Intracrine angiotensin II functions originate from noncanonical pathways in the human heart

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Ferrario CM, Ahmad S, Varagic J, Cheng CP, Groban L, Wang H, Collawn JF, Dell’Italia LJ. Intracrine angiotensin II functions originate from noncanonical pathways in the human heart. Am J Physiol Heart Circ Physiol 311: H404–H414, 2016. First published May 27, 2016; doi:10.1152/ajpheart.00219.2016.—Although it is well-known that excess renin angiotensin system (RAS) activity contributes to the pathophysiology of cardiac and vascular disease, tissue-based expression of RAS genes has given rise to the possibility that intracellularly produced angiotensin II (Ang II) may be a critical contributor to disease processes. An extended form of angiotensin I (Ang I), the dodecapeptide angiotensin-(1–12) [Ang-(1–12)], that generates Ang II directly from chymase, particularly in the human heart, reinforces the possibility that an alternative noncanonical renin independent pathway for Ang II formation may be important in explaining the mechanisms by which the hormone contributes to adverse cardiac and vascular remodeling. This review summarizes the work that has been done in evaluating the functional significance of Ang-(1–12) and how this substrate generated from angiotensinogen by a yet to be identified enzyme enhances knowledge about Ang II pathological actions.

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tensins becomes an important aspect of the mechanisms by which the intracellular RAS contributes to physiology and pathology (31).

Accumulating evidence showing that Ang II intracellular effects are not inhibited by AT₁ receptor blockade (11, 34) indicates the independence of the intracrine RAS from the external environment. Intracellular Ang II synthesis is augmented in rat cardiomyocytes exposed to high glucose; this effect is not prevented by the presence of the Ang II receptor blocker candesartan (118). In hamster myocytes, losartan did not block the inotropic actions of intracellular Ang II when added to the perfusion media (34). Of importance, chronic administration of losartan, lisinopril, or both drugs combined had no effect on cardiac Ang II content even though the robust antihypertensive effects of these treatments produced the expected changes in circulating angiotensins (47). Likewise, the antihypertensive effects of chronic administration of olmesartan to mRen2.Lewis transgenic hypertensive rats did not change cardiac Ang II content even though the treatment was associated with significant increases in plasma renin concentration and plasma Ang I and Ang II content (148). Additional studies supporting the existence of an independent intracrine RAS system has been reviewed recently (1, 36). Thus there is strong experimental evidence to support the view that intracrine Ang II activity may function independent of the circulating system (45, 49).

This review addresses how the recent identification of shorter forms of angiotensinogen that are expressed in human and rodent organs reinforces the importance of intracellular RAS intracrine mechanisms and the importance of intracrine signaling. New research demonstrates that noncanonical mechanisms regulate the synthesis and processing of intracellular angiotensins and brings to light a need to reconsider the acknowledged usefulness of ACE inhibitors and Ang II receptor blockers (ARBs) as the most effective approach to halt or prevent the adverse cardiac and vascular remodeling associated with increased Ang II activity.

Currently recognized pathways for RAS expression. The process of polypeptide biotransformation, first discussed by Burbach (20), was applied by our laboratory (46) to examine the biochemical processes associated with the formation and processing of biologically active angiotensin peptides. These guiding principles of polypeptide biotransformation, and the research that followed this methodological approach, led to the recognition that the RAS was a true example of a complex hormonal mechanism in which at least three identified angiotensinogen-derived peptides - angiotensin-(1–25) [Ang-(1–25)], angiotensin-(1–12) [Ang-(1–12)], and angiotensin I (Ang I) - act as intermediate precursors for the generation of the shorter amino acid sequences of the active hormones angiotensin-(1–9) [Ang-(1–9)], Ang II, and Ang-(1–7). These three biologically active peptides, in turn, become precursor molecules for the formation of two other active angiotensins, angiotensin III (Ang III) and angiotensin IV (Ang IV). An additional level of complexity, leading to potentially further biological selectivity, involves the decarboxylation of Ang II into des[Asp²]-[Ala¹]-Ang II (Ang A) (69) and, not unexpectedly, the formation of des[Asp²]-[Ala¹]-Ang-(1–7) (alamidine) from either Ang A or Ang-(1–7) (81). As ligands, angiotensin hormones bind to the G protein nuclear and transmembrane receptors AT₁, AT₂, mas (mas-R), and a component of the family of mas-related MRG receptors (MRG-D) (10). Several reviews have documented our current understanding of the biochemical pathways, enzymes, biologically active peptides, and receptors encompassing the RAS (3, 45, 149).

Upstream from Ang I, the diversity of substrates and enzymes contributing to the generation of Ang II and Ang-(1–7) had been relatively ignored since most research remains focused on the catalytic actions of prorenin, renin, and their action in hydrolyzing angiotensinogen into Ang I. Over the years, a number of renin-like enzymes have been reported to release Ang I from either the angiotensinogen molecule or the model substrate tetradecapeptide [Ang-(1–14)]. Cathepsin D and G, acid and neutral proteases, tonin, the serine protease esterase B, a kallikrein-like serine protease, and mouse γ-nerve growth factor (γ-NGF) are among the enzymes identified as Ang I-forming enzymes (16, 17, 23, 42, 56, 59, 73, 117, 157). Tonin, an enzyme discovered by Boucher et al. (17) and present at its highest concentration in the submaxillary gland, forms Ang II directly from the tetradecapeptide substrate [Ang-(1–14)] and from Ang I (117). A neutral protease with Ang I-forming activity that was readily separated from acid proteases and both plasma and renal renin was reported by Husain et al. (65, 66) in the canine brain. Mast cell-derived cathepsin G is an Ang II-forming enzyme implicated in the evolution of atherosclerosis and aortic aneurysms (15, 68, 111, 114, 134, 151). More recently, the characterization of the (pro)renin receptor (PRR) (100) has led to the demonstration that binding of (pro)renin to the PRR augments the catalytic activity of renin (100) as well as activating proinflammatory and profibrotic signaling pathways by both Ang II-dependent and independent mechanisms (44, 63, 105). Overexpression of the human PRR in Neuro-2A cells stimulated production of radical oxygen species via both Ang II and non-Ang II mediated mechanisms (105). In addition, the microinjection of human prorenin into the paraventricular nucleus of the rat’s hypothalamus stimulates splanchnic sympathetic nerve activity, whereas the increases in inducible nitric oxide synthase and cytb gene transcripts mediated by exposure to human prorenin were not blocked by losartan (63). The potential role of PRR as a potential source for non-Ang II-dependent actions in the heart remains to be investigated since the PRR is highly expressed in the heart (115).

The extended forms of angiotensin I. Since characterization in 1957 of the first 14 amino acids of horse angiotensinogen (119), this α₂-globulin member of the serpin family remains the sole known angiotensins forming substrate (93). Although high (H) and low (L) molecular weight (MW) forms of angiotensinogen have been described, the function of the HMW form of angiotensinogen remains poorly understood. According to Tewksbury and Tewksbury and Tryon (126, 128), levels of the HMW form of angiotensinogen are less than 5% that of the LMW form. When compared with the LMW form, both the \( K_m \) and \( V_{max} \) of the HMW form are lower at a physiological pH, and its blood concentration doubles as a result of estrogen treatment or pregnancy (127, 129). The major known function of angiotensinogen to date is to act as a substrate for the liberation of the decapeptide angiotensin I [Ang-(1–10)] through a renin-mediated cleavage of the Leu¹⁰-Val¹¹ amino acid bond in humans and the Leu¹⁰-Leu¹¹ bond in nonprimate species such as the rat (93). The substitution of histidine for tyrosine at residue 13 of the human angiotensinogen protein...
contributes to the species-specificity of the human renin angiotensinogen reaction (94). In recent years, the identification of extended molecular sequences of Ang I as potential intermediate sources of Ang II production, through nonrenin-dependent mechanisms, has revealed new paradigms regarding the biochemical mechanisms that account for the expression and action of angiotensins (3, 149).

In their searching for additional bioactive angiotensin family peptides, Nagata and collaborators (98) at Miyazaki University in Japan reported in 2006 the isolation of an extended form of Ang I composed of the first 12 amino acids of angiotensinogen from the rat small intestine. The peptide, termed proangiotensin-12, was present in multiple tissues with its highest concentrations in the rat intestine (Fig. 1). Interest in this peptide as a functional Ang II-forming substrate was strengthened by the demonstration that proangiotensin 12 constricted aortic strips and, when infused intravenously, raised blood pressure in Wistar rats (98). The vasoconstrictor responses to proangiotensin 12 could be abolished by either captopril or the Ang II type I receptor blocker candesartan (98). To maintain consistency with the accepted nomenclature, proangiotensin 12 was renamed Ang-(1–12) (70).

The discovery of Ang-(1–12) and the studies conducted on it to date provide an alternate explanation as to how tissues, independently from the circulation, may avail from shorter angiotensinogen-derived peptides to give rise to Ang II and Ang-(1–7) within the immediate surrounding extracellular microenvironment or the cell itself. This idea gained support by the demonstration that both immunoreactive Ang-(1–12) expression and the left ventricular content of the dodecapeptide were markedly increased in adult spontaneously hypertensive rats (SHRs) than in normotensive Wistar Kyoto (WKY) rat controls (70). The Ang-(1–12) immunoreactive products were primarily localized within the cytoplasm of myocytes, although some positive staining was observed in the medial layer and surrounding adventitia of intracoronary arteries (70). As related by Jessup et al. (70), clusters of Ang-(1–12) immunoreactivity were primarily visualized in the endocardium of the right and left ventricles of WKY rats. This patchy Ang-(1–12) expression in the normotensive rat contrasted sharply with the more diffuse distribution of Ang-(1–12) immunoreactive staining throughout the right and left ventricular myocardium of SHRs (70). In keeping with these histological observations, direct Ang-(1–12) assays revealed higher left ventricular Ang-(1–12) tissue concentrations in the SHR than in the control WKY strain (70). The higher Ang-(1–12) content in SHRs was associated with higher tissue concentrations of Ang I and Ang II (Fig. 2). The authors inferred that Ang-(1–12) may be a cardiac precursor for angiotensin peptide formation based on the minimal presence of angiotensinogen immunoreactivity staining in the hearts of both WKY rats and SHRs (70). Although these findings should not be interpreted as excluding intracellular angiotensinogen as a contributing source of Ang-(1–12), the available literature suggest that a significant fraction of the angiotensinogen found in the heart is derived from the circulation (32, 85, 103). These data suggest that Ang-(1–12) may be a critical precursor for the formation of cardiac angiotensins, although the intracellular origin of the propeptide needs to be investigated further.

The characterization of Ang-(1–12) as an important source for production of angiotensins has been derived from a series of studies in which the peptide was administered both systemically and to isolated organs. Extending the initial observations of Nagata et al. (98), Ang-(1–12) elicited coronary artery vasoconstriction and impaired recovery from global ischemia as reflected by a marked increase in creatine kinase and troponin I release from the isolated perfused heart of Sprague Dawley (SD) rats subjected to ischemia reperfusion injury (108). An additional report showed that vasoconstrictor responses to Ang-(1–12) administration could be elicited in both muscular and conduit arteries of SD rats, although no responses were observed in femoral and renal arteries (109). The
mechanism that accounts for the progressive reduction of vasoconstrictor responses of the isolated vessels further away from the heart remains to be fully investigated (109). Additional studies confirming the activity of Ang-(1–12) as an Ang II-forming substrate included the characterization of a modulatory action of the locally applied substrate on the central regulation of baroreflexes (9, 29) and the stimulation of neuronal circuits in both the rostral (7) and ventrolateral medulla (71). The demonstration of an antihypertensive effect of Ang-(1–12) immunoneutralization in the brain of transgenic hypertensive rats expressing the Ren-2 gene provided the first functional evidence for a role of Ang-(1–12) as a source of Ang II actions (67). In these studies, blockade of the biological response through the administration of ARBs confirmed that the substrate was converted to Ang II before having an effect (6, 7, 28, 29, 71).

Ang-(1–12) activity as an endogenous Ang II source was investigated in several studies, including an assessment of the chronic effects of continuous infusion of the dodecapeptide in normotensive Wistar rats (77), an investigation of changes in cardiac and systemic RAS components in WKY rats 48 h post-bilateral nephrectomy (48), and an examination of the effects of stimulation of endogenous renin activity by salt depletion (97) or administration of RAS inhibitors (96). Overall, these studies confirmed that Ang-(1–12) functions as an endogenous Ang II-forming substrate and showed that the substrate is regulated independently from the circulating RAS.

This conclusion is based on the increased cardiac Ang-(1–12) concentrations in anephric rats with depleted circulating renin and Ang II (48); the absence of changes in Ang-(1–12) concentrations in kidneys, small intestine, cardiac ventricles, and brain of Wistar rats fed a low-salt diet (97); and the decrease in tissue but not blood Ang-(1–12) of WKY rats and SHRs exposed to 1 wk of treatment with either the ARB losartan or the ACE inhibitor imidapril (96). The fall in cardiac and renal Ang-(1–12) content induced by a 7-day treatment with RAS inhibitors was interpreted by the authors to reflect a fall in cardiac and renal angiotensinogen gene expression (96). Ang-(1–12) functional responses appear to be primarily mediated through its conversion to Ang II (24). In this elegantly conducted study, Chan et al. (24) showed that Ang-(1–12) augmented intracellular Ca++ mobilization and phosphorylated ERK in COS-7 and Chinese Hamster Ovary cells transiently transfected with AT1 receptor cDNA at concentrations 40- to 100-fold higher than Ang II. The authors attributed the need of higher Ang-(1–12) doses to the absence of Ang-(1–12)-processing enzymes in the cells transfected with the AT1 receptor. Further studies showed that losartan but not the AT2 receptor antagonist PD123319 suppressed the Ang-(1–12) effects (24). The demonstration that Ang-(1–12)-mediated Ca++ mobilization failed to occur in Chinese Hamster Ovary cells expressing the AT2 receptor might be important in terms of differentiating responses by the two peptides (24).

It remained to be established whether tissue Ang-(1–12) is overexpressed in diseased states associated with activation of the RAS. The initial observation of augmented Ang-(1–12) expression and concentration in the heart of SHRs (70) was extended through the demonstration of increased immunohistochemical Ang-(1–12) and Ang II expression in the kidney of [mRen2]27 transgenic hypertensive rats that were suppressed by a 3-wk administration of a low-dose of a mineralocorticoid receptor (MR) antagonist (155). Because MR blockade augments Ang-(1–7) generation through ACE2 facilitation of Ang II metabolism (72), the findings in [mRen2]27 transgenic hypertensive rats implicate different pathways for Ang-(1–12) processing in tissues. Consistent with this interpretation, Westwood and Chappell (154) showed that the primary route for Ang-(1–12) metabolism in renal cortical membranes of congenic mRen2.Lewis hypertensive rats involves the formation of Ang-(1–7) and Ang-(1–4) by nephrilysin.

A recently completed study affirms the importance of this alternate pathway for Ang II formation in tissues. Past studies had reported that transgenic expression of the human angiotensinogen gene in SD rats [TGR(hAGT)I1623] did not result in hypertension or cardiovascular pathology because rat renin does not cleave human angiotensinogen (54). However, a more recent study demonstrated that these transgenic rats are hypertensive and display structural and functional indexes of cardiac hypertrophy and systolic dysfunction (49). Robust human Ang-(1–12) immunofluorescence within myocytes of TGR(hAGT)I1623 rats was associated with a fourfold increase in left ventricular cardiac Ang II content (49). Because rat renin has a very low affinity for human angiotensinogen, these new studies underscored the functional importance of alternate enzymatic processes that can lead to Ang II pathology through a nonrenin-dependent pathway.

In addressing the significance of Ang-(1–12) as an endogenous substrate for human tissue angiotensins, our laboratory documented the expression of the peptide in human left ventricles (5), the left atrial appendage of patients with resistant atrial fibrillation (2), and the right and left atrial tissue of subjects with left-heart disease (99). The localization of Ang-(1–12) immunoreactive products in atrial myocytes of subjects with either resistant atrial fibrillation (2) or diseases of the mitral and aortic valves, or ischemic heart disease (99), suggests that increased myocyte stretch secondary to atrial enlargement is a stimulus for the expression of the intracellular substrate. In keeping with this interpretation, Ang-(1–12) expression was significantly higher in the enlarged left atrial appendages of these subjects (99). Of importance, the increased left atrial appendage expression of Ang-(1–12) immunoactivity was associated with increased chymase mRNA and enzymatic activity (99).

Fig. 2. Left ventricular tissue content of angiotensin-(1–12) [Ang-(1–12)], angiotensin I (Ang I), and angiotensin II (Ang II) is higher in adult male spontaneously hypertensive rats (SHRs) than in Wistar-Kyoto (WKY) control rats. Data are reported by our laboratory and published elsewhere (70). *P < 0.05.
Ang-(1–12) and chymase. Ang-(1–12) metabolism follows noncanonical pathways that seem to be independent of renin and involves enzymatic pathways that are both tissue and species specific. Trask et al. (131) investigated Ang-(1–12) processing in the isolated perfused heart of three control rat strains (SD, Lewis, and WKY) and two other hypertensive rat strains (congenic mRen2.Lewis and SHRs). Over a 60-min perfusion period of rat Ang-(1–12), a rapid appearance of both Ang I and Ang II demonstrated that the heart has the enzymes to process the substrate to the smaller peptides. A delayed appearance of Ang-(1–7) in the Ang-(1–12) perfused hearts suggested that it was generated from the accumulated Ang II. All rat strains showed a similar pattern of Ang-(1–12) processing into Ang I and Ang II, whereas addition of a rat renin-specific inhibitor [Ac-His-Pro-Phe-Val-Sta-Leu-Phe-NH2 (WFML-1)] (47) had no effect on the rate and magnitude of Ang-(1–12) metabolism in WKY rats and SHRs (131). To further evaluate a potential role of renin in hydrolyzing Ang-(1–12), a second study investigated the effects of excluding endogenous renin on the circulating and cardiac tissue concentrations of angiotensins (48). In bilaterally nephrectomized WKY rats, a marked fall in circulating Ang I and Ang II levels was associated with a significant increase in the cardiac content of Ang-(1–12), Ang I, and Ang II (48). The accumulation of angiotensins in the heart of anephric WKY rats was confirmed by the visualization of increased immunopositive fluorescence intensities for angiotensinogen, Ang II, and AT1 receptors and no differences in cardiac Ang-(1–12) expression between sham-operated and anephric WKY rats (48).

Although renin is not necessary for Ang-(1–12) processing, studies by our laboratory (2, 3, 5, 92) and others (19, 96, 97, 107–109, 116) demonstrated that Ang-(1–12) hydrolysis to Ang I and Ang II is achieved primarily through the proteolytic activities of ACE and chymase. Additional studies implicate the existence of alternate Ang-(1–12) processing in renal and vascular tissues, whereby neprilysin yields direct formation of Ang-(1–7) in rat renal cortical membranes (154) and carboxypeptidases A2 (cathepsin A) cleaves Ang-(1–12) into Ang I and anephric WKY rats (48).

This noncanonical Ang-(1–12) metabolism pathway is different in rodents and human cardiac tissue (Fig. 3). Experiments investigating Ang-(1–12) metabolism in plasma membranes obtained from the left atria of patients undergoing
cardiac surgery for the treatment of resistant atrial fibrillation (2) or from the left ventricle of normal subjects dying from vehicular motor accidents (5) found that chymase was almost entirely accountable for the biotransformation of this alternative substrate directly into Ang II. In these studies ACE and nephrilysin displayed negligible hydrolytic activity (2). In plasma membranes isolated from the ventricles of SHR, Ang-(1–12) rather than Ang I showed a higher hydrolytic processing activity by chymase in the generation of Ang II (Km, 57.1 μM and Vmax, 12.14 μM·mg⁻¹·min⁻¹ vs. Km, 88.66 μM and Vmax, 4.07 μM·mg⁻¹·min⁻¹, respectively), whereas the opposite is apparently true in human cardiac tissue (2, 4). Two factors may account for the differential affinities of chymase in Ang-(1–12) processing between rodents and humans. Differences in the amino acid sequence of Ang-(1–12) at the COOH terminus between rats and humans (Leu¹⁰-Leu¹¹-Tyr¹² in the rat versus Leu¹⁰-Val¹¹-Ile¹² in humans) may influence the enzyme active site at which chymase processes the peptide (2, 4, 45). In addition, multiple β-chymase isofoms are present in the rat, whereas humans express only α-chymase (25, 39). These species differences are of considerable importance since the predominant β-chymase species found in rats cleaves the Tyr²-Ile³ bond of Ang I rather than the Phe⁸-His⁹ of the decapeptide (122).

The same group of investigators from Miyazaki University in Japan (95) isolated another Ang II-forming peptide from the urine of healthy volunteers. This alternative Ang II-forming substrate, named Big angiotensin-25 [hereafter abbreviated as Ang-(1–25)], consists of the first 25 amino acids of angiotensinogen and is N-glycosylated on Asn¹⁴ with a cysteine linked to Cys¹⁸ (95). In their studies, human recombinant renin rapidly degraded angiotensinogen into Ang I, whereas production of Ang I from Ang-(1–25) was much slower. Nagata et al. (95) reported that the Km for Ang-(1–25) hydrolysis by renin averaged 95 μM compared with 1.2 μM for angiotensinogen. On the other hand, chymase has no catalytic activity on human angiotensinogen (143), mast cell chymase digested Ang-(1–25) more efficiently than human recombinant renin, with Km and Vmax values averaging 9.6 μM and 129 pmol·min⁻¹·ml⁻¹, respectively (95). An antibody directed to the COOH terminus of human Ang-(1–25) stained a wide range of tissues with robust expression in kidney, heart, pancreas, adrenal medulla, testicular Sertoli cells, fallopian tubes, and placenta extravillous trophoblasts (95). The strong expression of Ang-(1–25) immunoreactivity in human pancreas, adrenal medulla, and placenta led the Japanese investigators to suggest that Ang-(1–25) may function as a direct source of intracellular Ang II activity. Whether Ang-(1–25) acts as a substrate for direct Ang II production or requires prior conversion into Ang-(1–12) remains to be investigated. Nevertheless, identification of Ang-(1–25) and Ang-(1–12) as extended Ang I-forms and precursors has revealed the possibility of new mechanisms that describe how organs such as the heart activate intracellular actions of angiotensins.

The predominant role of chymase in cardiac tissue Ang-(1–12) processing, particularly in human cardiac tissue, is consistent with a robust literature documenting a singular role for chymase in the production of Ang II from Ang I in humans (156). In their examination of the finding that serine proteinase inhibitors were more potent than captopril in preventing Ang II formation from Ang I in human left ventricular tissue (142), Urata and coworkers (143) purified and characterized cardiac chymase as the major enzyme in the production of Ang II in the human heart. Human chymase was found to display heterogeneous and widespread tissue distribution throughout the human body with the highest expression in alimentary tract tissue, uterus, and tonsil (144). According to Urata et al. (144), the highest levels of chymase-like enzymatic activity were found in the skin, gastrointestinal track, and uterus, whereas human ventricular tissue, the lung, and renal cortex express lower levels of chymase activity. Cardiac sources of chymase in the human heart were reported in cytosolic granules of mast and endothelial cells, as well as mesenchymal interstitial cells (140). Using specific antibodies, we showed high chymase expression in atrial myocytes of patients with diverse cardiac pathology (2, 99). In addition, expression of Ang-(1–12), chymase mRNA, and enzymatic activity were significantly higher in the left than in the right atrial appendage of human diseased hearts (99).

Other studies have documented the role of chymase as the key enzyme forming Ang II from Ang I in humans (13, 39, 40, 64, 84, 130, 136–138). Excellent reviews by Takai (121, 122) and Dell’Italia and Husain (39) provide a comprehensive analysis of chymase contribution to cardiovascular disease. A schematic diagram of chymase contribution to cardiovascular function is summarized in Fig. 4. For the specific objectives of this review article, we would point out that: (1) chymase shows no enzymatic activity in the circulation partly because of the presence of internal serine protease inhibitors (53) and strict intracellular localization; (2) chymase inhibition has no direct antihypertensive or vasodilator effects nor does it increase plasma renin activity (122); (3) although secretory granules of mast cells are the recognized principal source of chymase, our recent studies identified rat cardiac fibroblasts as enriched with chymase (51, 52); and (4) chymase is a potent intermediate of collagen deposition as it activates the precursor of TGF-β to its active form (120, 158) and promotes an increase in matrix metallopeptidase (MMP-9) activity (27, 101, 125). Fu et al. (51, 52) reported recently that chymase of fibroblast origin induced autophagic degradation of newly synthesized intracellular procollagen I in an experimental model of cardiac volume overload. Mechanisms associated with chymase-mediated cardiac Ang II production may be influenced by multiple factors other than stretch since chronic estrogen treatment attenuates the increases in cardiac Ang II, Ang-(1–7), chymase mRNA, and mast cell number induced in oophorectomized mRen2.Lewis hypertensive rats (150, 159). In addition, Li et al. (83) reported recently that chymase and tryptase induction of cardiac fibrosis may require the protease-activated receptor-2 (PAR-2).

**CLINICAL PERSPECTIVE**

Further investigation of the noncanonical pathways through which tissue Ang II may lead to cardiac and vascular pathology is warranted given the possibility that enzymes and precursor proteins currently been characterized as existing between the primary angiotensinogen substrate and Ang I may explain why ACE inhibitors or even ARBs have limited efficacy in reversing or halting the progression of cardiovascular disease (12, 18, 132, 133). Arguments in favor of this conclusion have been advanced by us (45, 49) and others (60, 90, 156) previously.
These issues were recently underscored in a report summarizing the current limitations for heart failure therapies as elaborated upon a broad representation of health care providers that included clinical and basic science investigators, regulators, and pharmaceutical industry representatives attending a meeting facilitated by the Food and Drug Administration (55). Although it may be argued that the limited effectiveness of RAS blockers may reflect the limited contribution of this system to cardiovascular disease progression, this interpretation does not agree with the overwhelming knowledge that continues to accumulate regarding the cellular and molecular mechanisms by which Ang II contributes to adverse cardiac and vascular remodeling (55). Rigorous analysis of the reduction in important clinical cardiovascular end points in hypertensive patients (18, 132, 133), ischemic heart disease (12), and heart failure (60) are in keeping with this interpretation. In a recently reported meta-analysis of the impact of ACE and ARBs in cardiovascular mortality, Brugts et al. (18) stated that “The annual incidence rate of all-cause mortality was 0.0233 in patients randomized to RAAS inhibitors versus 0.0252 in controls (hazard ratio, 0.95; 95% confidence interval, 0.91 to 0.99).” Furthermore, although ACE inhibitors reduced the combined end point of all-cause and cardiovascular mortality and myocardial infarction, the number of patients that needed to be treated (NNT) for 4.3 years to avoid one event averaged 116 for patients medicated with an ACE inhibitor and 409 for those treated with an ARB (18). A separate analysis of all-cause mortality demonstrated a minor improvement in patients medicated with ACE inhibitors [hazard ratio (HR), 0.90; 95% CI, 0.84 to 0.97] and no differences in those treated with ARBs (HR, 0.99; 95% CI, 0.94 to 1.04) (18). Another comprehensive analysis of the comparative effectiveness of ACE inhibitors and ARBs in ischemic heart disease found a relative risk (RR) reduction of 0.87 (95% CI, 0.81 to 0.94) for total mortality and a RR reduction of 0.83 (CI, 0.73 to 0.94) for nonfatal myocardial infarction with ACE inhibitors (12). Although these data show a benefit from the use of ACE inhibitors in the treatment of heart disease, the residual risk of events (1.0 minus RR) documented in these studies is close to or greater than 70%. Additional studies from well-conducted clinical trials shows that the “residual risk” for cardiovascular events is substantially greater than the risk reduction achieved by these agents (1, 12, 18, 36, 60, 75, 132, 133, 145–147).

We suggest that this discrepancy may be in part accounted for by 1) the existence of tissue-specific noncanonical mechanisms in which ACE is not the obligatory processing pathway for Ang II formation (137); and 2) the almost exclusive role of chymase, rather than ACE, as the Ang II forming enzyme in the human heart (156) and blood vessels (8). It is also possible that ACE inhibition may upregulate the alternate expression of other Ang II-forming enzymes as observed by cardiac chymase upregulation during ACE inhibition in mice (153) or increased vascular rat elastase-2 expression in enalapril-treated rats (14). As noted by Takai et al. (121, 123), a renewed effort for the discovery of better tolerated, orally active chymase inhibitors should be undertaken to effectively and specifically prevent the pathological actions of Ang II in humans.

As documented in this review, chymase is the major Ang II-forming mechanism in humans. Mast cells contain a plethora of mediators, of which the most important in tissue injury are mast cell proteases including tryptase, chymase, and dipeptidyl peptidase I (DPI I), in addition to TNF-α, and stem cell factor (SCF), which are responsible for recruitment of macrophages and neutrophils (22, 104). Targeting mast cells with mast cell stabilizers resulted in left ventricular and cardiomyocyte dysfunction in dogs with mitral regurgitation due to inherent calcium-entry blocking effects of mast cell stabilizers (102). As indicated by chymase’s multiple actions (Fig. 4), cardiac chymase has been identified to contribute to left ventricular remodeling post-myocardial infarction (61, 153), atherosclerosis (8, 135), type 2 diabetes (62, 76), heart failure (87), and cardiac ischemia reperfusion injury (160).

As demonstrated in Fig. 4, blockade of chymase has implications beyond the effects on Ang II formation because of the many other direct protease actions of chymase that have major effects on tissue remodeling, especially in the breakdown of matricellular connections. Our recent studies showed that dual inhibition of ACE and chymase was significantly more effective in decreasing infarct size and left ventricular dilatation, as well as improving cardiac function, compared with ACE inhibitor or chymase inhibitor alone in hamsters 1 mo after coronary occlusion (153). These results underscore the fact that...
chymase inhibitors can target other enzymes in the pathophysiology of left ventricular remodeling and thus may synergize with ACE inhibition or AT1 receptor blockade in the treatment of heart failure.

Finally, our new finding of chymase within the cardiomyocyte during volume overload in the rat opens up an entirely new potential mechanism for Ang II formation and protease-mediated destructive actions within the cell that are untouched by ACE inhibitors or AT1 receptor blockers that act on the cell surface (102). The presence of the chymase-Ang-(1–12) pathway is further exemplified by the failure to achieve additional benefit when more complete blockade of the canonical pathway was obtained with the renin inhibitor aliskerin in patients with heart failure (88). The immediacy of translation of this work is underscored by an ongoing clinical trial of a new type of chymase inhibitor. BAY1142524 (Bayer Health Care) is in phase III clinic trial (NCT02452515; www.clinicaltrials.gov) to treat patients with left ventricular dysfunction after myocardial infarction. In view of the relative plateau in efficacy of neurohormonal blockade in cardiovascular disease including the improvement obtained with the combination of valsartan and the nephrilisyn inhibitor sacubitril (88, 89), we look forward to future applications for the addition of chymase inhibition as an adjunct to existing pharmacotherapy in cardiovascular disease.

Although further research is clearly needed to assess the importance of a nonrenin-dependent Ang II-forming pathway from Ang-(1–12) in human cardiovascular disease, the data reported thus far demonstrate the existence of this functional alternate substrate in cardiovascular tissues, its increased expression and content in the heart of hypertensive strains and the left atrial appendage of heart disease patients, and a singular role for chymase in forming Ang II directly from Ang-(1–12), particularly in human subjects.

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AUTHOR CONTRIBUTIONS

REFERENCES
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