preliminary answer to this question may be derived from the demonstration of increased expression and cardiac content of Ang-(1–12) in the myocytes of SHR, (80) increased expression of cardiac Ang-(1–12) and Ang II in both anephric rats, (82) and in those in which hypertension was abated by administration of a mineralocorticoid receptor antagonist. (87) In addition, the recent demonstration that endogenous neutralization of Ang-(1–12) via infusion of a selective Ang-(1–12) antibody into the cerebrospinal fluid of transgenic hypertensive rats lowers blood pressure provides further evidence for its role as a precursor for the formation of Ang II. (88)

Summary

Even from this limited overview of the intricate internal mechanisms regulating the pathways determining the production of angiotensin peptides, it is obvious that the RAS is embodied with a great capacity to use alternate mechanisms in bypassing blockade of primary pathways. In unraveling the complexity of the biochemical physiology of the system it is also apparent that formation of angiotensin peptides within the cellular environment may not follow what has been characterized in the circulation or even the extracellular compartment.

A new level of regulatory complexity is added with the demonstration of Ang-(1–12) as an alternate substrate contributing to forming angiotensin peptides by a non renin-dependent mechanism. The tissue formation and processing of the biological active products of the renin angiotensin system may follow those outlined in Figure 5, whereby synthesis of Ang II and Ang-(1–7) may be determined by availability of Ang-(1–12) either formed through intracellular processing of Aogen, uptake from the extracellular compartment, or both. While there are still many fundamental gaps in uncovering the conditions and processes that determine the enzyme (s) accounting for either the cleavage of Ang-(1–12) from Aogen or the processing of Ang-(1–12) to Ang II and Ang-(1–7), the fact remains that this substrate occurs endogenously, is altered in a genetic model of hypertension, and can clearly produce angiotensin peptides by a non renin dependent mechanism. As the ubiquitous functions of tissue RAS extends outside the cardiovascular system, the detection of high concentrations of Ang-(1–12) in the rat gut needs to be explored since Ang II contributes to jejunal motility and fluid transport. (89;90) Indeed, the finding that high doses of the direct renin inhibitor aliskiren are associated in humans with diarrhea (91;92) posits the question as to whether this side-effect of the drug might be related to increases in the gut content of Ang-(1–12).

Perspectives

Understanding the structure and the dynamics of the complex intercellular web of interactions that contribute to the function of a living cell requires acceptance that a discrete biological action can rarely be attributed to an individual molecule. The intertwined relationship between the ACE/Ang II/AT₁ receptor axis and its counter lever ACE2/Ang-(1–7)/mas receptor axis is now buttressed on a firm literature. A further investigation of the potential clinical impact of pursuing the hypothesis of whether genetic or acquired deficiency in one or more of the components of the Ang-(1–7)/ACE2/mas-R axis may explain the progression of hypertensive vascular disease ought to be pursued with greater vigor as research in this field is extending knowledge of disease processes both within and outside the cardiovascular system.

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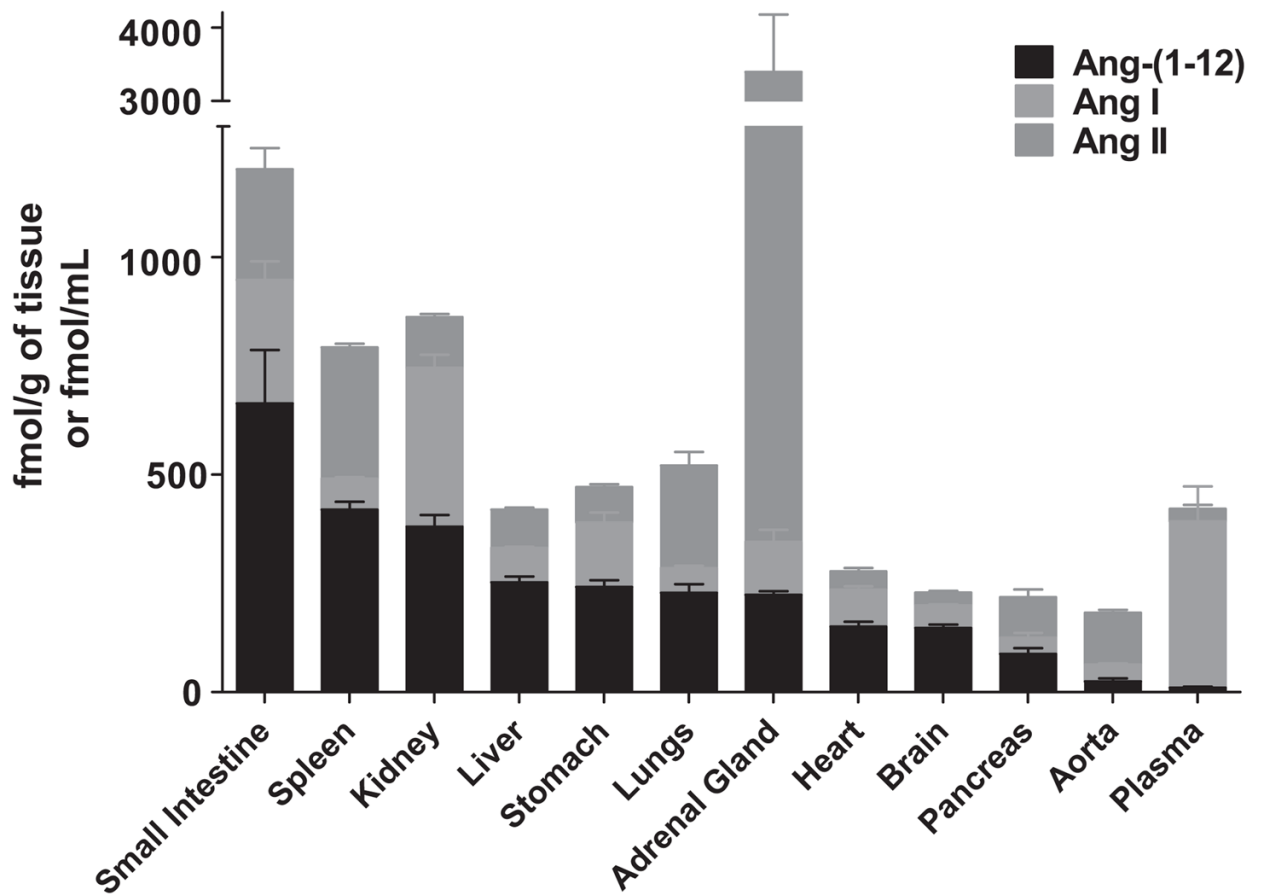


Figure 1.

Data (Means \pm SEM), redrawn from Nagata et al (77), illustrate the relative concentrations of angiotensin-(1-12) [Ang-(1-12)], angiotensin I [Ang I], and angiotensin II [Ang II] in tissues and plasma from the Japanese-derived strain of normotensive Wistar rats.

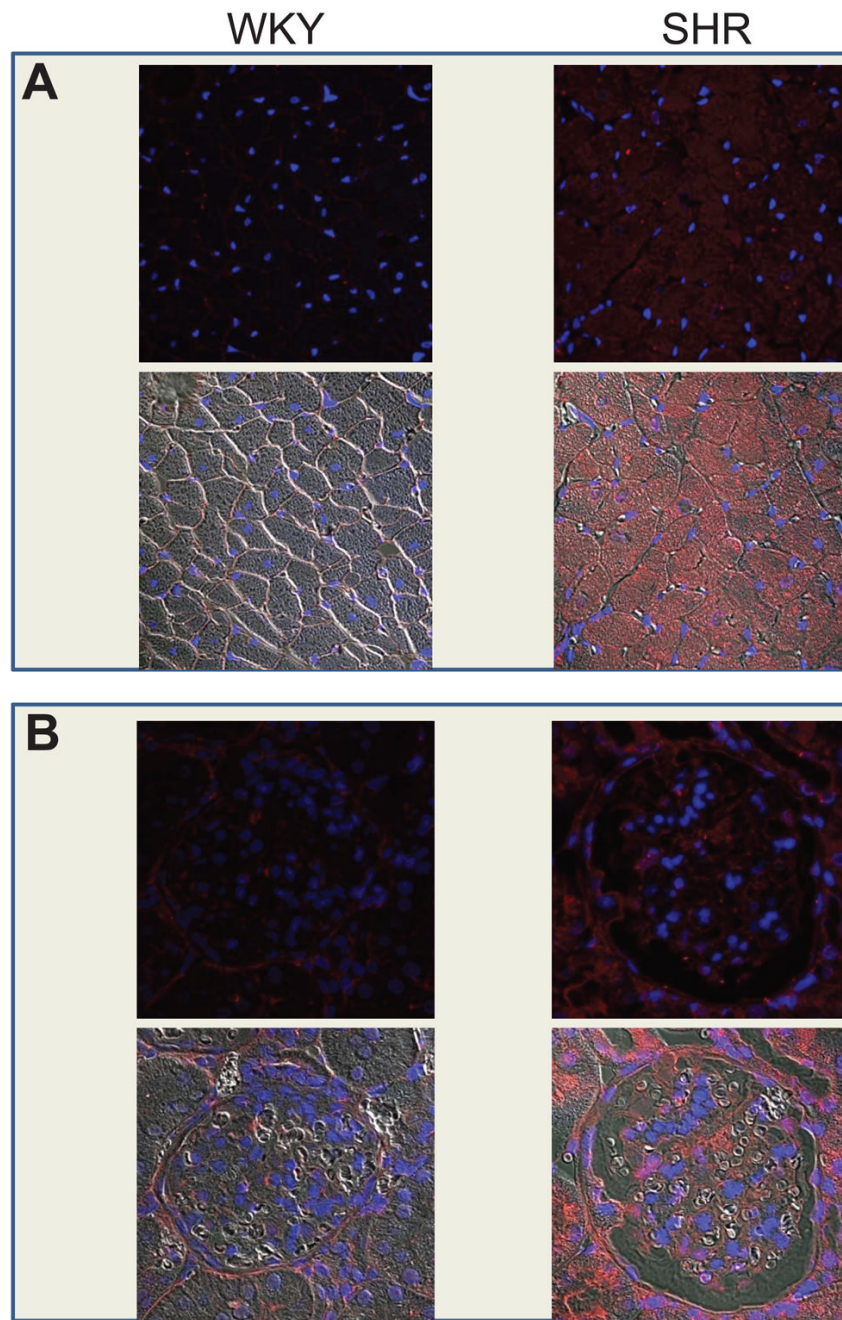


Figure 2. Representative micrographs of angiotensin-(1–12) immunostaining from cardiac (A) and renal (B) tissue of WKY and SHR (upper images in each panel). In panel A, faint immunostaining for Ang-(1–12) is observed in the myocardium of WKY while intense punctuate staining is expressed in left ventricular myocytes of SHR. Panel B illustrates the expression of Ang-(1–12) immunostaining in sections of kidney cortex from WKY and SHR. Fluorescent staining for Ang-(1–12) found in renal tubules is increased in sections obtained from SHR. Nuclei are indicated by 4',6-diamidino-2-phenylindole (DAPI) blue fluorescence staining. Lower images in each panel illustrate merged confocal and phase contrast immunostaining for each organ. From studies reported in our reference. (80)

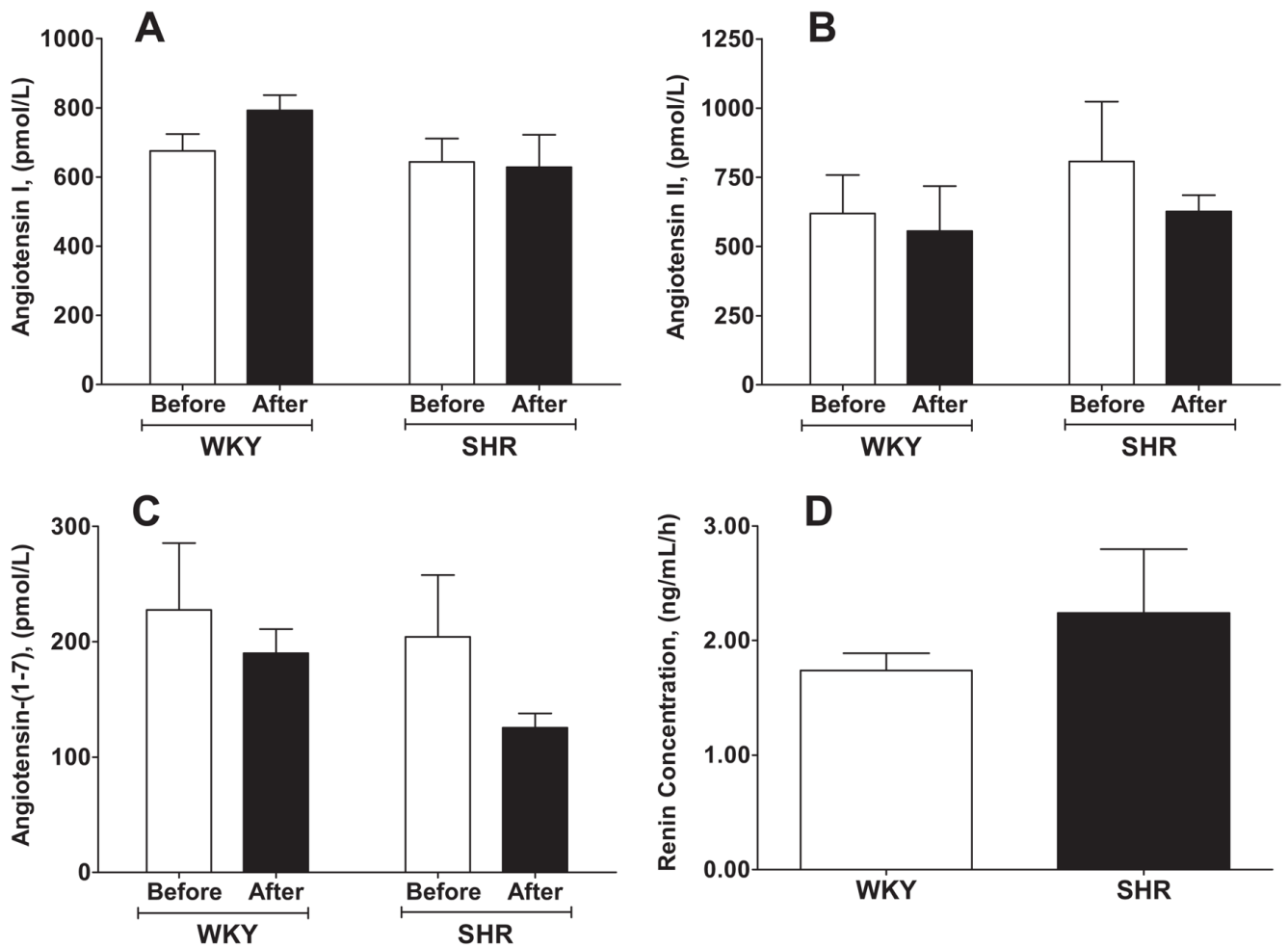


Figure 3.

Panels A to C are peak concentrations of angiotensin peptides resulting from the addition of 10 nmol Ang-(1–12) to the perfusate of isolated hearts from Wistar Kyoto (WKY) and spontaneously hypertensive (SHR) adult rats. Panel D illustrated renin concentration in the effluent of isolated perfused hearts from WKY and SHR. Before, peak angiotensin values obtained 60 min after administration of Ang-(1–12); After, shows the absence of a change in the peak values of Ang-(1–12) following administration of 1 μ mol/L of the rat specific renin inhibitor WFML-1. Data (Means \pm SEM) redrawn from our reference. (81)

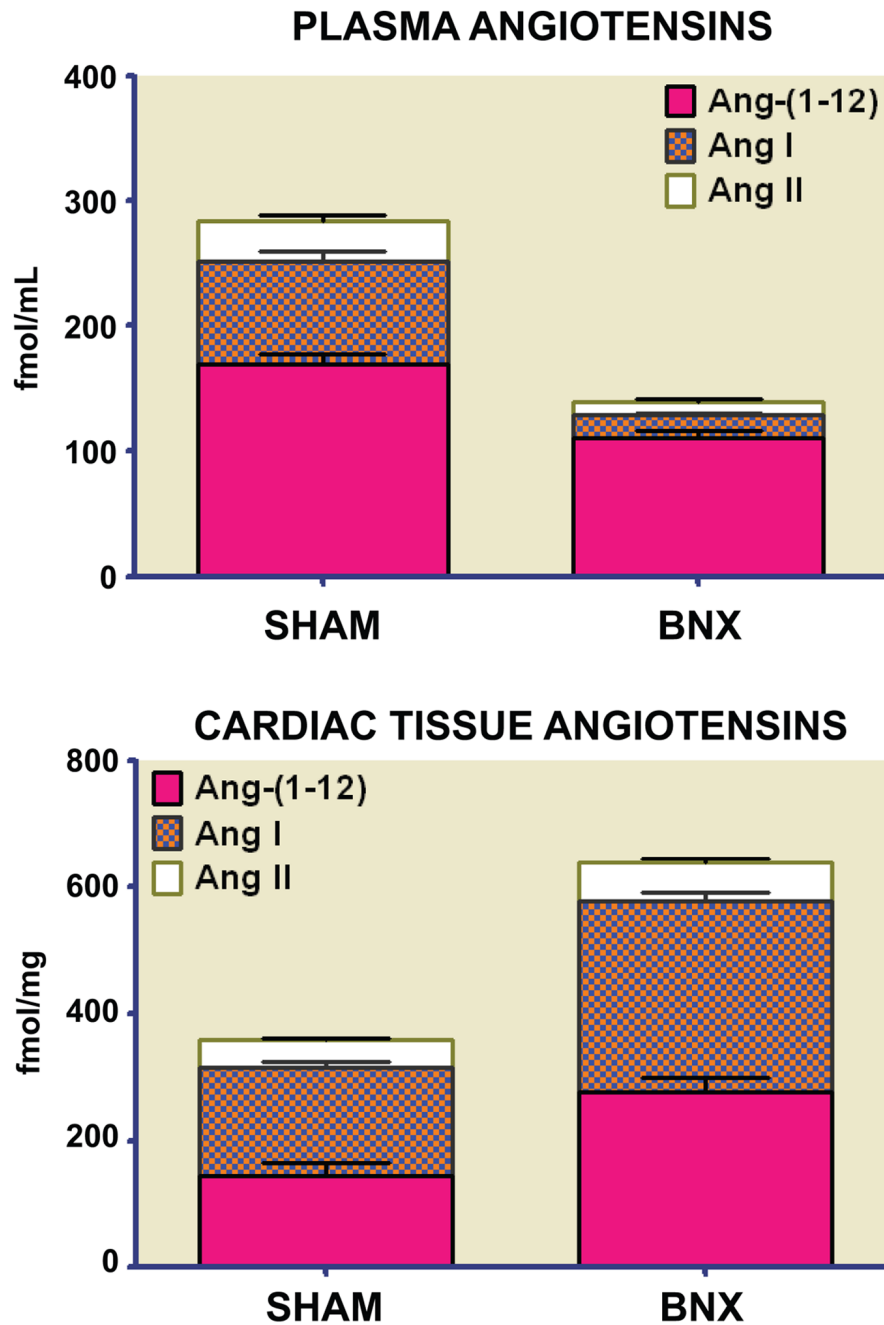


Figure 4. Plasma and cardiac tissue concentrations of angiotensin peptides in sham and 48 h bilateral nephrectomized (BNX) rats reveals differential expression of angiotensin-(1-12) [Ang-(1-12)], angiotensin I (Ang I) and angiotensin II (Ang II) in adult Wistar Kyoto rats. Data redrawn from our reference (82)

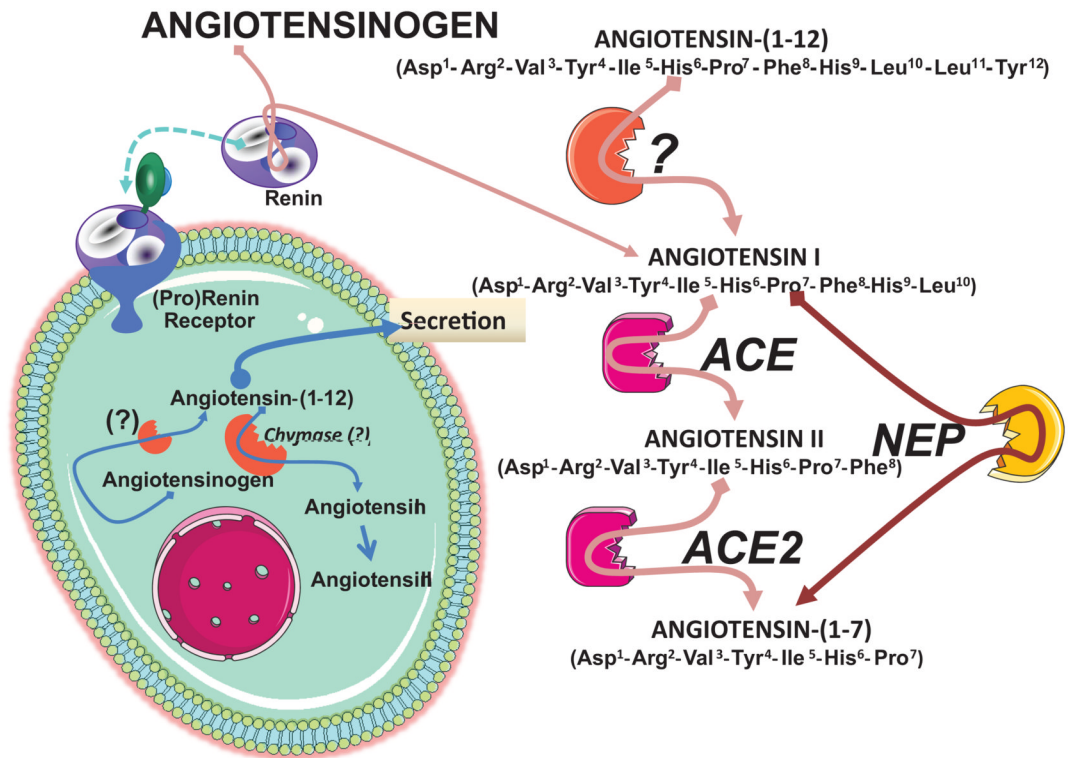


Figure 5. Diagram of the biochemical pathways leading to the formation of angiotensin peptides in tissues. Abbreviations are: ACE, angiotensin converting enzyme; ACE2, angiotensin converting enzyme 2; NEP, neutral endopeptidase 24.11 and related endopeptidases contributing to formation of Ang-(1-7) from Ang I. (12)

Table 1

Amino Acid Sequences of Main Angiotensin Peptides

Peptides	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Ang-(1-14)	Asp	Arg	Val	Tyr	Ile	His	Pro	Phe	His	Leu	Leu	Val	Tyr	Ser
Human Ang-(1-12)	NH ₂	Arg	Val	Tyr	Ile	His	Pro	Phe	His	Leu	Leu	Val	Ile	COOH
Rat Ang-(1-12)	NH ₂	Arg	Val	Tyr	Ile	His	Pro	Phe	His	Leu	Leu	Tyr	Tyr	COOH
Ovine Ang-(1-12)	NH ₂	Arg	Val	Tyr	Ile	His	Pro	Phe	His	Leu	Leu	Val	His	COOH
Canine Ang-(1-12)	NH ₂	Arg	Val	Tyr	Ile	His	Pro	Phe	His	Leu	Leu	Val	Tyr	COOH
Equine Ang-(1-12)	NH ₂	Arg	Val	Tyr	Ile	His	Pro	Phe	His	Leu	Leu	Val	Tyr	COOH
Ang-(1-10)	NH ₂	Arg	Val	Tyr	Ile	His	Pro	Phe	His	Leu	Leu	Val	Tyr	COOH
Ang-(1-8)	NH ₂	Arg	Val	Tyr	Ile	His	Pro	Phe	His	Leu	Leu	Val	Tyr	COOH
Ang-(1-7)	NH ₂	Arg	Val	Tyr	Ile	His	Pro	Phe	His	Leu	Leu	Val	Tyr	COOH
Ang-(2-8)	NH ₂	Arg	Val	Tyr	Ile	His	Pro	Phe	His	Leu	Leu	Val	Tyr	COOH
Ang-(3-8)	NH ₂		Val	Tyr	Ile	His	Pro	Phe	His	Leu	Leu	Val	Tyr	COOH