Published in final edited form as:

Curr Hypertens Rep. 2014 May ; 16(5): 429. doi:10.1007/s11906-014-0429-9.

Angiotensin-(1–12): A Chymase-Mediated Cellular Angiotensin II Substrate

Sarfaraz Ahmad, Ph.D.¹, Jasmina Varagic, MD, Ph.D.^{1,2,3}, Leanne Groban, MD^{2,4}, Louis J Dell'Italia, MD⁷, Sayaka Nagata, Ph.D.¹, Neal D. Kon, MD⁵, and Carlos M. Ferrario, MD^{1,3,6,*}

¹Division of Surgical Sciences, Winston Salem, NC

²Hypertension and Vascular Research Center, Winston Salem, NC

³Department of Physiology/Pharmacology, Winston Salem, NC

⁴Department of Anesthesiology, Winston Salem, NC

⁵Cardiothoracic Surgery, Winston Salem, NC

⁶Department of Internal Medicine/Nephrology Wake Forest School of Medicine, Winston Salem, NC

⁷Division of Cardiovascular Disease, Department of Medicine, University of Alabama Medical Center, Birmingham, AL

Abstract

The classical view of biochemical pathways for the formation of biologically active angiotensins continues to undergo significant revision as new data uncovers the existence of important species differences between humans and rodents. The discovery of two novel substrates that, cleaved from angiotensinogen, can lead to direct tissue angiotensin II formation has the potential of radically altering our understanding of how tissues source angiotensin II production and explain the relative lack of efficacy that characterizes the use of angiotensin converting enzyme inhibitors in cardiovascular disease. This review addresses the discovery of angiotensin-(1–12) as an endogenous substrate for the production of biologically active angiotensin peptides by a non-renin dependent mechanism and the revealing role of cardiac chymase as the angiotensin II convertase in the human heart. This new information provides a renewed argument for exploring the role of chymase inhibitors in the correction of cardiac arrhythmias and left ventricular systolic and diastolic dysfunction.

Keywords

Angiotensin-(1–12); Angiotensin II; Angiotensin-(1–7); Cardiac chymase; Angiotensin-converting enzyme; Metabolism; Renin-angiotensin system; Angiotensinogen

*Corresponding Author: Carlos M Ferrario, MD Division of Surgical Sciences Wake Forest School of Medicine Medical Center Blvd. Winston-Salem, NC 27157 Phone: 336-716-3512 Fax: 336-716-6644 cferrari@wakehealth.edu.

Compliance with Ethics Guidelines

Conflict of Interest Sarfaraz Ahmad, Jasmina Varagic, and Sayaka Nagata declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

Introduction

The renin-angiotensin system (RAS) is a major physiological regulatory hormonal system of the basic mechanisms that determine tissue perfusion pressure, body fluid volumes, electrolyte balances, and cardiovascular homeostasis [1–3]. The classic biochemical pathways leading to the generation of biologically active angiotensins has been extensively described in multiple publications. As an endocrine hormonal system, angiotensin II (Ang II) formation in the circulation results from the linear processing of the substrate angiotensinogen (Aogen) produced by the liver, by the renal enzyme renin. The angiotensin I (Ang I) generated product is subsequently converted into Ang II, primarily by angiotensin-converting enzyme (ACE). In the later part of the 1980s, Ferrario's laboratory challenged this universally accepted bio-transformative process with the characterization of angiotensin-(1–7) [Ang-(1–7)] biological actions [4]. The functions of Ang-(1–7) as an endogenous inhibitor of the vasoconstrictor, neurogenic, trophic, prothrombotic, and profibrotic actions of Ang II are extensively reviewed elsewhere [3, 5–9].

The further demonstration that cells have the intrinsic ability to express the genes accounting for Ang II production greatly expanded knowledge of the importance of the system in modulating cellular functions in both health and disease. As reviewed by Paul [10], the expression of tissue-angiotensin peptides' generation led to a significant expansion of knowledge regarding their function as paracrine/intracrine/autocrine regulators of physiological functions and their role in human diseases. The expanded tissue RAS vocabulary now includes the characterization of ACE2 as an enzyme cleaving Ang I to form Ang-(1–9) and Ang-(1–7) from Ang II [11, 12], the opposing actions of the AT₂-receptor on Ang II AT₁ mediated actions [13, 14*, 15], and more recently, the detection of a new Ang-(1–7)-derivative that couples to a Mas-related type D (MrgD) receptor [16*, 17]. A new and intriguing observation is the demonstration that Ang-(1–9) may have biological actions comparable to those associated with Ang-(1–7) [18–20].

While knowledge of non-renin dependent alternate enzymatic mechanisms for Ang I formation, as well as non-ACE pathways for Ang II from Ang I, are described in the literature, scant attention has been paid to their significance. The overwhelming assumption that the beneficial clinical results achieved with the use of inhibitors of ACE or prevention of Ang II binding through the use of AT₁ receptor antagonists has led to the opinion that these alternate mechanisms for Ang II production are not relevant. Although a rich literature shows benefits of blockade using direct renin inhibitors (DRI), ACE inhibitors or Ang II receptor blockers (ARBs), the overall results related to a delay or reversal of target organ damage or morbid events has fallen short of expectations. This issue has come of age from the analysis of large clinical trials for hypertension, strokes, and heart failure [21], as well as atrial fibrillation (AF) [22]. Turnbull et al.'s [21] report, based on the analysis of 31 trials 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.” These findings were further underscored by a more focused analysis of potential differences in cardiovascular outcomes between ACE inhibitors and ARBs. In this meta-regression analysis of data from 26 large-scale trials, the investigators found no

evidence of any blood pressure-independent effects of either ACE inhibition or Ang II receptor blockade [23]. The potential for these treatment approaches to account for incomplete blockade of Ang II actions or synthesis is not an explanation for these outcomes, because combination of ARB and ACE inhibitors showed no further benefits in the large ONgoing Telmisartan Alone and in combination with Ramipril Global Endpoint Trial, in which ramipril was combined with telmisartan [24, 25], the Altitude trial which combined aliskiren with valsartan [26, 27], or in a systematic large meta-analysis of studies of patients with symptomatic left ventricular dysfunction [28]. While we are not denying the proven benefits of ACE inhibitors and ARBs in the reduction of target-organ damage and the occurrence of clinical events, the benefit appears to be primarily the results of their antihypertensive effects (i.e., magnitude of blood pressure lowering), rather than the additional benefit that could be gained from blockade of tissue Ang II. These findings suggest that the Ang II pathological actions may occur at sites that are not effectively reached by the current RAS blockers, or that for the case of renin and ACE, these enzymes are not critical in the formation of Ang II in humans. This review will bring these questions to the forefront by asking:

- 1) Is chymase in humans more important than ACE in Ang II production?
- 2) Is renin the only enzyme initiating Aogen biotransformation toward angiotensin peptides generation?

The relevance of these questions has been brought to bear by the recent discoveries of alternate substrates for Ang II production, BigAng25 [Ang-(1–25)] [29**] and Ang-(1–12) [30]. The intermediate precursor—named angiotensin-(1–12) [Ang-(1–12)]—contains the human sequence of Ang I and Ang II within the 12 amino acid chain of the molecule (Asp¹-Arg²-Val³-Tyr⁴-Ile⁵-His⁶-Pro⁷-Phe⁸-His⁹-Leu¹⁰-Val¹¹-Ile¹²), while Ang-(1–25) extends the C-terminus of Ang-(1–12) by the presence of an additional 13 amino acids.

Ang-(1–12) Actions and Metabolism

In 2006, Nagata and colleagues [30] identified Ang-(1–12) in the plasma and several tissues of a strain of Japanese Wistar rats. The amino acid sequence of this novel rat peptide is similar to the sequence of Ang I plus –Leu¹¹-Tyr¹²– positions of the C-terminus. It is our opinion that the differences in the amino acid composition at positions 11 and 12 between rodents (Leu¹¹-Tyr¹²) and humans (Val¹¹-Ile¹²) should not be overlooked, as it may be important in terms of the bio-enzymatic processes by which this alternate substrate is degraded into Ang II. Research from our laboratory, and that of our collaborators at the Miyazaki University in Japan, suggest that Ang-(1–12) is a source for tissue Ang II formation. In the pursuit of this hypothesis, we were rewarded by finding that Ang-(1–12) expression and content was selectively augmented in the left ventricle of SHR compared to WKY, while no differences in Ang-(1–12) expression were apparent in the kidneys of both SHR and WKY [31]. Several studies further demonstrated that Ang-(1–12) serves as a functional substrate for Ang II formation, as the peptide showed vasoconstrictor actions in isolated rat aortic strips and the potent pressor responses produced by systemic Ang-(1–12) administration were blocked by either captopril or candesartan [30]. Similar conclusions were obtained when administration of either losartan or the ACE inhibitor perindopril

abolished the pressor, aldosterone, and cardiac hypertrophic actions of a 14-day Ang-(1–12) systemic infusion [32*]. To address whether Ang-(1–12) functions as an endogenous source of Ang II production, Isa et al. [33] administered antibodies directed against the C-terminal end of the rat's Ang-(1–12) molecule into a lateral cerebral ventricle of (mRen2)27 transgenic hypertensive rats. Endogenous neutralization of Ang-(1–12) elicited a prompt blood pressure reduction that was associated with a transient anti-dipsogenic behavior [33]. More selective assessments of Ang-(1–12)'s physiological role were done through microinjections of the dodecapeptide in the nucleus of the tractus solitarius (nTS) [34, 35], the paraventricular hypothalamic nuclei [36], the hypothalamic arcuate nuclei [37] or the rostral ventrolateral medulla (RVLM) [38*]. In these conditions, the dodecapeptide caused blood pressure responses consistent with modulation of baroreflexes or sympathoexcitation. Additional studies of Ang-(1–12) actions in the heart and vasculature supported its role as a functional source for tissue Ang II production, as the peptide constricted coronary arteries with no significant effect on left-ventricular contractility and also impaired recovery from global ischemia [39]. In the periphery, Prosser et al. [40] showed Ang-(1–12) vasoconstrictor activity in the isolated descending thoracic aorta, right and left common carotid arteries, abdominal aorta and superior mesenteric artery. Altogether, these studies demonstrated that Ang-(1–12) serves as a pathway for the generation of Ang peptides, a biotransformation mechanism that may be of relevance in situations of suppressed renin activity, as well as functioning as an intracellular precursor for the generation of biologically active angiotensin peptides.

While early studies seem to indicate that Ang-(1–12) conversion into Ang II was mediated by ACE, other studies implicated chymase as contributing to its metabolism [34, 39, 40]. This dual processing may be related to the site for Ang-(1–12) metabolism, with ACE having a significant role in the systemic circulation [41] and chymase a more prominent role in the heart [39, 42*]. The divergency of Ang-(1–12) metabolic pathways might be highly tissue specific, as a report suggested that neprilysin could also function as Ang-(1–12) convertase in the kidney [43]. Neprilysin is a major metalloproteinase member of the M13 family of proteases that also includes endothelin-converting enzyme (ECE) [44]. Since neprilysin activity in the kidney is very high compared to renal ACE activity [45*], the data suggesting that neprilysin converts Ang-(1–12) into Ang I will need to be verified in studies in which more selective inhibitors of neprilysin are utilized. On the other hand, divergency in metabolic pathways is not surprising, since past work showed the existence of different enzymatic routes for the generation of angiotensin peptides, different receptors distribution patterns in different tissues, and alternate mechanisms influencing receptor regulation in cardiovascular functions and remodeling [10, 14*]. These characteristics support the dual role of the RAS as both a circulating hormonal and a tissue-specific system, wherein the generation of angiotensin substrates serves not only autocrine/paracrine, but also intracrine functions [14*, 46, 47, 48*].

The discovery of Ang-(1–12) as an Ang peptides forming substrate has generated new directions in exploring the role of non-renin pathways for Ang II production. Studies suggest that Ang II is produced both extracellularly and intracellularly to exert tropic and profibrotic actions in the heart [49–51]. Ang II actions in the heart would depend upon the site at which the peptide is generated, the proximity of the enzyme and substrate needed for the

production of the peptide, as well as the turnover of these components [52]. One additional consideration is whether or not the processes occur outside or inside the cardiac myocytes. This is a critical factor, as ACE inhibitors or ARBs do not reach the intracellular compartment at which Ang II acts [49, 53, 54•, 55, 56]. In keeping with these findings, we showed that administration of lisinopril, losartan or both drugs combined did not alter myocardial Ang II content while having the expected effects on plasma Ang II [57]. The studies showing the presence of Ang-(1–12) and chymase protein in cardiac human and rodent myocytes suggest the possibility that this is an intracellular mechanism for Ang II production that will not be interrupted by RAS blockade.

Species differences in the enzymatic mechanisms by which Ang-(1–12) is processed into Ang II are now strongly suggested by a series of comparative studies that explored this issue in heart tissue from normal and diseased subjects [5, 58•, 59•]. In these studies, we showed that chymase was the sole enzyme converting Ang-(1–12) into Ang I and Ang II. Of particular interest was the finding that Ang-(1–12) was directly converted to Ang II in plasma membranes obtained from left ventricle (LV) of normal subjects [59]. These data buttressed our concept that Ang II formation is compartmentalized, with chymase being the major Ang II forming mechanism in the cardiac interstitium while ACE acts as the major Ang II forming mechanism in the intravascular space.

Cardiac chymase is important in adverse LV remodeling post-myocardial infarction [60, 61], atherosclerosis [62, 63], type 2 diabetes [64], and heart failure [65–67]. Furthermore, increased LV mast cells and chymase were found in experimental models of pure volume overload induced by an aortocaval fistula in rats or isolated mitral regurgitation (MR) in dogs [68–71, 72•] and estrogen-sensitive diastolic dysfunction in ovariectomized mRen2.Lewis rats [73•, 74]. All mammals have the Ang II-forming α -chymase isoform [75, 76]. However, only the baboon, macaque, and dog have a single α -chymase; while the mouse, rat, pig, and rabbit have the additional β -chymase isoform that degrades Ang II [75, 77, 78]. There are numerous studies demonstrating the beneficial effects of chymase inhibitors that target the cleavage site on the human chymase, in species having the β -chymase isoform. We have shown such an effect in the mouse, where an orally active human chymase inhibitor decreases interstitial fluid Ang II formation in the C56B6 mouse [61]. Thus, species with the β -chymase isoform do have baseline net chymase-mediated Ang II formation that increases with cardiovascular stress. Nevertheless, much has been written about the suitability and clinical relevance of rodent species, in particular, for the study of chymase-mediated effects on cardiovascular remodeling because of the lack of the β -chymase isoform in the human and the specificity of the various human targeted chymase inhibitors across various mammalian species. These points are underscored by our studies, as chymase was shown to be the enzyme cleaving Ang-(1–12) into Ang II in humans [58, 59], while ACE primarily accounted for the Ang-(1–12) cleavage in WKY and SHR rats [42•].

It is important to stress that chymase activity is only detected in tissues and not in the circulation. It has been suggested that chymase released from activated mast cells is mostly trapped as a cage-like structure by an α 2-macroglobulin (α 2-M). This trapping may prevent its interactions with large protein substrates and inhibitors (like serpins) [79]. This interaction does not seem to interfere with the ability of chymase to access small peptides

such as Ang I, since it has been reported that α 2-M trapped chymase is able to convert Ang I into Ang II [79]. Therefore, the biochemical and signaling mechanisms forming the Ang II within the cellular microenvironment are accounted for alternate enzymatic pathways that are different than those in the circulation and unable to be blocked by either ACE inhibitors or ARBs. Our recent studies in neonatal rat myocytes [42•] and human atrial appendages [58] documented the presence of Ang-(1–12) and chymase in the diseased cardiocytes. We postulated that the intracellular formation of Ang II via this chymase pathway is independent of and unaffected by inhibition of Ang II production by ACE inhibitors or blockade of AT1 receptors [5]. Consequently, neither of these approaches will exert complete inhibition of Ang II cellular activities, as these agents do not penetrate beyond the cell membrane.

Synthesis and Cellular Ang-(1–12) Expression

Our previous studies showed high cardiac concentration of Ang-(1–12) in SHR as compared to normotensive WKY [31], increased Ang-(1–12) content in anephric normotensive rats [80] and cardiac formation of Ang I, Ang II and Ang-(1–7) through cleavage of Ang-(1–12) via a non-renin pathway in isolated hearts of three normotensive (Sprague Dawley, Lewis and WKY) and two hypertensive (congenic mRen2.Lewis and SHR) rat strains [81]. Increased concentrations of endogenously expressed Ang-(1–12) and chymase, as well as chymase activity, were also found in neonatal cultured cardiac myocytes of SHR compared to WKY [42•].

Existence of an intrinsic mechanism for the intracellular generation of Ang II in cardiac myocytes that is independent from uptake from the circulation or the interstitial environment has been demonstrated by others [55, 82–84], but no data exists as to whether intracrine Ang II formation follows the canonical enzymatic pathways and intermediate peptides described in the circulation, or whether it is generated by alternate non-canonical mechanism whereby Aogen is processed to intermediate substrates [i.e., Ang-(1–12)] independent of renin and ACE pathways. While both renin and Aogen are not highly expressed in heart tissue, cardiac content of Ang II has been reported to be > 100 time higher than in the plasma [75]. Whether these high concentrations are a reflection of local synthesis in the immediate interstitial microenvironment or intracellularly remains to be firmly established [52]. We have shown increased incorporation of intact Ang-(1–12) in cardiac myocytes from neonatal SHR [42•]. Ang-(1–12) internalization may be accounted for by receptor mediated endocytosis, as the amount internalized represented a small fraction of the total loaded Ang-(1–12) [42•].

In the characterization of the non-canonical pathways accounting for the intracrine or paracrine production of Ang II, there is a need to uncover what enzymes and intermediate substrates account for the generation of Ang-(1–12) from Aogen. This area is just beginning to be explored. Our past studies demonstrate that renin is not involved in Ang-(1–12) metabolism, but these findings do not exclude that renin may serve as an Ang-(1–12) forming enzyme. Past studies showed that several serine proteases, such as tonin and cathepsin G, hydrolyze Ang II precursors [85–88]. Of relevance to the current topic, Ang-(1–12) was shown to be generated from the synthetic tetradecapeptide Ang-(1–14) in rat aorta tissue [89]. Ang-(1–12) formation from Ang-(1–14) was almost 7-fold higher than the formation of Ang I by rat aorta tissue [89]. Work in progress from our laboratory suggests

that cardiac Ang-(1–12) may be cleaved from Aogen by a member of the kallikrein enzyme system [5, 90]. This observation is in keeping with the early demonstration that trypsin could generate Ang II directly from human plasma protein in the absence of converting enzyme [91, 92]. This may not be the sole pathway for tissue formation of bioactive angiotensin peptides, as Nagata and collaborators [29**] have recently reported the existence of another precursor named (Big-angiotensin-(1–25)). This new member (BAng-25) of the RAS isolated from human urine, consists of the first 25 amino acids of the Aogen molecule, and contains a glycosyl chain and added cysteine. BAng-25 is rapidly cleaved by chymase and was detected in a wide range of human organs and tissues (including left ventricle cardiocytes). This new finding raises the question as to whether BAng-25 may be an immediate precursor for Ang-(1–12) generation. Preliminary observations from our laboratory that the glycosylated form of BAng-25 is not expressed in rodents are in keeping with the overall hypothesis that the biochemical mechanisms for tissue Ang II formation in humans are distinctly different than those in rodents. That this point has not been sufficiently documented and understood by the scientific community is an important issue, as it has direct implications in terms of present and future approaches to treatment of cardiovascular disease.

Summary and Conclusions

Advances in the biochemical physiology of tissue RAS brought to the forefront the presence of alternate pathways for the generation and metabolism of angiotensin peptides. Adding to this knowledge, our new findings demonstrate the existence of additional alternate mechanisms for the formation of angiotensin peptides upstream from Ang I. The two questions posed in the Introduction can thus be appropriately answered. Chymase is the critical Ang II forming enzyme in humans, and renin is not the sole enzyme forming Ang II.

A highly productive effort to dissect the functional role of Ang-(1–12) as an alternate substrate for Ang II generation has led us to uncover highly specific tissue/cellular dependent mechanisms that through non-renin pathways mediate the intracellular actions of Ang II. The translational research in rodents and human cardiac tissue reveals species-specific regulatory substrates for Ang II formation. These findings may explain the relative failure of current approaches using ACE inhibitors and ARBs to suppress the pathological actions of Ang II in cardiovascular disease, as these drugs acting on the cell surface do not reach the intracellular sites at which biotransformation of the substrates [Ang-(1–12)] occurs.

Acknowledgments

Leanne Groban and Carlos M. Ferrario have declared that this work was supported by grants HL-051952 from the National Heart, Lung and Blood Institute and AG042758 (LG) and AG033727 (LG) from the National Institute on Aging of the NIH.

References

1. Ferrario CM, Chappell MC. Novel angiotensin peptides. *Cell Mol Life Sci.* 2004; 61(21):2720–2727. [PubMed: 15549173]

2. Ferrario CM. Role of angiotensin II in cardiovascular disease therapeutic implications of more than a century of research. *J Renin Angiotensin Aldosterone Syst.* 2006; 7(1):3–14. [PubMed: 17083068]
3. Ferrario CM. New physiological concepts of the renin-angiotensin system from the investigation of precursors and products of angiotensin I metabolism. *Hypertension.* 2010; 55(2):445–452. [PubMed: 20026757]
4. Schiavone MT, Santos RA, Brosnihan KB, et al. Release of vasopressin from the rat hypothalamo-neurohypophyseal system by angiotensin-(1–7) heptapeptide. *Proc Natl Acad Sci U S A.* 1988; 85(11):4095–4098. [PubMed: 3375255]
5. Ferrario CM, Ahmad S, Nagata S, et al. An evolving story of angiotensin-II-forming pathways in rodents and humans. *Clin Sci (Lond).* 2014; 126(7):461–469. [PubMed: 24329563]
6. 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(6):H2281–H2290. [PubMed: 16055515]
7. Ferrario CM. Angiotensin-converting enzyme 2 and angiotensin-(1–7): an evolving story in cardiovascular regulation. *Hypertension.* 2006; 47(3):515–521. [PubMed: 16365192]
8. Ferrario CM, Varagic J. The ANG-(1–7)/ACE2/mas axis in the regulation of nephron function. *Am J Physiol Renal Physiol.* 2010; 298(6):F1297–F1305. [PubMed: 20375118]
9. Santos RA, Ferreira AJ, Verano-Braga T, et al. Angiotensin-converting enzyme 2, angiotensin-(1–7) and Mas: new players of the renin-angiotensin system. *J Endocrinol.* 2013; 216(2):R1–R17. [PubMed: 23092879]
10. Paul M, Poyan MA, Kreutz R. Physiology of local renin-angiotensin systems. *Physiol Rev.* 2006; 86(3):747–803. [PubMed: 16816138]
11. Rice GI, Thomas DA, Grant PJ, et al. Evaluation of angiotensin-converting enzyme (ACE), its homologue ACE2 and neprilysin in angiotensin peptide metabolism. *Biochem J.* 2004; 383(Pt 1): 45–51. [PubMed: 15283675]
12. Rice GI, Jones AL, Grant PJ, et al. Circulating activities of angiotensin-converting enzyme, its homolog, angiotensin-converting enzyme 2, and neprilysin in a family study. *Hypertension.* 2006; 48(5):914–920. [PubMed: 17000927]
13. Carey RM. Cardiovascular and renal regulation by the angiotensin type 2 receptor: the AT2 receptor comes of age. *Hypertension.* 2005; 45(5):840–844. [PubMed: 15738342]
14. Carey RM. Newly discovered components and actions of the renin-angiotensin system. *Hypertension.* 2013; 62(5):818–822. [PubMed: 24082058] •This review article provides a timely perspective of the components and actions of the renin angiotensin system, including receptor mechanisms
15. Carey RM, Padia SH. Role of angiotensin AT(2) receptors in natriuresis: Intrarenal mechanisms and therapeutic potential. *Clin Exp Pharmacol Physiol.* 2013; 40(8):527–534. [PubMed: 23336117]
16. Lautner RQ, Villela DC, Fraga-Silva RA, et al. Discovery and characterization of alamandine: a novel component of the renin-angiotensin system. *Circ Res.* 2013; 112(8):1104–1111. [PubMed: 23446738] •Biological effects are demonstrated for an heptapeptide where the sequence of Ang-(1–7) is modified at the N-terminus by the substitution of aspartic by alanine in position 1. The heptapeptide has a preference for the Mas-related G-protein–coupled receptor, member D
17. Villela DC, Passos-Silva DG, Santos RA. Alamandine: a new member of the angiotensin family. *Curr Opin Nephrol Hypertens.* 2014; 23(2):130–134. [PubMed: 24389733]
18. Clarke C, Flores-Munoz M, McKinney CA, et al. Regulation of cardiovascular remodeling by the counter-regulatory axis of the renin-angiotensin system. *Future Cardiol.* 2013; 9(1):23–38. [PubMed: 23259473]
19. Flores-Munoz M, Godinho BM, Almalik A, et al. Adenoviral delivery of angiotensin-(1–7) or angiotensin-(1–9) inhibits cardiomyocyte hypertrophy via the mas or angiotensin type 2 receptor. *PLoS One.* 2012; 7(9):e45564. [PubMed: 23029101]
20. Ocaranza MP, Moya J, Barrientos V, et al. Angiotensin-(1–9) reverses experimental hypertension and cardiovascular damage by inhibition of the angiotensin converting enzyme/Ang II axis. *J Hypertens.* 2014

21. Turnbull F, Neal B, Ninomiya T, et al. 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(7653):1121–1123. [PubMed: 18480116]
22. Disertori M, Barlera S, Staszewsky L, et al. Systematic review and meta-analysis: renin-Angiotensin system inhibitors in the prevention of atrial fibrillation recurrences: an unfulfilled hope. *Cardiovasc Drugs Ther*. 2012; 26(1):47–54. [PubMed: 22009136]
23. Turnbull F, Neal B, Pfeffer M, et al. Blood pressure-dependent and independent effects of agents that inhibit the renin-angiotensin system. *J Hypertens*. 2007; 25(5):951–958. [PubMed: 17414657]
24. 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(4):213–219. [PubMed: 18575077]
25. Yusuf S, Teo KK, Pogue J, et al. Telmisartan, ramipril, or both in patients at high risk for vascular events. *N Engl J Med*. 2008; 358(15):1547–1559. [PubMed: 18378520]
26. 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(3):e1–e3. [PubMed: 23332648]
27. 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):1–2. [PubMed: 23418282]
28. Phillips CO, Kashani A, Ko DK, et al. 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(18):1930–1936. [PubMed: 17923591]
29. Nagata S, Hatakeyama K, Asami M, et al. Big angiotensin-25: A novel glycosylated angiotensin-related peptide isolated from human urine. *Biochem Biophys Res Commun*. 2013; 441(4):757–762. [PubMed: 24211583] ••By screening human urine gel filtration fractions for bioactive peptides using an antibody raised against the N-terminal portion of Ang II, these investigators discovered the existence of an extended form of Ang I consisting of the first 25 amino acids of the angiotensinogen molecule. Ang-(1–25) is expressed in most human tissues and it is cleaved into Ang II by chymase. The demonstration that Ang-(1–25) is N-glycosylated on its 14th amino acid (Asn) and has a cysteine linked to its 18th amino acid (Cys) is used as evidence that the peptide is expressed in humans but not rodents
30. Nagata S, Kato J, Sasaki K, et al. Isolation and identification of proangiotensin-12, a possible component of the renin-angiotensin system. *Biochem Biophys Res Commun*. 2006; 350(4):1026–1031. [PubMed: 17045572]
31. Jessup JA, Trask AJ, Chappell MC, et al. 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(6):H2614–H2618. [PubMed: 18408132]
32. Komatsu Y, Kida N, Nozaki N, et al. Effects of proangiotensin-12 infused continuously over 14 days in conscious rats. *Eur J Pharmacol*. 2012; 683(1–3):186–189. [PubMed: 22414812] •This well conducted study shows that Ang-(1–12) has long-term pressor activity, a finding that suggest that this extended form of Ang I is able to generate Ang II endogenously
33. Isa K, Garcia-Espinosa MA, Arnold AC, et al. 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(1):R111–R115. [PubMed: 19403863]
34. Arnold AC, Isa K, Shaltout HA, et al. 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(3):H763–H771. [PubMed: 20562338]
35. 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(1):H230–H240. [PubMed: 21076017]
36. 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(9):1001–1017. [PubMed: 22125313]

37. 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(3):H951–H960. [PubMed: 21186269]
38. 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(1):94–108. [PubMed: 22707504] •Extending the series of studies showing Ang-(1–12) biological effects in brain nuclei influencing the brain regulation of blood pressure, these investigators describe that Ang-(1–12) elicits pressor response associated with increased splanchnic nerve sympathetic discharges during microinjections of the peptide in this critical region of the rostral medulla oblongata. Adding weight to the dual role of ACE and chymase, the investigators showed that both enzymes are required for Ang-(1–12) metabolism into Ang II
39. Prosser HC, Forster ME, Richards AM, et al. Cardiac chymase converts rat proAngiotensin-12 (PA12) to angiotensin II: effects of PA12 upon cardiac haemodynamics. *Cardiovasc Res*. 2009; 82(1):40–50. [PubMed: 19147651]
40. Prosser HC, Richards AM, Forster ME, et al. Regional vascular response to ProAngiotensin-12 (PA12) through the rat arterial system. *Peptides*. 2010; 31(8):1540–1545. [PubMed: 20493224]
41. Moniwa N, Varagic J, Simington SW, et al. Primacy of angiotensin converting enzyme in angiotensin-(1–12) metabolism. *Am J Physiol Heart Circ Physiol*. 2013; 305(5):H644–H650. [PubMed: 23812388]
42. Ahmad S, Varagic J, Westwood BM, et al. Uptake and metabolism of the novel peptide angiotensin-(1–12) by neonatal cardiac myocytes. *PLoS One*. 2011; 6(1):e15759. [PubMed: 21249217] •This study represents the first publication that identifies Ang-(1–12) metabolic pathways in cardiac myocytes and shows increased Ang-(1–12) uptake in SHR compared with WKY
43. Westwood BM, Chappell MC. Divergent pathways for the angiotensin-(1–12) metabolism in the rat circulation and kidney. *Peptides*. 2012; 35(2):190–195. [PubMed: 22490446]
44. Ouimet T, Facchinetti P, Rose C, et al. Neprilysin II: A putative novel metalloprotease and its isoforms in CNS and testis. *Biochem Biophys Res Commun*. 2000; 271(3):565–570. [PubMed: 10814502]
45. Varagic J, Ahmad S, Vonnannon JL, et al. Predominance of AT(1) blockade over mas-mediated angiotensin-(1–7) mechanisms in the regulation of blood pressure and reninangiotensin system in mRen2.Lewis rats. *Am J Hypertens*. 2013; 26(5):583–590. [PubMed: 23459599] •While the focus of this study is on the potential contribution of the ACE2/Ang-(1–7)/mas-axis to the antihypertensive actions of the AT1-receptor antagonist, olmesartan, Table 2 documents that renal neprilysin activity is greater than 95-fold above those measured for renal ACE activity in a transgenic model of renin-dependent hypertension
46. Cook JL, Re RN. Lessons from in vitro studies and a related intracellular angiotensin II transgenic mouse model. *Am J Physiol Regul Integr Comp Physiol*. 2012; 302(5):R482–R493. [PubMed: 22170617]
47. Re RN. Tissue renin angiotensin systems. *Med Clin North Am*. 2004; 88(1):19–38. [PubMed: 14871049]
48. Re RN, Cook JL. Noncanonical intracrine action. *J Am Soc Hypertens*. 2011; 5(6):435–448. [PubMed: 21890449] •A strong position paper detailing the biological significance of intracrine mechanisms in health and disease from one of the major leaders in this field
49. Kumar R, Singh VP, Baker KM. The intracellular renin-angiotensin system: a new paradigm. *Trends Endocrinol Metab*. 2007; 18(5):208–214. [PubMed: 17509892]
50. Kumar R, Singh VP, Baker KM. The intracellular renin-angiotensin system: implications in cardiovascular remodeling. *Curr Opin Nephrol Hypertens*. 2008; 17(2):168–173. [PubMed: 18277150]
51. Kumar R, Singh VP, Baker KM. The intracellular renin-angiotensin system in the heart. *Curr Hypertens Rep*. 2009; 11(2):104–110. [PubMed: 19278599]
52. Dostal DE, Baker KM. The cardiac renin-angiotensin system: conceptual, or a regulator of cardiac function? *Circ Res*. 1999; 85(7):643–650. [PubMed: 10506489]

53. De Mello WC, Frohlich ED. On the local cardiac renin angiotensin system. Basic and clinical implications. *Peptides*. 2011; 32(8):1774–1779. [PubMed: 21729730]
54. De Mello WC. Mechanical stretch reduces the effect of angiotensin II on potassium current in cardiac ventricular cells of adult Sprague Dawley rats. On the role of AT1 receptors as mechanosensors. *J Am Soc Hypertens*. 2012; 6(6):369–374. [PubMed: 23063532] •This important paper reviews De Mello original studies showing that AT1 receptors functioning as mechanoreceptors sensing changes in cell volume can become a major source for cardiac arrhythmias
55. Dell'Italia LJ, Meng QC, Balcells E, et al. Increased ACE and chymase-like activity in cardiac tissue of dogs with chronic mitral regurgitation. *Am J Physiol*. 1995; 269(6 Pt 2):H2065–H2073. [PubMed: 8594918]
56. Ferrario CM. Addressing the theoretical and clinical advantages of combination therapy with inhibitors of the renin-angiotensin-aldosterone system: antihypertensive effects and benefits beyond BP control. *Life Sci*. 2010; 86(9–10):289–299. [PubMed: 19958778]
57. Ferrario CM, Jessup J, Chappell MC, et al. Effect of angiotensin-converting enzyme inhibition and angiotensin II receptor blockers on cardiac angiotensin-converting enzyme 2. *Circulation*. 2005; 111(20):2605–2610. [PubMed: 15897343]
58. Ahmad S, Simmons T, Varagic J, et al. Chymase-dependent generation of angiotensin II from angiotensin-(1–12) in human atrial tissue. *PLoS One*. 2011; 6(12):e28501. [PubMed: 22180785] ••This study in human atrial tissue from patients undergoing heart surgery for the treatment of resistant atrial fibrillation created the underpinning for a pathogenic role of myocyte Ang-(1–12) as the principal source accounting for the role of Ang II in the generation of cardiac arrhythmias. The studies include demonstration of chymase as the Ang-(1–12) converting enzyme in human atrial tissue
59. Ahmad S, Wei CC, Tallaj J, et al. Chymase mediates angiotensin-(1–12) metabolism in normal human hearts. *J Am Soc Hypertens*. 2013; 7(2):128–136. [PubMed: 23312967] ••Extending the prior conclusions obtained in human disease atrial tissue, the study confirms that chymase is the sole enzyme accounting for Ang-(1–12) metabolism to Ang II directly
60. Hoshino F, Urata H, Inoue Y, et al. Chymase inhibitor improves survival in hamsters with myocardial infarction. *J Cardiovasc Pharmacol*. 2003; 41(Suppl 1):S11–S18. [PubMed: 12688390]
61. Wei CC, Hase N, Inoue Y, et al. Mast cell chymase limits the cardiac efficacy of Ang I-converting enzyme inhibitor therapy in rodents. *J Clin Invest*. 2010; 120(4):1229–1239. [PubMed: 20335663]
62. Arakawa K, Urata H. Hypothesis regarding the pathophysiological role of alternative pathways of angiotensin II formation in atherosclerosis. *Hypertension*. 2000; 36(4):638–641. [PubMed: 11040250]
63. Ihara M, Urata H, Kinoshita A, et al. Increased chymase-dependent angiotensin II formation in human atherosclerotic aorta. *Hypertension*. 1999; 33(6):1399–1405. [PubMed: 10373223]
64. Rafiq K, Sherajee SJ, Fan YY, et al. Blood glucose level and survival in streptozotocin-treated human chymase transgenic mice. *Chin J Physiol*. 2011; 54(1):30–35. [PubMed: 21786536]
65. Hara M, Ono K, Hwang MW, et al. Evidence for a role of mast cells in the evolution to congestive heart failure. *J Exp Med*. 2002; 195(3):375–381. [PubMed: 11828013]
66. Kokkonen JO, Lindstedt KA, Kovanen PT. Role for chymase in heart failure: angiotensin II-dependent or -independent mechanisms? *Circulation*. 2003; 107(20):2522–2524. [PubMed: 12777313]
67. Matsumoto T, Wada A, Tsutamoto T, et al. Chymase inhibition prevents cardiac fibrosis and improves diastolic dysfunction in the progression of heart failure. *Circulation*. 2003; 107(20):2555–2558. [PubMed: 12742989]
68. Balcells E, Meng QC, Johnson WH Jr. et al. Angiotensin II formation from ACE and chymase in human and animal hearts: methods and species considerations. *Am J Physiol*. 1997; 273(4 Pt 2):H1769–H1774. [PubMed: 9362242]
69. Pat B, Chen Y, Killingsworth C, et al. Chymase inhibition prevents fibronectin and myofibrillar loss and improves cardiomyocyte function and LV torsion angle in dogs with isolated mitral regurgitation. *Circulation*. 2010; 122(15):1488–1495. [PubMed: 20876440]

70. Wei CC, Meng QC, Palmer R, et al. Evidence for angiotensin-converting enzyme- and chymase-mediated angiotensin II formation in the interstitial fluid space of the dog heart in vivo. *Circulation*. 1999; 99(19):2583–2589. [PubMed: 10330392]
71. Wei CC, Lucchesi PA, Tallaj J, et al. Cardiac interstitial bradykinin and mast cells modulate pattern of LV remodeling in volume overload in rats. *Am J Physiol Heart Circ Physiol*. 2003; 285(2):H784–H792. [PubMed: 12663259]
72. Wei CC, Chen Y, Powell LC, et al. Cardiac kallikrein-kinin system is upregulated in chronic volume overload and mediates an inflammatory induced collagen loss. *PLoS One*. 2012; 7(6):e40110. [PubMed: 22768235] ••Treatment of left heart valve insufficiency that leads to cardiac volume overload remains a formidable issue for which effective treatments are not available. In an experimental model of pure volume overload induced by the creation of an aorto-caval fistula, the authors demonstrate a significant contribution of the cardiac cellular kallikrein-kinin system and beneficial effects of blockade with aprotinin
73. Wang H, Jessup JA, Zhao Z, et al. Characterization of the cardiac renin angiotensin system in oophorectomized and estrogen-replete mRen2.Lewis rats. *PLoS One*. 2013; 8(10):e76992. [PubMed: 24204720] •By exploring the role of estrogen on the cardiac renin angiotensin system, the authors demonstrate a beneficial action of this sex steroid in blocking cardiac chymase Ang II formation
74. Zhao Z, Wang H, Jessup JA, et al. The Role of Estrogen in Diastolic Dysfunction. *Am J Physiol Heart Circ Physiol*. 2014
75. Dell'Italia LJ, Meng QC, Balcells E, et al. Compartmentalization of angiotensin II generation in the dog heart. Evidence for independent mechanisms in intravascular and interstitial spaces. *J Clin Invest*. 1997; 100(2):253–258. [PubMed: 9218500]
76. 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(4):374–379. [PubMed: 12151872]
77. Baker KM, Booz GW, Dostal DE. Cardiac actions of angiotensin II: Role of an intracardiac renin-angiotensin system. *Annu Rev Physiol*. 1992; 54:227–241. [PubMed: 1562174]
78. Takai S, Sakaguchi M, Jin D, et al. Different angiotensin II-forming pathways in human and rat vascular tissues. *Clin Chim Acta*. 2001; 305(1–2):191–195. [PubMed: 11249939]
79. Raymond WW, Su S, Makarova A, et al. Alpha 2-macroglobulin capture allows detection of mast cell chymase in serum and creates a reservoir of angiotensin II-generating activity. *J Immunol*. 2009; 182(9):5770–5777. [PubMed: 19380825]
80. Ferrario CM, Varagic J, Habibi J, et al. Differential regulation of angiotensin-(1–12) in plasma and cardiac tissue in response to bilateral nephrectomy. *Am J Physiol Heart Circ Physiol*. 2009; 296(4):H1184–H1192. [PubMed: 19218503]
81. Trask AJ, Jessup JA, Chappell MC, et al. Angiotensin-(1–12) is an alternate substrate for angiotensin peptide production in the heart. *Am J Physiol Heart Circ Physiol*. 2008; 294(5):H2242–H2247. [PubMed: 18359898]
82. Baker KM, Kumar R. Intracellular angiotensin II induces cell proliferation independent of AT1 receptor. *Am J Physiol Cell Physiol*. 2006; 291(5):C995–1001. [PubMed: 16774988]
83. Dell'Italia LJ, Balcells E, Meng QC, et al. Volume-overload cardiac hypertrophy is unaffected by ACE inhibitor treatment in dogs. *Am J Physiol*. 1997; 273(2 Pt 2):H961–H970. [PubMed: 9277516]
84. Singh VP, Baker KM, Kumar R. Activation of the intracellular renin-angiotensin system in cardiac fibroblasts by high glucose: role in extracellular matrix production. *Am J Physiol Heart Circ Physiol*. 2008; 294(4):H1675–H1684. [PubMed: 18296558]
85. Gondo M, Maruta H, Arakawa K. Direct formation of angiotensin II without renin or converting enzyme in the ischemic dog heart. *Jpn Heart J*. 1989; 30(2):219–229. [PubMed: 2470929]
86. Ikeda M, Sasaguri M, Maruta H, et al. Formation of angiotensin II by tonin-inhibitor complex. *Hypertension*. 1988; 11(1):63–70. [PubMed: 2448241]
87. Wintroub BU, Klickstein LB, Kaempfer CE, et al. A human neutrophil-dependent pathway for generation of angiotensin II: purification and physicochemical characterization of the plasma protein substrate. *Proc Natl Acad Sci U S A*. 1981; 78(2):1204–1208. [PubMed: 6785751]

88. Wintroub BU, Klickstein LB, Dzau VJ, et al. Granulocyte-angiotensin system. Identification of angiotensinogen as the plasma protein substrate of leukocyte cathepsin G. *Biochemistry*. 1984; 23(2):227–232. [PubMed: 6696878]
89. Bujak-Gizycka B, Olszanecki R, Suski M, et al. Angiotensinogen metabolism in rat aorta: robust formation of proangiotensin-12. *J Physiol Pharmacol*. 2010; 61(6):679–682. [PubMed: 21224498]
90. Simington SW, Moniwa N, Ahmad S, VonCannon J, Dell Italia LJ, Varagic J, et al. Renin does not participate in the production of plasma angiotensin-(1–12) from angiotensinogen. *Hypertension*. 2012; 60:A628.
91. Arakawa K, Ikeda M, Fukuyama J, et al. A pressor formation by trypsin from renindenatured human plasma protein. *J Clin Endocrinol Metab*. 1976; 42(3):599–602. [PubMed: 1254698]
92. Arakawa K, Maruta H. Ability of kallikrein to generate angiotensin II-like pressor substance and a proposed 'kinin-tensin enzyme system'. *Nature*. 1980; 288(5792):705–706. [PubMed: 6905924]