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An Evolving Story of Angiotensin II Forming Pathways in Rodents and Humans

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Abstract

Lessons learned from the characterization of the biological roles of angiotensin-(1-7) in opposing the vasoconstrictor, proliferative, and prothrombotic actions of angiotensin II created an underpinning for a more comprehensive exploration of the multiple pathways by which the blood and tissues renin angiotensin system regulates homeostasis and its altered state in disease processes. This review summarizes the progress that has been made in the novel exploration of intermediate shorter forms of angiotensinogen through the characterization of the expression and functions of the dodecapeptide angiotensin-(1-12) in the cardiac production of angiotensin II. The studies reveal significant differences in humans versus rodents regarding the enzymatic pathway by which angiotensin-(1-12) undergoes metabolism. Highlights of the research include the demonstration of chymase-direct formation of angiotensin II from angiotensin-(1-12) in human left atrial myocytes and left ventricular tissue, the presence of robust expression of angiotensin-(1-12) and chymase in the atrial appendage of subjects with resistant atrial fibrillation, and the preliminary observation of significantly higher angiotensin-(1-12) expression in human left atrial appendages.

Keywords

angiotensin converting enzyme; angiotensin-(1-7); angiotensin-(1-12); atrial fibrillation; chymase; proangiotensin-12; renin

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Author contributions

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INTRODUCTION –The Yesteryears–

During the last 25 years knowledge of the biochemical physiology of the circulating and tissue renin angiotensin system has grown exponentially through the discovery and characterization of the vasodilator and anti-proliferative actions of angiotensin-(1-7) [Ang-(1-7)] [1], the characterization of angiotensin converting enzyme 2 (ACE2) as an Ang-(1-7)-forming enzyme from angiotensin II (Ang II) [2–4], and the demonstration of the mas receptor as the endogenous binding protein for Ang-(1-7) [5]. On the 25th anniversary of the publication of the first report describing the actions of Ang-(1-7) [6], the science presented at the XI International Symposium on Vasoactive Peptides (May 2–4, 2013, Belo Horizonte, Brazil) attested to the impressive progress achieved in deciphering the complexity of the biochemical and physiological processes by which the renin angiotensin system contributes to organ and cell homeostasis. As the original participant in the discovery of Ang-(1-7) function [6], we ask for the readers' indulgence for taking the liberty of reflecting on how this all began.

The 1980s was a period of active research in the field of arterial hypertension given the effective introduction of ACE inhibitors, knowledge and identification of Ang II receptors, and the extensive characterization of Ang II analogs and antagonists by chemical substitution of its amino acids [7;8]. These efforts led to the recognition that the phenyl group in position eight of Ang II conveyed the information for biological response, whereas the aromatic side chain in positions 4 and 6, the guanido group in position 2, and the C-terminal carboxyl were involved in binding to the receptor [7]. In sharing an environment of scientific giants at the Cleveland Clinic –Merlin F Bumpus, Mahesh Khosla, and Robert Smeby– Robson Santos's observation in my laboratory that canine brain homogenates contained high amounts of the Ang-(1-7) heptapeptide was received with significant skepticism since this fragment had been reported previously to be devoid of any biological activity [9;10]. This interpretation was based on studies showing that truncation of the C-terminus of Ang II, particularly the removal of phenylalanine at position 8 prevented the peptide from binding to the Ang II receptor in isolated blood vessels or the adrenal medulla [7]. Despite these significant odds, we chose to pursue whether Ang-(1-7) may possess biological activity since the high concentrations of this fragment in canine brain homogenates suggested otherwise. Since we were pursuing at that time the study of neuroendocrine functions of Ang II, the availability in our laboratory of a rat hypothalamic-hypophysial explant preparation [11;12] allowed testing whether Ang-(1-7) had any action in vasopressin secretion. These studies, initiating the era of Ang-(1-7) research, showed that the heptapeptide stimulated the secretion of vasopressin with an agonistic activity equal to comparable Ang II doses [6]. The evidence that Ang-(1-7) stimulated vasopressin release despite the fact that this N-terminal peptide had lost the eight amino acid residue at the carboxy-terminal epitope of Ang II stimulated a series of critical studies showing that the heptapeptide augmented neuronal activity of circuits that in the dorsal medulla oblongata regulate baroreflexes [13–15], enhanced the production of prostanoids in the rabbit isolated vas deferens [16], and elicited dose-dependent vasodepressor responses in areflexic rats [17]. The latter studies established the foundation for the hypothesis that Ang-(1-7) opposes the actions of Ang II [18;19], a proposal that became the cornerstone of the research that

followed these initial observations as lucidly demonstrated in the excellent science presented in the Symposium organized by Dr. Santos. For the interested reader, several reviews provide a comprehensive analysis of the status of this field and the role of Ang-(1-7) in cardiovascular regulation, organ system physiology and pathology, and novel functions as an anti-tumoral and anti-inflammatory agent [1;5;20–25].

Novel Pathways Upstream from Angiotensin I –The New Research–

In continuing to explore the complexity and diversity of the biochemical pathways associated with the production of the main biologically active angiotensin peptides, we are investigating whether the diversity in the biotransformation processes leading to the formation of Ang II and Ang-(1-7) is also present upstream from Ang I. Past research supports the view of: (i) different enzymatic routes for the generation of angiotensin peptides; (ii) different receptor expression in tissues; (iii) different mechanisms influencing receptor regulation; and (iv) different signal transduction pathways for conveying activation of the system. These factors explain the dual role of the RAS as both a circulating hormonal and tissue-specific regulatory system wherein the expression of angiotensins subserves not only autocrine/paracrine but also intracrine functions.

The *raison d'être* for exploring the biochemical pathways upstream from Ang I is based on accumulating evidence suggesting that the long-term effects of RAS blockade using direct renin inhibitors (DRI), angiotensin converting enzyme (ACE) inhibitors, and Ang II receptor blockers (ARBs) has fallen short of expectations when compared to other antihypertensive classes. A metaanalysis of 31 trials [26], with 190,606 participants, showed “*no clear difference between age groups in the effects of lowering blood pressure or any difference between the effects of the drug classes on major cardiovascular events.*” Another meta-regression analysis from 26 large-scale trials, found no evidence of any blood pressure-independent effects of either ACEI or ARB [27]. The potential for these treatment approaches to be accounted for incomplete blockade of Ang II actions or synthesis cannot explain these findings because the combination of ARB and ACE inhibitors showed no further benefits in the large ONgoing Telmisartan Alone and in combination with Ramipril Global Endpoint Trial (ONTARGET) [28;29], the Aliskiren Trial in Type 2 Diabetes Using Cardiorenal Endpoints (Altitude) trial which combined aliskiren [direct renin inhibitor (DRI)] with valsartan [30;31] or another systematic large meta-analysis of patients with symptomatic left ventricular dysfunction [32].

All of these contrasting observations pose the following question, if ACE inhibitors and ARBs have so well-proven biological effects in preventing the cardiovascular consequences of excess Ang II expression or activity, why are clinical hard end-point benefits not superior to what can be achieved by other classes of antihypertensive agents? To answer this question, we are pursuing the hypothesis that the discrepancy might be explained by proposing that: 1)- ACE inhibitors and ARBs are not able to access the cellular sites at which Ang II acts; 2)- Ang II production in tissues may involve pathways that neither require ACE nor renin for its generation; or 3)- a combination of both.

Accumulating evidence suggests that Ang II, either produced within the cell or incorporated from the extracellular microenvironment, drive the processes by which the peptide acts as a trophic, proinflammatory and profibrotic factor [33–35]. Convincing evidence for intracrine Ang II actions in the heart are provided by De Mello [36–39] who showed that intracellular Ang II augments electrophysiological indices of myocyte excitability. Others have reached similar conclusions. Baker's laboratory showed that cardiac Ang II formation and action was not altered by ACE inhibitors or ARBs [33;35;40;41]. In our own studies [42], the content of left ventricular Ang II did not change in response to chronic inhibition of Ang II synthesis or activity (Figure 1). Other studies by Dell'Italia and collaborators [43;44], Urata et al. [45–47] and Kumar et al. [48–51] demonstrated that intracellular Ang II formation in myocytes is independent of ACE. Therefore, it is plausible that intracellular Ang II formation or action escapes inhibition from drugs which act either in the extracellular compartment or the plasma membrane.

The second possibility, formation of Ang II by mechanisms that do not involve renin or ACE, is gaining new momentum with the isolation of proangiotensin-12 [angiotensin-1-12; Ang-(1-12)] by Nagata et al. [52] from the small intestine of a Japanese strain of Wistar rats. This peptide, containing the first 12 amino acids of the N-terminus of the angiotensinogen (Aogen) molecule [human sequence: N-Asp¹-Arg²-Val³-Tyr⁴-Ile⁵-His⁶-Pro⁷-Phe⁸-His⁹-Leu¹⁰-Val¹¹-Ile¹²-] is present at high concentrations in the kidney, heart and brain of Wistar rats [52]. These authors [52] further showed that Ang-(1-12) was readily converted to Ang II as the vasoconstrictor response produced by Ang-(1-12) in either the systemic circulation or aortic strips was blocked by prior administration of captopril or candesartan.

The discovery of this extended form of Ang I raised our interest since we had previously reported the existence of a family of extended Ang I peptides in the dog's cerebrospinal fluid [53]. In a series of correlative studies we showed increased expression and content of Ang-(1-12) in the left ventricle of SHR compared with WKY [54]. Furthermore, a 42% higher Ang-(1-12) uptake was found in cultured neonatal myocytes from SHR compared to WKY. Increased incorporation of the radiolabelled Ang-(1-12) in SHR was associated with a faster rate of peptide degradation in the media collected from the neonatal myocytes [55]. As illustrated in Figure 2 there were significant differences in Ang-(1-12) metabolism in the cultured media obtained from WKY and SHR neonatal cardiomyocytes. Major differences in ¹²⁵I-Ang-(1-12) hydrolysis between the two strains included a greater catalytic activity of the media for Ang-(1-12) metabolism by ACE, neprilysin (NEP) and chymase [Figure 2 and reference [55]]. Importantly, the catalytic activity of chymase in the media collected from cultured cardiac myocytes was markedly augmented in SHR compared with WKY (Figure 2). The importance of ACE as an Ang-(1-12)-degrading enzyme in rodents is documented in studies in which the pressor effects of a 14-day infusion of Ang-(1-12) was mitigated in rats medicated with either losartan or the ACE inhibitor, perindopril [56]. In agreement with this study, we also showed that ACE is the primary pathway for Ang II production from Ang-(1-12) in the circulation of WKY and SHR [57].

Since the original identification of chymase as an Ang II-forming enzyme in the human heart [58–60] the involvement of this enzyme in forming Ang II from Ang I has been reported to be crucially important in the pathogenesis of adverse cardiac remodeling

associated with volume overload, development of cardiomyopathies, heart failure, vascular atherogenesis, and diabetic nephropathy [58;61–64]. Although ACE was identified as the primary enzyme accounting for Ang-(1-12) metabolism in the circulation of Wistar rats [52], isolated rat arteries [65], the serum of a congenic model of hypertension expressing high tissue renin [66], and the circulation of both WKY and SHR [57], the demonstration that chymase is an Ang II-forming enzyme from Ang-(1-12) in the hypertrophied heart of SHR [55] and a model of ischemia/reperfusion injury [67] suggested that chymase may be recruited in conditions of tissue strain or injury. This was not to be the complete interpretation of our previous observations.

Given the fact that prior studies suggested that chymase was particularly important as an Ang I converting enzyme in humans [62;64], two studies addressed the expression and metabolic pathways for Ang-(1-12) in the human heart [68;69]. Human atrial tissue, obtained from patients undergoing heart surgery for the correction of resistant atrial fibrillation (AF) expressed immunoreactive (ir-) Ang-(1-12) and chymase within atrial myocytes [68]. Incubation of plasma membranes from these atrial myocytes with ^{125}I -Ang-(1-12) resulted in the rapid production of Ang II that was prevented in the presence of chymostatin and not inhibited by lisinopril (Figure 3). As discussed in our study [68], while chymase cleaving activity in rodents occurs at amino acid residues containing the sequence of Tyr⁴-Ile⁵ [70], human chymase hydrolytic activity is specific for the Phe⁸-His⁹ sequence. The latter sequence is present within the amino acid composition of human Ang-(1-12) [H-Asp¹-Arg²-Val³-Tyr⁴-Ile⁵-His⁶-Pro⁷-Phe⁸-His⁹-Leu¹⁰-Val¹¹-Ile¹²-OH]. These data agree with the finding that Ang I formation represented less than 2% of the Ang-(1-12) hydrolysis (Figure 3). To exclude the possibility that the primacy of chymase as an Ang-(1-12) converting enzyme was influenced by the existence of cardiac pathology, a second study characterized Ang-(1-12) expression and biotransformation in human left ventricular tissue obtained from normal subjects [69]. Like in the study from subjects with resistant AF, processing of Ang-(1-12) into Ang II was entirely due to the catalytic activity of chymase [69].

The studies describe above are in keeping with the tenet that chymase mediates the majority of Ang II production in the human heart, and that Ang II formation is compartmentalized: with chymase being the major Ang II forming mechanism in the cardiac interstitium and both chymase and ACE acting as Ang II forming mechanism in the intravascular space (Figure 4). Investigation of the alternate pathway for Ang II production from Ang-(1-12) also shows that chymase is an important Ang II forming mechanism within the cardiomyocytes. These findings have important clinical implications because intracellular chymase-mediated Ang II formation is unaffected by AT₁ receptor antagonists, as these drugs act on the cell surface [49;62;64;71;72]. The mechanism by which chymase becomes a primary enzymatic pathway for Ang-(1-12) metabolism remains under investigation. Current studies show that mast cells are a predominant source for chymase [73–75]. Mast cell chymase is increased in the dilated left atrium of patients with mitral regurgitation with evidence for an intracellular presence of chymase in the atrial myocytes (Figure 5). This was associated with the detection of Ang-(1-12) in myocytes from the left atrial of these patients (Figure 6). In addition, recent work showed that ir-Ang-(1-12) expression is significantly higher in human left atria appendage (35.37 ± 6.24 units) compared to the expression of the

peptide in the right atrium (19.38 ± 2.38 units, $p=0.031$). The increased Ang-(1-12) expression was associated with high left atrium chymase activity [76]. A differential content or expression of endocrine peptides in the heart has been reported in several species [77;78]. In all species studied including humans, right atrial tissue concentrations of atrial natriuretic peptide are at least two-fold higher in the right compared to the left atrium [77]. On the other hand, the content of neuropeptide Y (NPY) is significantly higher in the left atria of the rat, rabbit, and guinea pig. Although NPY content was similar in the right and left atrial appendages of humans, much higher concentrations were present in the pulmonary veins [77]. Since NPY colocalizes with sympathetic nerve terminals, the higher expression of Ang-(1-12) in the human left atria suggest an interplay between Ang-(1-12) and NPY in the modulation of atrial contractility and the production of atrial arrhythmias. Further research will be required to answer these questions.

Studies exploring the role of Ang-(1-12) and its interaction with cardiac chymase in the regulation of cardiac function as a primary or alternate Ang II-forming substrate shed new and potentially exciting information as to mechanisms by which Ang II, formed through an intracrine pathway, contributes to cardiovascular pathology. The studies showing chymase and Ang-(1-12) in the left atria tissue obtained from subjects with chronic AF explains how this non-ACE dependent pathway for intracellular proarrhythmic Ang II actions will be non-responsive to therapies using ACE inhibitors or ARBs in humans [1]. The universally accepted cardiorenal protective effects of ACE are limited by the return of plasma and tissue Ang II levels to pretreatment levels [79], while the benefits derived from ARBs or even aliskiren are restricted by the inability of these agents to reach intracellular sites of Ang II action or prevent the activation of alternate non-renin pathways for Ang II synthesis. Indeed, characterization of the processing pathways mediating Ang-(1-12) metabolism clearly demonstrate both species-specific (humans versus rodents) and compartment selectivity (i.e., blood, organ, and cellular sites) while other studies from this laboratory excluded renin from having any catalytic activity on Ang-(1-12) [80;81]. The demonstration of Ang-(1-12) expression and chymase in atrial myocytes from patients with AF [68] is a critical finding as AF is the most common clinically significant cardiac arrhythmia, a potent risk factor for stroke, and it is associated with higher medical costs and increased risk of death [82;83].

In summary, the diversity of the biochemical pathways leading to the production of Ang II and Ang-(1-7) now extends to the existence of intermediate substrate peptides upstream from Ang I. The dodecapeptide Ang-(1-12) is a functional substrate for the production of Ang II as shown in metabolism studies [55;65–69;81;84;85] and physiological conditions [80;86–89]. A primacy of chymase as a cardiac Ang II-forming enzyme in humans should generate cautionary notes as to the direct applicability of characterized biochemical pathways for angiotensin peptides formation in rodents versus humans. While these issues have been eloquently demonstrated in numerous past studies, the characterization of a chymase/Ang-(1-12) axis, as an alternate or primary mechanism for Ang II production in tissues, should provide impetus to furthering the understanding of the differential regulatory pathways by which Ang II exerts trophic and profibrotic effects on the heart through intracellular mechanisms that are renin-, and to a certain extent, ACE-independent. While renin has been excluded from generating Ang II from Ang-(1-12), the question of what enzymatic pathway accounts for the formation of Ang-(1-12) from Aogen remains

unanswered. In pursuit of this objective, our recent studies have identified kallikrein or a kallikrein related enzyme as capable of forming Ang-(1-12) from a synthetic form of Aogen containing the first 20 amino acids of the molecule [90].

While this review has focused on the biochemical physiology of Ang II production from Ang-(1-12) in the heart, accumulating evidence shows that Ang-(1-12) is also a functional important substrate for Ang II actions in the kidney [54;66] as well as the brain [86–89;91;92]. The later studies show an important effect of Ang-(1-12) as an Ang II forming substrate in neuronal hypothalamic and brain stem circuits regulating the central control of arterial pressure and baroreceptor reflexes.

Clinical Perspective

Mechanisms of Ang II production vary significantly between tissues and blood, and new data show that these differences may be species-specific.

In human subjects, cardiac Ang II synthesis originates from the hydrolytic activity of chymase, which released from mast cells acts on an angiotensinogen-derived extended form of Ang I [Ang-(1-12)] to generate Ang II directly.

Characterization of this pathway in human left atrial appendage and in subjects with resistant atrial fibrillation implicates Ang-(1-12) as the intracrine substrate for the pro-arrhythmogenic actions of Ang II. These studies demonstrate a need to develop new treatment strategies, based on either selective inhibition of cardiac chymase activity or suppression of intracellular Ang II production as a most effective approach to reversal of adverse cardiac remodeling or arrhythmias.

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References

1. Ferrario CM, Ahmad S, Joyner J, Varagic J. Advances in the renin angiotensin system focus on angiotensin-converting enzyme 2 and angiotensin-(1-7). *Adv Pharmacol.* 2010; 59:197–233. [PubMed: 20933203]
2. Donoghue M, Hsieh F, Baronas E, Godbout K, Gosselin M, Stagliano N, Donovan M, Woolf B, Robison K, Jeyaseelan R, Breitbart RE, Acton S. A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. *Circ Res.* 2000; 87:E1–E9. [PubMed: 10969042]
3. Rice GI, Thomas DA, Grant PJ, Turner AJ, Hooper NM. Evaluation of angiotensin-converting enzyme (ACE), its homologue ACE2 and neprilysin in angiotensin peptide metabolism. *Biochem J.* 2004; 383:45–51. [PubMed: 15283675]
4. Turner AJ, Tipnis SR, Guy JL, Rice G, Hooper NM. ACEH/ACE2 is a novel mammalian metallocarboxypeptidase and a homologue of angiotensin-converting enzyme insensitive to ACE inhibitors. *Can J Physiol Pharmacol.* 2002; 80:346–353. [PubMed: 12025971]
5. Santos RA, Simoes e Silva AC, Maric C, Silva DM, Machado RP, de BI, Heringer-Walther S, Pinheiro SV, Lopes MT, Bader M, Mendes EP, Lemos VS, Campagnole-Santos MJ, Schultheiss

- HP, Speth R, Walther T. Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas. *Proc Natl Acad Sci U S A*. 2003; 100:8258–8263. [PubMed: 12829792]
6. Schiavone MT, Santos RA, Brosnihan KB, Khosla MC, Ferrario CM. Release of vasopressin from the rat hypothalamo-neurohypophyseal system by angiotensin-(1-7) heptapeptide. *Proc Natl Acad Sci U S A*. 1988; 85:4095–4098. [PubMed: 3375255]
 7. Bumpus FM. Mechanisms and sites of action of newer angiotensin agonists and antagonists in terms of activity and receptor. *Fed Proc*. 1977; 36:2128–2132. [PubMed: 194800]
 8. Douglas JG, Khosla MC, Bumpus FM. Efficacy of octa- and heptapeptide antagonists of angiotensin II as inhibitors of angiotensin III binding in the rat adrenal glomerulosa. *Endocrinology*. 1985; 116:1598–1602. [PubMed: 3971929]
 9. Nussberger J, Matsueda GR, Re R, Haber E. Selectivity of angiotensin II antisera. *J Immunol Methods*. 1983; 56:85–96. [PubMed: 6827092]
 10. Tonnaer JA, Engels GM, Wiegant VM, Burbach JP, De JW, De WD. Proteolytic conversion of angiotensins in rat brain tissue. *Eur J Biochem*. 1983; 131:415–421. [PubMed: 6299738]
 11. Gregg CM, Sladek CD. A compartmentalized, organ-cultured hypothalamo-neurohypophyseal system for the study of vasopressin release. *Neuroendocrinology*. 1984; 38:397–402. [PubMed: 6728121]
 12. Sladek CD, Johnson AK. Effect of anteroventral third ventricle lesions on vasopressin release by organ-cultured hypothalamo-neurohypophyseal explants. *Neuroendocrinology*. 1983; 37:78–84. [PubMed: 6888661]
 13. Barnes KL, Knowles WD, Ferrario CM. Angiotensin II and angiotensin (1-7) excite neurons in the canine medulla in vitro. *Brain Res Bull*. 1990; 24:275–280. [PubMed: 2322862]
 14. Campagnole-Santos MJ, Diz DI, Santos RA, Khosla MC, Brosnihan KB, Ferrario CM. Cardiovascular effects of angiotensin-(1-7) injected into the dorsal medulla of rats. *Am J Physiol*. 1989; 257:H324–H329. [PubMed: 2750946]
 15. Campagnole-Santos MJ, Diz DI, Ferrario CM. Actions of angiotensin peptides after partial denervation of the solitary tract nucleus. *Hypertension*. 1990; 15:I34–I39. [PubMed: 2298474]
 16. Trachte GJ, Meixner K, Ferrario CM, Khosla MC. Prostaglandin production in response to angiotensin-(1-7) in rabbit isolated vasa deferentia. *Prostaglandins*. 1990; 39:385–394. [PubMed: 2343143]
 17. Benter IF, Diz DI, Ferrario CM. Cardiovascular actions of angiotensin(1-7). *Peptides*. 1993; 14:679–684. [PubMed: 8234010]
 18. Ferrario CM, Brosnihan KB, Diz DI, Jaiswal N, Khosla MC, Milsted A, Tallant EA. Angiotensin-(1-7): a new hormone of the angiotensin system. *Hypertension*. 1991; 18:III126–III133. [PubMed: 1937675]
 19. Ferrario CM, Chappell MC, Tallant EA, Brosnihan KB, Diz DI. Counterregulatory actions of angiotensin-(1-7). *Hypertension*. 1997; 30:535–541. [PubMed: 9322978]
 20. Ferrario CM, Trask AJ, Jessup JA. Advances in biochemical and functional roles of angiotensin-converting enzyme 2 and angiotensin-(1-7) in regulation of cardiovascular function. *Am J Physiol Heart Circ Physiol*. 2005; 289:H2281–H2290. [PubMed: 16055515]
 21. Ferrario CM. New physiological concepts of the renin-angiotensin system from the investigation of precursors and products of angiotensin I metabolism. *Hypertension*. 2010; 55:445–452. [PubMed: 20026757]
 22. Gallagher PE, Tallant EA. Inhibition of human lung cancer cell growth by angiotensin-(1-7). *Carcinogenesis*. 2004; 25:2045–2052. [PubMed: 15284177]
 23. Gallagher PE, Cook K, Soto-Pantoja D, Menon J, Tallant EA. Angiotensin peptides and lung cancer. *Curr Cancer Drug Targets*. 2011; 11:394–404. [PubMed: 21395552]
 24. Santos RA, Ferreira AJ, Simoes e Silva AC. Recent advances in the angiotensin-converting enzyme 2-angiotensin(1-7)-Mas axis. *Exp Physiol*. 2008; 93:519–527. [PubMed: 18310257]
 25. Santos RA, Ferreira AJ, Verano-Braga T, Bader M. Angiotensin-converting enzyme 2, angiotensin-(1-7) and Mas: new players of the renin-angiotensin system. *J Endocrinol*. 2013; 216:R1–R17. [PubMed: 23092879]
 26. Turnbull F, Neal B, Ninomiya T, Algert C, Arima H, Barzi F, Bulpitt C, Chalmers J, Fagard R, Gleason A, Heritier S, Li N, Perkovic V, Woodward M, MacMahon S. Effects of different

- regimens to lower blood pressure on major cardiovascular events in older and younger adults: meta-analysis of randomised trials. *BMJ*. 2008; 336:1121–1123. [PubMed: 18480116]
27. Turnbull F, Neal B, Pfeffer M, Kostis J, Alpert C, Woodward M, Chalmers J, Zanchetti A, MacMahon S. Blood pressure-dependent and independent effects of agents that inhibit the renin-angiotensin system. *J Hypertens*. 2007; 25:951–958. [PubMed: 17414657]
 28. Scheen AJ, Krzesinski JM. ONTARGET: similar protection of telmisartan and ramipril and lack of benefit of combined therapy in patients at high risk for vascular events. *Rev Med Liege*. 2008; 63:213–219. [PubMed: 18575077]
 29. Yusuf S, Teo KK, Pogue J, Dyal L, Copland I, Schumacher H, Dagenais G, Sleight P, Anderson C. Telmisartan, ramipril, or both in patients at high risk for vascular events. *N Engl J Med*. 2008; 358:1547–1559. [PubMed: 18378520]
 30. Messerli FH, Bangalore S. ALTITUDE trial and dual RAS blockade: the alluring but soft science of the surrogate end point. *Am J Med*. 2013; 126:e1–e3. [PubMed: 23332648]
 31. Sever P. Hypotension and ischaemic stroke associated with aliskiren in the ALTITUDE trial: sensitisation of the Bezold-Jarisch reflex? *J Renin Angiotensin Aldosterone Syst*. 2013; 14:1–2. [PubMed: 23418282]
 32. Phillips CO, Kashani A, Ko DK, Francis G, Krumholz HM. Adverse effects of combination angiotensin II receptor blockers plus angiotensin-converting enzyme inhibitors for left ventricular dysfunction: a quantitative review of data from randomized clinical trials. *Arch Intern Med*. 2007; 167:1930–1936. [PubMed: 17923591]
 33. Baker KM, Kumar R. Intracellular angiotensin II induces cell proliferation independent of AT1 receptor. *Am J Physiol Cell Physiol*. 2006; 291:C995–1001. [PubMed: 16774988]
 34. Danser AH. Cardiac angiotensin II: does it have a function? *Am J Physiol Heart Circ Physiol*. 2010; 299:H1304–H1306. [PubMed: 20833955]
 35. Kumar R, Singh VP, Baker KM. The intracellular renin-angiotensin system: implications in cardiovascular remodeling. *Curr Opin Nephrol Hypertens*. 2008; 17:168–173. [PubMed: 18277150]
 36. De Mello WC. Cardiac intracrine renin angiotensin system. Part of genetic reprogramming? *Regul Pept*. 2006; 133:10–12. [PubMed: 16325280]
 37. De Mello WC. Intracellular and extracellular renin have opposite effects on the regulation of heart cell volume. Implications for myocardial ischaemia. *J Renin Angiotensin Aldosterone Syst*. 2008; 9:112–118. [PubMed: 18584588]
 38. De Mello WC. Cell swelling, impulse conduction, and cardiac arrhythmias in the failing heart. Opposite effects of angiotensin II and angiotensin (1-7) on cell volume regulation. *Mol Cell Biochem*. 2009; 330:211–217. [PubMed: 19484190]
 39. De Mello WC. Intracrine action of angiotensin II in the intact ventricle of the failing heart: angiotensin II changes cardiac excitability from within. *Mol Cell Biochem*. 2011; 358:309–315. [PubMed: 21744071]
 40. Baker KM, Chernin MI, Schreiber T, Sanghi S, Haiderzaidi S, Booz GW, Dostal DE, Kumar R. Evidence of a novel intracrine mechanism in angiotensin II-induced cardiac hypertrophy. *Regul Pept*. 2004; 120:5–13. [PubMed: 15177915]
 41. Singh VP, Le B, Bhat VB, Baker KM, Kumar R. High-glucose-induced regulation of intracellular ANG II synthesis and nuclear redistribution in cardiac myocytes. *Am J Physiol Heart Circ Physiol*. 2007; 293:H939–H948. [PubMed: 17483239]
 42. Ferrario CM, Jessup J, Chappell MC, Averill DB, Brosnihan KB, Tallant EA, Diz DI, Gallagher PE. Effect of angiotensin-converting enzyme inhibition and angiotensin II receptor blockers on cardiac angiotensin-converting enzyme 2. *Circulation*. 2005; 111:2605–2610. [PubMed: 15897343]
 43. Dell'italia LJ, Meng QC, Balcells E, Wei CC, Palmer R, Hageman GR, Durand J, Hanks GH, Oparil S. Compartmentalization of angiotensin II generation in the dog heart. Evidence for independent mechanisms in intravascular and interstitial spaces. *J Clin Invest*. 1997; 100:253–258. [PubMed: 9218500]
 44. Dell'italia LJ, Ferrario CM. The Never-ending Story of Angiotensin Peptides: Beyond Angiotensin I and II. *Circ Res*. 2013; 112:1086–1087. [PubMed: 23580768]

45. Urata H, Ganten D. Cardiac angiotensin II formation: the angiotensin-I converting enzyme and human chymase. *Eur Heart J.* 1993; 14(Suppl I):177–182. [PubMed: 8293772]
46. Urata H, Hoffmann S, Ganten D. Tissue angiotensin II system in the human heart. *Eur Heart J.* 1994; 15(Suppl D):68–78. [PubMed: 7713117]
47. Urata H, Nishimura H, Ganten D. Mechanisms of angiotensin II formation in humans. *Eur Heart J.* 1995; 16(Suppl N):79–85. [PubMed: 8682066]
48. Kumar R, Singh VP, Baker KM. The intracellular renin-angiotensin system: a new paradigm. *Trends Endocrinol Metab.* 2007; 18:208–214. [PubMed: 17509892]
49. Kumar R, Singh VP, Baker KM. The intracellular renin-angiotensin system in the heart. *Curr Hypertens Rep.* 2009; 11:104–110. [PubMed: 19278599]
50. Kumar R, Boim MA. Diversity of pathways for intracellular angiotensin II synthesis. *Curr Opin Nephrol Hypertens.* 2009; 18:33–39. [PubMed: 19077687]
51. Kumar R, Thomas CM, Yong QC, Chen W, Baker KM. The intracrine renin-angiotensin system. *Clin Sci (Lond).* 2012; 123:273–284. [PubMed: 22590974]
52. Nagata S, Kato J, Sasaki K, Minamino N, Eto T, Kitamura K. Isolation and identification of proangiotensin-12, a possible component of the renin-angiotensin system. *Biochem Biophys Res Commun.* 2006; 350:1026–1031. [PubMed: 17045572]
53. Husain A, Bumpus FM, Smeby RR, Brosnihan KB, Khosla MC, Speth RC, Ferrario CM. Evidence for the existence of a family of biologically active angiotensin I-like peptides in the dog central nervous system. *Circ Res.* 1983; 52:460–464. [PubMed: 6339108]
54. Jessup JA, Trask AJ, Chappell MC, Nagata S, Kato J, Kitamura K, Ferrario CM. Localization of the novel angiotensin peptide, angiotensin-(1-12), in heart and kidney of hypertensive and normotensive rats. *Am J Physiol Heart Circ Physiol.* 2008; 294:H2614–H2618. [PubMed: 18408132]
55. Ahmad S, Varagic J, Westwood BM, Chappell MC, Ferrario CM. Uptake and metabolism of the novel peptide angiotensin-(1-12) by neonatal cardiac myocytes. *PLoS One.* 2011; 6:e15759. [PubMed: 21249217]
56. Komatsu Y, Kida N, Nozaki N, Kuwasako K, Nagata S, Kitamura K, Kato J. Effects of proangiotensin-12 infused continuously over 14 days in conscious rats. *Eur J Pharmacol.* 2012; 683:186–189. [PubMed: 22414812]
57. Moniwa N, Varagic J, Simington SW, Ahmad S, Nagata S, Von cannon JL, Ferrario CM. Primacy of Angiotensin Converting Enzyme in Angiotensin-(1-12) Metabolism. *Am J Physiol Heart Circ Physiol.* 2013 (in press).
58. Hirakata H, Fouad-Tarazi FM, Bumpus FM, Khosla M, Healy B, Husain A, Urata H, Kumagai H. Angiotensins and the failing heart. Enhanced positive inotropic response to angiotensin I in cardiomyopathic hamster heart in the presence of captopril. *Circ Res.* 1990; 66:891–899. [PubMed: 2180588]
59. Urata H, Kinoshita A, Misono KS, Bumpus FM, Husain A. Identification of a highly specific chymase as the major angiotensin II-forming enzyme in the human heart. *J Biol Chem.* 1990; 265:22348–22357. [PubMed: 2266130]
60. Urata H, Kinoshita A, Perez DM, Misono KS, Bumpus FM, Graham RM, Husain A. Cloning of the gene and cDNA for human heart chymase. *J Biol Chem.* 1991; 266:17173–17179. [PubMed: 1894611]
61. Arakawa K, Urata H. Hypothesis regarding the pathophysiological role of alternative pathways of angiotensin II formation in atherosclerosis. *Hypertension.* 2000; 36:638–641. [PubMed: 11040250]
62. Dell'italia LJ, Husain A. Dissecting the role of chymase in angiotensin II formation and heart and blood vessel diseases. *Curr Opin Cardiol.* 2002; 17:374–379. [PubMed: 12151872]
63. Takai S, Shiota N, Kobayashi S, Matsumura E, Miyazaki M. Induction of chymase that forms angiotensin II in the monkey atherosclerotic aorta. *FEBS Lett.* 1997; 412:86–90. [PubMed: 9257695]
64. Urata H. Pathological involvement of chymase-dependent angiotensin II formation in the development of cardiovascular disease. *J Renin Angiotensin Aldosterone Syst.* 2000; 1:S35–S37. [PubMed: 17199219]

65. Prosser HC, Richards AM, Forster ME, Pemberton CJ. Regional vascular response to ProAngiotensin-12 (PA12) through the rat arterial system. *Peptides*. 2010; 31:1540–1545. [PubMed: 20493224]
66. Westwood BM, Chappell MC. Divergent pathways for the angiotensin-(1-12) metabolism in the rat circulation and kidney. *Peptides*. 2012; 35:190–195. [PubMed: 22490446]
67. Prosser HC, Forster ME, Richards AM, Pemberton CJ. Cardiac chymase converts rat proAngiotensin-12 (PA12) to angiotensin II: effects of PA12 upon cardiac haemodynamics. *Cardiovasc Res*. 2009; 82:40–50. [PubMed: 19147651]
68. Ahmad S, Simmons T, Varagic J, Moniwa N, Chappell MC, Ferrario CM. Chymase-dependent generation of angiotensin II from angiotensin-(1-12) in human atrial tissue. *PLoS One*. 2011; 6:e28501. [PubMed: 22180785]
69. Ahmad S, Wei CC, Tallaj J, Dell'italia LJ, Moniwa N, Varagic J, Ferrario CM. Chymase mediates angiotensin-(1-12) metabolism in normal human hearts. *J Am Soc Hypertens*. 2013; 7:128–136. [PubMed: 23312967]
70. Raymond WW, Ruggles SW, Craik CS, Caughey GH. Albumin is a substrate of human chymase. Prediction by combinatorial peptide screening and development of a selective inhibitor based on the albumin cleavage site. *J Biol Chem*. 2003; 278:34517–34524. [PubMed: 12815038]
71. De Mello WC. Intracellular angiotensin II increases the total potassium current and the resting potential of arterial myocytes from vascular resistance vessels of the rat. *Physiological and pathological implications*. *J Am Soc Hypertens*. 2013 (in press).
72. Wei CC, Hase N, Inoue Y, Bradley EW, Yahiro E, Li M, Naqvi N, Powell PC, Shi K, Takahashi Y, Saku K, Urata H, Dell'italia LJ, Husain A. Mast cell chymase limits the cardiac efficacy of Ang I-converting enzyme inhibitor therapy in rodents. *J Clin Invest*. 2010; 120:1229–1239. [PubMed: 20335663]
73. Caughey GH, Raymond WW, Vanderslice P. Dog mast cell chymase: molecular cloning and characterization. *Biochemistry*. 1990; 29:5166–5171. [PubMed: 2378872]
74. Caughey GH, Raymond WW, Wolters PJ. Angiotensin II generation by mast cell alpha- and beta-chymases. *Biochim Biophys Acta*. 2000; 1480:245–257. [PubMed: 10899625]
75. Caughey GH. Mast cell tryptases and chymases in inflammation and host defense. *Immunol Rev*. 2007; 217:141–154. [PubMed: 17498057]
76. Nagata S, Varagic J, Simington SW, Ahmad S, VonCannon J, Wang H, Groban L, Kon N, Dell Italia LJ, Ferrario CM. Differential Expression Of Angiotensin-(1-12)/chymase In Human Atrial Tissue. *Hypertension*. 2013 (in press).
77. Onuoha GN, Nicholls DP, Alpar EK, Ritchie A, Shaw C, Buchanan K. Regulatory peptides in the heart and major vessels of man and mammals. *Neuropeptides*. 1999; 33:165–172. [PubMed: 10657487]
78. Onuoha GN, Nugent AM, Hunter SJ, Alpar EK, McEneaney DJ, Campbell NP, Shaw C, Buchanan KD, Nicholls DP. Neuropeptide variability in man. *Eur J Clin Invest*. 2000; 30:570–577. [PubMed: 10886296]
79. Lorenz JN. Chymase: the other ACE? *Am J Physiol Renal Physiol*. 2010; 298:F35–F36. [PubMed: 19923404]
80. Ferrario CM, Varagic J, Habibi J, Nagata S, Kato J, Chappell MC, Trask AJ, Kitamura K, Whaley-Connell A, Sowers JR. Differential regulation of angiotensin-(1-12) in plasma and cardiac tissue in response to bilateral nephrectomy. *Am J Physiol Heart Circ Physiol*. 2009; 296:H1184–H1192. [PubMed: 19218503]
81. Trask AJ, Jessup JA, Chappell MC, Ferrario CM. Angiotensin-(1-12) is an alternate substrate for angiotensin peptide production in the heart. *Am J Physiol Heart Circ Physiol*. 2008; 294:H2242–H2247. [PubMed: 18359898]
82. Maggioni AP, Latini R, Carson PE, Singh SN, Barlera S, Glazer R, Masson S, Cere E, Tognoni G, Cohn JN. Valsartan reduces the incidence of atrial fibrillation in patients with heart failure: results from the Valsartan Heart Failure Trial (Val-HeFT). *Am Heart J*. 2005; 149:548–557. [PubMed: 15864246]

83. Van den Berg MP, Crijns HJ, Van Veldhuisen DJ, Griep N, de Kam PJ, Lie KI. Effects of lisinopril in patients with heart failure and chronic atrial fibrillation. *J Card Fail.* 1995; 1:355–363. [PubMed: 12836710]
84. Moniwa N, Varagic J, Nagata S, Ahmad S, Simington SW, VonCannon J, Hasegawa K, Takizawa H, Ra N, Uruhashi M, Pshida H, Himamoto K, Iura T, Ferrario CM. Renin Independent Ang-(1-12) Production in both the Systemic and Coronary Circulation. *Hypertension.* 2013; 60
85. Nagata S, Kato J, Kuwasako K, Asami M, Kitamura K. Plasma and tissue concentrations of proangiotensin-12 in rats treated with inhibitors of the renin-angiotensin system. *Hypertens Res.* 2012; 35:234–238. [PubMed: 21993212]
86. Arnold AC, Isa K, Shaltout HA, Nautiyal M, Ferrario CM, Chappell MC, Diz DI. Angiotensin-(1-12) requires angiotensin converting enzyme and AT1 receptors for cardiovascular actions within the solitary tract nucleus. *Am J Physiol Heart Circ Physiol.* 2010; 299:H763–H771. [PubMed: 20562338]
87. Chitravanshi VC, Sapru HN. Cardiovascular responses elicited by a new endogenous angiotensin in the nucleus tractus solitarius of the rat. *Am J Physiol Heart Circ Physiol.* 2011; 300:H230–H240. [PubMed: 21076017]
88. Chitravanshi VC, Proddutur A, Sapru HN. Cardiovascular actions of angiotensin-(1-12) in the hypothalamic paraventricular nucleus of the rat are mediated via angiotensin II. *Exp Physiol.* 2012; 97:1001–1017. [PubMed: 22125313]
89. Isa K, Garcia-Espinosa MA, Arnold AC, Pirro NT, Tommasi EN, Ganten D, Chappell MC, Ferrario CM, Diz DI. Chronic immunoneutralization of brain angiotensin-(1-12) lowers blood pressure in transgenic (mRen2)27 hypertensive rats. *Am J Physiol Regul Integr Comp Physiol.* 2009; 297:R111–R115. [PubMed: 19403863]
90. Simington SW, Moniwa N, Ahmad S, VonCannon J, Dell'italia LJ, Varagic J, Ferrario CM. Renin Does not Participate in the Production of Plasma Ang-(1-12) from Angiotensinogen. *Hypertension.* 2013; 60:A628.
91. Arakawa H, Chitravanshi VC, Sapru HN. The hypothalamic arcuate nucleus: a new site of cardiovascular action of angiotensin-(1-12) and angiotensin II. *Am J Physiol Heart Circ Physiol.* 2011; 300:H951–H960. [PubMed: 21186269]
92. Arakawa H, Kawabe K, Sapru HN. Angiotensin-(1-12) in the rostral ventrolateral medullary pressor area of the rat elicits sympathoexcitatory responses. *Exp Physiol.* 2013; 98:94–108. [PubMed: 22707504]
93. Wei CC, Lucchesi PA, Tallaj J, Bradley WE, Powell PC, Dell'italia LJ. Cardiac interstitial bradykinin and mast cells modulate pattern of LV remodeling in volume overload in rats. *Am J Physiol Heart Circ Physiol.* 2003; 285:H784–H792. [PubMed: 12663259]
94. Wei CC, Chen Y, Powell LC, Zheng J, Shi K, Bradley WE, Powell PC, Ahmad S, Ferrario CM, Dell'italia LJ. Cardiac kallikrein-kinin system is upregulated in chronic volume overload and mediates an inflammatory induced collagen loss. *PLoS One.* 2012; 7:e40110. [PubMed: 22768235]
95. Welches WR, Brosnihan KB, Ferrario CM. A comparison of the properties and enzymatic activities of three angiotensin processing enzymes: angiotensin converting enzyme, prolyl endopeptidase and neutral endopeptidase 24.11. *Life Sci.* 1993; 52:1461–1480. [PubMed: 8387132]

Figure 1.

Correlative changes in plasma (top panel) and left ventricular (bottom panel) content of angiotensin II (Ang II) at the completion of a 14-day therapy with either vehicle, lisinopril, losartan or both drugs combined shows significant divergency of the effects of the therapies on plasma versus cardiac Ang II levels. Data are means $\pm$ SEM in Wistar rats. *P < 0.05 vs. vehicle; #P < 0.05 vs. lisinopril; ♦P < 0.05 vs losartan. Adapted from our reference 34.

Figure 2. Metabolism of ^{125}I -Ang-(1-12) (1 nmol/L) expressed as percent of the peptides remaining in the cultured medium of neonatal cardiac myocytes following 60 min incubation at 37°C in Wistar Kyoto and Spontaneously Hypertensive Rats. RAS cocktail includes lisinopril, a neprilysin inhibitor (SCH39370), an ACE2 inhibitor (MLN-4760), and a chymase inhibitor (chymostatin) all added at a concentration of 10 μM . Data from our reference 2.

Figure 3.

Processing of ^{125}I -Ang-(1-12) by plasma membranes isolated from the human left atrial appendage of subjects undergoing heart surgery for the treatment of resistant atrial fibrillation shows a primary role of chymase as the Ang-(1-12) processing enzyme. Data drawn from Table 1 of our reference 1.

Figure 4.

Schematic diagram of biotransformation pathways for Ang-(1-12) in the human circulation and cardiac myocytes. The sequence of Ang-(1-12) is that of the human form which differs from that expressed in rodents in terms of the substitution of valine (Val) for leucine (Leu) in position 11 and the presence of histidine (His) instead of tyrosine (Tyr) in position 12. Chymase either produced in cardiac myocytes or released by mast cells during ischemia reperfusion/injury (I/R) or increased oxidative stress generates Ang II intracellularly from Ang-(1-12) [93;94]. ACE, angiotensin converting enzyme; NEPs, angiotensin-(1-7) forming enzymes (prolyl endopeptidase 24.26 and neprilysin) [95].

Figure 5.

Representative example of immunohistochemistry of left atria (LA) from a subject with mitral regurgitation. From left to right, the LA demonstrates infiltration of mast cells with chymase (red) in various stages of degranulation. A) intact mast cell, B) mast cell release of chymase into interstitium, C) mast cell chymase located within atrial myocyte as well as in the interstitium in magnified inset with degranulating mast cell in lower right corner. Green: desmin, Blue: 4',6-diamidino-2-phenylindole (DAPI).

Figure 6.

Immunohistochemistry fluorescent staining for desmin and Ang-(1-12) in normal and mitral regurgitation (MR) left atrium (LA). MR LA has a loss of desmin (green) in the intercalated disc (white arrow) along with an increase in Ang-(1-12) (red) compared to normal LA. DAPI for nucleus: blue