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Review Article

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 Ang-(1–7) [angiotensin-(1–7)] in opposing the vasoconstrictor, proliferative and prothrombotic actions of AngII (angiotensin II) created an underpinning for a more comprehensive exploration of the multiple pathways by which the RAS (renin–angiotensin system) of blood and tissues regulates homoeostasis and its altered state in disease processes. The present 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 Ang-(1–12) [angiotensin-(1–12)] in the cardiac production of AngII. The studies reveal significant differences in humans compared with rodents regarding the enzymatic pathway by which Ang-(1–12) undergoes metabolism. Highlights of the research include the demonstration of chymase-directed formation of AngII from Ang-(1–12) in human left atrial myocytes and left ventricular tissue, the presence of robust expression of Ang-(1–12) and chymase in the atrial appendage of subjects with resistant atrial fibrillation, and the preliminary observation of significantly higher Ang-(1–12) expression in human left atrial appendages.

Key words: angiotensin-(1–7), angiotensin-(1–12), angiotensin-converting enzyme (ACE), atrial fibrillation, chymase, pro-angiotensin-12, renin

THE YESTERYEARS

Over 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 Ang-(1–7) [angiotensin-(1–7)] [1], the characterization of ACE2 (angiotensin-converting enzyme 2) as an Ang-(1–7)-forming enzyme from AngII (angiotensin 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 (2–4 May 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 homoeostasis. 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 AngII receptors, and the extensive characterization of AngII analogues and antagonists by chemical substitution of its amino acids [7,8]. These efforts led to the recognition that the phenyl group in the eighth residue of AngII conveyed the information for biological response, whereas the aromatic side chain in residues four and six, the guanido group in the second residue, and the C-terminal carboxyl were involved in binding to the receptor [7]. In sharing an environment of scientific giants at the Cleveland Clinic, including 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

Abbreviations: ACE, angiotensin-converting enzyme; AF, atrial fibrillation; Ang-(1–7), angiotensin-(1–7); Ang-(1–12), angiotensin-(1–12); AngI etc., angiotensin I etc.; ARB, angiotensin II type 1 receptor blocker; DRI, direct renin inhibitor; NPY, neuropeptide Y; SHR, spontaneously hypertensive rat; WKY, Wistar–Kyoto.

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interpretation was based on studies showing that truncation of the C-terminus of AngII, particularly the removal of Phe^8, prevented the peptide from binding to the AngII 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 AngII, the availability in our laboratory of a rat hypothalamic-hypophysial explant preparation [11,12] allowed the testing of 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 AngII doses [6]. The evidence that Ang-(1–7) stimulated vasopressin release, despite the fact that this N-terminal peptide had lost the eighth amino acid residue at the C-terminal epitope of AngII, 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 AngII [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: 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 AngII and Ang-(1–7) is also present upstream from AngI (angiotensin 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 (renin–angiotensin system) 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 AngI is based on accumulating evidence suggesting that the long-term effects of RAS blockade using DRIs (direct renin inhibitors), ACE inhibitors and ARBs (AngII type 1 receptor blockers) has fallen short of expectations when compared with other antihypertensive classes. A meta-analysis of 31 trials [26], with 190606 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 account for incomplete blockade of AngII actions or synthesis cannot explain these findings, because the combination of ARB and ACE inhibitors showed no further benefits in the large ONTARGET (ONgoing Telmisartan Alone and in combination with Ramipril Global Endpoint Trial) [28,29], Altitude (Aliskiren Trial in Type 2 Diabetes Using Cardiorenal Endpoints) which combined aliskiren (a 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 such well-proven biological effects in preventing the cardiovascular consequences of excess AngII expression or activity, why are clinical hard endpoint 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: (i) ACE inhibitors and ARBs are not able to access the cellular sites at which AngII acts; (ii) AngII production in tissues may involve pathways that neither require ACE nor renin for its generation; or (iii) a combination of both.

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

The second possibility, formation of AngII by mechanisms that do not involve renin or ACE, is gaining new momentum with the isolation of pro-angiotensin-12 {Ang-(1–12) [angiotensin-(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 molecule (human sequence: N-Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8-His^9-Leu^{10}-Val^{11}-Ile^{12}-) is present at high concentrations in the kidney, heart and brain of Wistar rats [52]. Nagata et al. [52] showed further that Ang-(1–12) was readily converted into AngII, as the vasoconstrictor response produced by Ang-(1–12) in either

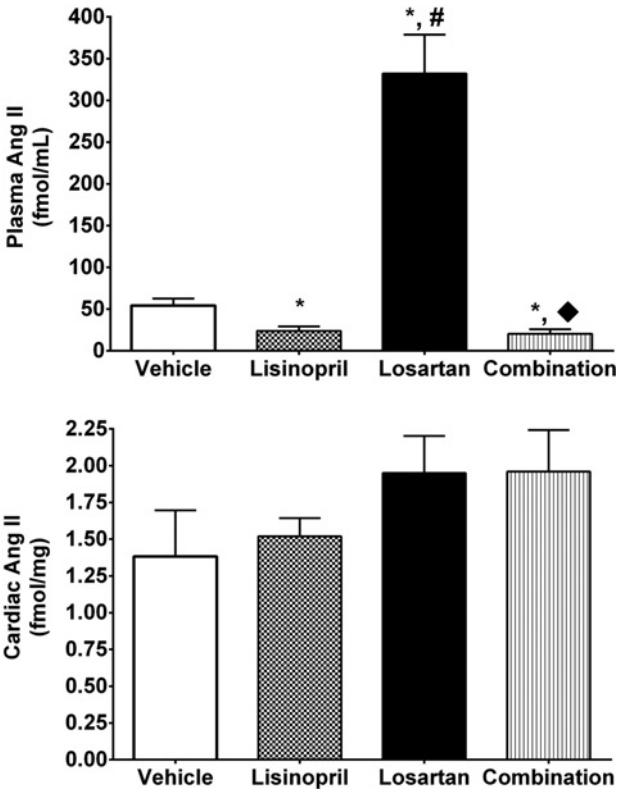

Figure 1 Correlative changes in plasma (top panel) and left ventricular (bottom panel) content of AngII at the completion of a 14-day therapy with either vehicle, lisinopril, losartan or both drugs combined shows significant divergency of the effects of the therapies on plasma compared with cardiac AngII levels

The results are means ± S.E.M. in Lewis rats. $^{*}P < 0.05$ compared with vehicle; $\#P < 0.05$ compared with lisinopril; $\blacklozenge P < 0.05$ compared with losartan. Reprinted with permission from 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, 111, 20, 2605–2610 [42]. Copyright © 2005 American Heart Association Inc.

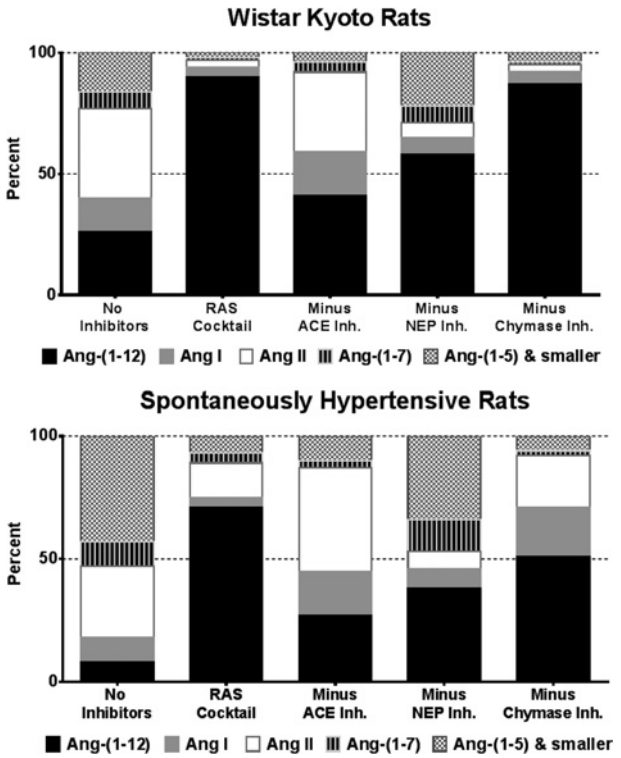

Figure 2 Metabolism of ^{125}I-labelled Ang-(1–12) (1 nmol/l) expressed as a percentage of the peptides remaining in the cultured medium of neonatal cardiac myocytes following 60 min incubation at 37 °C in WKY rats and SHRs

RAS cocktail includes lisinopril, a neprilysin (NEP) inhibitor (SCH39370), an ACE2 inhibitor (MLN-4760) and a chymase inhibitor (chymostatin) all added at a concentration of 10 μM. Inh., inhibitor. This Figure was drawn from results appearing in Table 1 in [55].

the systemic circulation or aortic strips was blocked by prior administration of captopril or candesartan.

The discovery of this extended form of AngI raised our interest since we had previously reported the existence of a family of extended AngI peptides in dog cerebrospinal fluid [53]. In a series of correlative studies we showed increased expression and content of Ang-(1–12) in the left ventricle of SHRs (spontaneously hypertensive rats) compared with WKY (Wistar–Kyoto) rats [54]. Furthermore, a 42% higher Ang-(1–12) uptake was found in cultured neonatal myocytes from SHRs compared with WKY rats. Increased incorporation of the radiolabelled Ang-(1–12) in SHRs was associated with a faster rate of peptide degradation in the medium collected from the neonatal myocytes [55]. As illustrated in Figure 2, there were significant differences in Ang-(1–12) metabolism in the cultured medium obtained from WKY rat and SHR neonatal cardiomyocytes. Major differences in ^{125}I-labelled Ang-(1–12) hydrolysis between the two strains included a greater catalytic activity of the medium for Ang-(1–12) metabolism by ACE, neprilysin and chymase (Figure 2, and see [55]). Importantly, the catalytic activity of chymase in the medium collected from cultured cardiac myocytes was markedly augmented in SHRs compared with WKY rats (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 that study, we also showed that ACE is the primary pathway for AngII production from Ang-(1–12) in the circulation of WKY rats and SHRs [57].

Since the original identification of chymase as an AngII-forming enzyme in the human heart [58–60], the involvement of this enzyme in forming AngII from AngI has been reported to be crucially important in the pathogenesis of adverse cardiac remodelling 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 rats and SHRs [57], the demonstration that chymase is an AngII-forming enzyme from Ang-(1–12) in the hypertrophied heart of SHRs [55]

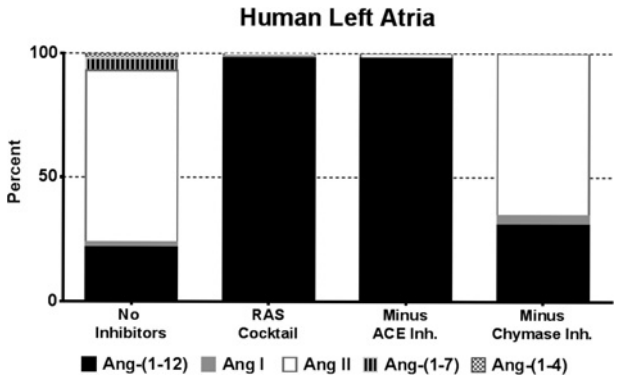

Figure 3 Processing of ^{125}I-labelled Ang-(1–12) by plasma membranes isolated from the human left atrial appendage of subjects undergoing heart surgery for the treatment of resistant AF shows a primary role of chymase as the Ang-(1–12)-processing enzyme
This Figure was drawn from results appearing in Table 1 in [68].

and a model of ischaemia/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 AngI-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 AF (atrial fibrillation) expressed immunoreactive Ang-(1–12) and chymase within atrial myocytes [68]. Incubation of plasma membranes from these atrial myocytes with ^{125}I-labelled Ang-(1–12) resulted in the rapid production of AngII that was prevented in the presence of chymostatin and not inhibited by lisinopril (Figure 3). As discussed in our previous study [68], although chymase-cleaving activity in rodents occurs at amino acid residues containing the sequence of Tyr^4-Ile^5 [70], human chymase hydrolytic activity is specific for the Phe^8-His^9 sequence. The latter sequence is present within the amino acid composition of human Ang-(1–12) [H-Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8-His^9-Leu^{10}-Val^{11}-Ile^{12}-OH]. These data agree with the finding that AngI 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]. Similar to the study from subjects with resistant AF, processing of Ang-(1–12) into AngII was entirely due to the catalytic activity of chymase [69].

The studies described above are in keeping with the tenet that chymase mediates the majority of AngII production in the human heart, and that AngII formation is compartmentalized, with chymase being the major AngII-forming mechanism in the cardiac interstitium and both chymase and ACE acting as AngII-forming mechanisms in the intravascular space (Figure 4). Investigation of the alternate pathway for AngII production from Ang-(1–12) also shows that chymase is an important AngII-forming mechanism within the cardiomyocytes. These findings have important clinical implications because intracellular chymase-mediated AngII formation is unaffected by AngII type 1 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 atrium of these patients (Figure 6). In addition, recent work showed that immunoreactive Ang-(1–12) expression is significantly higher in the human left atrial appendage (35.37 ± 6.24 units) compared with 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 2-fold higher in the right compared with the left atrium [77]. On the other hand, the content of NPY (neuropeptide Y) 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 atrium suggests 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 address these observations.

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 AngII-forming substrate have shed new and potentially exciting light on mechanisms by which AngII, formed through an intracrine pathway, contributes to cardiovascular pathology. The studies showing chymase and Ang-(1–12) in the left atrial tissue obtained from subjects with chronic AF explains how this non-ACE-dependent pathway for intracellular pro-arrhythmic AngII 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 AngII levels to pretreatment levels [79], whereas the benefits derived from ARBs or even aliskiren are restricted by the inability of these agents to reach intracellular sites of AngII action or prevent the activation of alternate non-renin pathways for AngII synthesis. Indeed, characterization of the processing pathways mediating Ang-(1–12) metabolism clearly demonstrate both species-specific (humans compared with rodents) and compartment (i.e., blood, organ and cellular sites) selectivity, whereas other studies excluded renin from having any catalytic effect 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

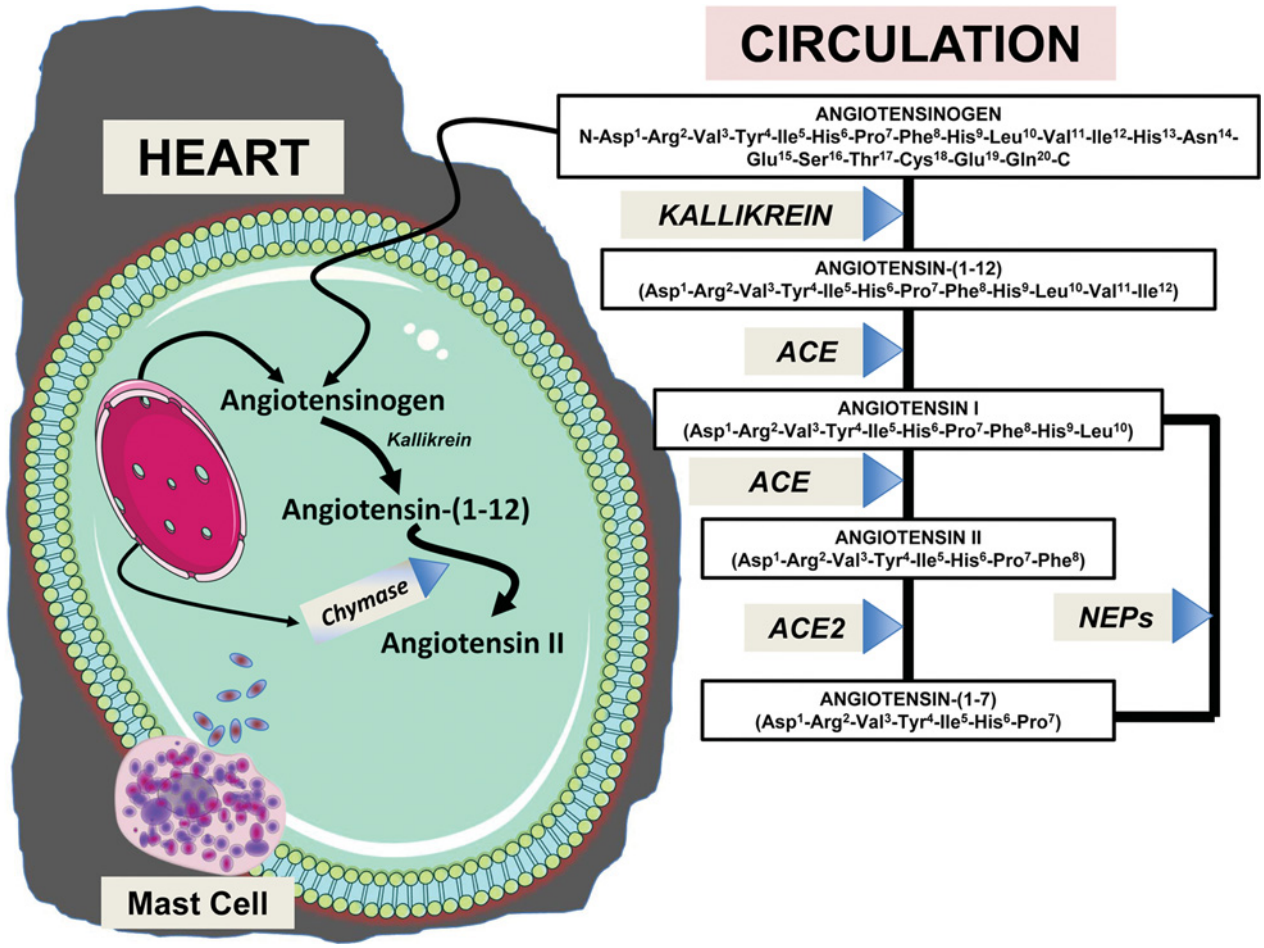

Figure 4 **Schematic diagram of biotransformation pathways for Ang-(1–12) in the human circulation and cardiac myocytes**
The sequence of Ang-(1–12) is that of the human form which differs from that expressed in rodents in terms of the substitution of valine for leucine at residue 11 and the presence of histidine instead of tyrosine at residue 12. Chymase either produced in cardiac myocytes or released by mast cells during ischaemia/reperfusion injury (I/R) or increased oxidative stress generates AngII intracellularly from Ang-(1–12) [93,94]. NEPs include angiotensin-(1–7)-forming enzymes (prolyl endopeptidase 24.26 and neprilysin) [95].

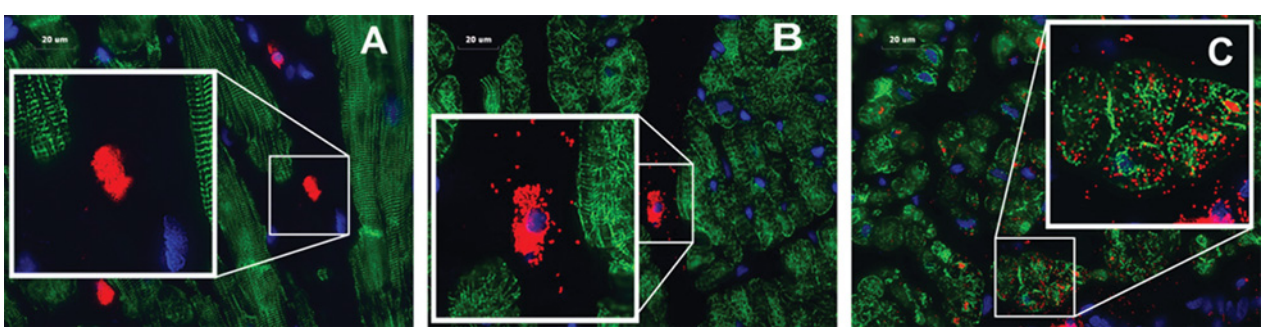

Figure 5 **Representative example of immunohistochemistry of the left atrium from a subject with mitral regurgitation**
From left to right, the left atrium demonstrates infiltration of mast cells with chymase (red) in various stages of degranulation. (**A**) Intact mast cell; (**B**) mast cell release 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. Blue, DAPI; green, desmin.

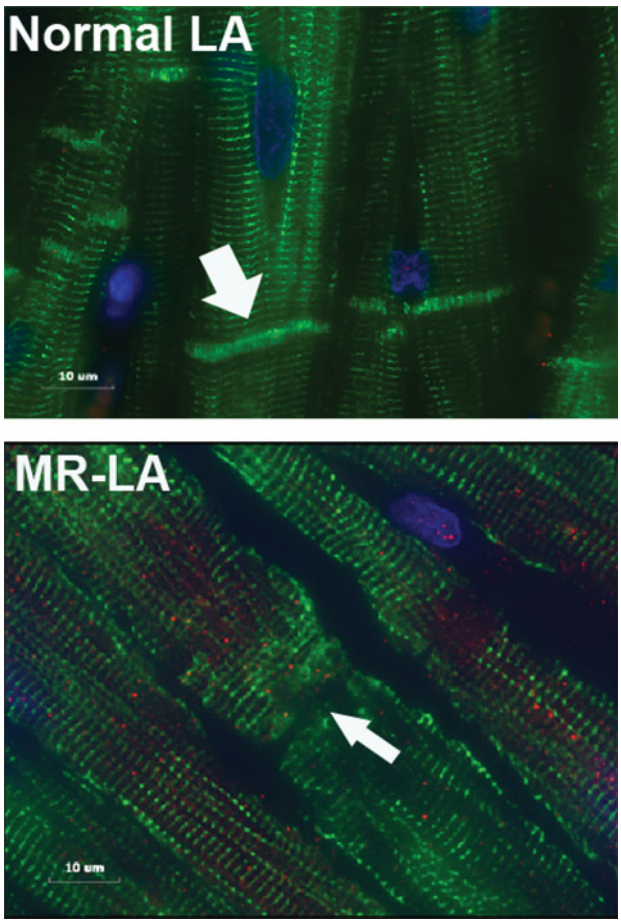

Figure 6 Immunohistochemistry fluorescent staining for desmin and Ang-(1–12) in normal and mitral regurgitation (MR) left atrium (LA)

MR LA has a loss of desmin (green) in the intercalated disc (white arrow) along with an increase in Ang-(1–12) (red) compared with normal LA. The nuclei are stained with DAPI (blue).

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 AngII and Ang-(1–7) now extends to the existence of intermediate substrate peptides upstream from AngI. The dodecapeptide Ang-(1–12) is a functional substrate for the production of AngII, as shown in metabolism studies [55,65–69,81,84,85] and physiological conditions [80,86–89]. The primacy of chymase as a cardiac AngII-forming enzyme in humans should generate cautionary notes as to the direct applicability of characterized biochemical pathways for angiotensin peptide formation in rodents compared with humans. Although 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 AngII production in tissues, should provide impetus to furthering the understanding of the differential regulatory pathways by which AngII exerts trophic and profibrotic effects on the heart through intracellular mechanisms that are renin- and, to a certain extent, ACE-independent. Although renin has been excluded from generating AngII from Ang-(1–12), the question of what enzymatic pathway accounts for the formation of Ang-(1–12) from angiotensinogen 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 angiotensinogen containing the first 20 amino acids of the molecule [90].

Although the present review has focused on the biochemical physiology of AngII production from Ang-(1–12) in the heart, accumulating evidence shows that Ang-(1–12) is also a functionally important substrate for AngII 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 AngII-forming substrate in neuronal hypothalamic and brain stem circuits regulating the central control of arterial pressure and baroreceptor reflexes.

CONCLUSIONS

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

In human subjects, cardiac AngII synthesis originates from the hydrolytic activity of chymase, which when released from mast cells acts on an angiotensinogen-derived extended form of AngI [Ang-(1–12)] to generate AngII directly. Characterization of this pathway in human left atrial appendages and in subjects with resistant AF implicates Ang-(1–12) as the intracrine substrate for the pro-arrhythmogenic actions of AngII. These studies demonstrate a need to develop new treatment strategies, on the basis of either selective inhibition of cardiac chymase activity or suppression of intracellular AngII production, as a most effective approach to reversal of adverse cardiac remodelling or arrhythmias.

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