Published in final edited form as:
J Am Soc Hypertens. 2013 March ; 7(2): 128–136. doi:10.1016/j.jash.2012.12.003.

Chymase Mediates Angiotensin-(1-12) Metabolism in Normal Human Hearts

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Abstract

Background—Identification of angiotensin-(1-12) [Ang-(1-12)] in forming Ang II by a non-renin dependent mechanism has increased knowledge on the paracrine/autocrine mechanisms regulating cardiac expression of Ang peptides. This study now describes in humans the identity of the enzyme accounting for Ang-(1-12) metabolism in the left ventricular (LV) tissue of normal subjects.

Methods and Results—Reverse phase HPLC characterized the products of ^{125}I-Ang-(1-12) metabolism in plasma membranes (PMs) from human LV in the absence and presence of inhibitors for chymase (chymostatin), angiotensin converting enzyme (ACE) 1 (lisinopril) and 2 (MLN-4760) and neprilysin (SHC39370). In the presence of the inhibitor cocktail ≥ 98 ± 2% of cardiac ^{125}I-Ang-(1-12) remained intact, whereas exclusion of chymostatin from the inhibitor cocktail led to significant conversion of Ang-(1-12) into angiotensin II. In addition, chymase-mediated hydrolysis of ^{125}I-Ang I was higher compared to Ang-(1-12). Negligible Ang-(1-12) hydrolysis occurred by ACE, ACE2, and neprilysin. A high chymase activity was detected for both ^{125}I-Ang-(1-12) and ^{125}I-Ang I substrates.

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There are no financial disclosures or conflict of interests declared by any of the authors.

Conclusions—Chymase accounts for the conversion of Ang-(1-12) and Ang I to Ang II in normal human LV. These novel findings expand knowledge of the alternate mechanism by which Ang-(1-12) contributes to the production of cardiac angiotensin peptides.

Keywords

ACE2; cardiac myocytes; angiotensin converting enzyme inhibitors; angiotensin II; heart disease; proangiotensin 12; angiotensin-(1-7)

Introduction

The concept that angiotensin II (Ang II) synthesis occurs in multiple organs is now established and construed as primary evidence to explain the role of the renin angiotensin system (RAS) in adverse cardiovascular remodeling, stroke, and renal failure (1). Previous seminal studies demonstrating the existence of divergent biochemical mechanisms, that within the circulating and tissue RAS, account for the formation of the vasodilator and anti-trophic heptapeptide angiotensin-(1-7) [Ang-(1-7)] provided newer insights as to the mechanisms by which the system in the tissues regulates cardiac and vascular homeostasis (2). The further identification of an angiotensin converting enzyme (ACE) homologue [ACE2], insensitive to blockade with ACE inhibitors, and functioning to primarily metabolize Ang II into Ang-(1-7) (3–6) revealed that this servo control system is more complex than originally suspected, as both Ang-(1-7) and ACE2 acts to negatively influence the vasoconstrictor, trophic, and pro-inflammatory actions of Ang II (7).

While past studies showed that Ang II formation from angiotensin I (Ang I) in humans occurs through a non-ACE pathway (8), the importance of this alternate Ang II-forming mechanism in terms of therapeutic approaches has remained relatively ignored. The recent description of an alternate processing pathway for Ang II production upstream from Ang I now expands current concepts of the biochemical mechanisms engaged in the tissue production of Ang II. Nagata et al. (9) first identified in rats an extended form of Ang I, [Angiotensin-(1-12), Ang-(1-12)], able to generate Ang II through ACE. In realizing the potential impact of their observations to the understanding of the tissue actions of Ang II, a series of studies from our laboratory extended Nagata et al. (9) observations by showing: a)- increased expression of Ang-(1-12) in cardiac myocytes of spontaneously hypertensive rats (SHR) compared with the WKY normotensive strain (10); b)- that renin did not contribute to cardiac Ang II formation from Ang-(1-12) (11); and c)- that increased cardiac chymase expression in the hypertrophy heart of adult SHR (10) was associated with increased Ang-(1-12) metabolism by chymase in neonatal cardiac myocytes from the same hypertensive rat strain (12). The potential relevance of these findings to human cardiac pathology was further strengthened by our recent demonstration of Ang-(1-12) expression in left atrial tissue of patients undergoing open-heart surgery for the correction of resistant atrial fibrillation (13). The objective of the current study was to ascertain the enzymatic pathway accounting for the biotransformation of Ang-(1-12) into angiotensin peptides in normal cardiac tissue. This study was warranted by the necessity to uncover whether increased chymase expression occurred only in conditions of cardiac pathology or whether it is the fundamental pathway for Ang II formation in both normal and disease human hearts.

Methods

Ethic Statement

Samples of left ventricular (LV) tissue were obtained from Imgenex Laboratories (San Diego, CA) from six normal humans involved in motor vehicle accidents (MVA). All MVA patients had normal histological examination of the tissue, no history of cardiovascular

disease, and no reported evidence of medications at the time of death (Table 1). The use of these tissues was approved by the University of Alabama at Birmingham Institutional Review Board.

Reagents

Human Ang-(1-12) (>99% purity) was purchased from GenScript USA Inc. (Piscataway, NJ). Chymostatin (chymase inhibitor), lisinopril (ACE inhibitor), amastatin, bestatin, benzyl succinate and *p*-chloromercuribenzoate (PCMB) were purchased from Sigma-Aldrich Co. (St. Louis, MO). SCH39370 (neprilysin inhibitor) was obtained from Merck Inc. (West Point, PA). MLN-4760 (ACE2 inhibitor) was obtained from Millennium Pharmaceuticals (Cambridge, MA). ^{125}Iodine was purchased from PerkinElmer Life and Analytical Sciences, Inc. (Waltham, MA). All other chemicals used in this study were of analytical grade and were obtained from Sigma (St. Louis, MO) and Fisher Scientific (Atlanta, GA).

Iodination (^{125}I) of Ang peptides

The iodination of Ang peptides [Ang-(1-12), Ang I, Ang II and Ang-(1-7)] using the oxidant chloramine-T were done in small plastic tubes at room temperature as described elsewhere (12;13). Briefly, 10 μL of 1 mM angiotensin peptides were added to 20 μL of PBS and 10 μL of 1 mCi of Na[^{125}I] (Perkin-Elmer, Waltham, MA). The iodination reaction was started by adding 10 μL of chloramines-T solution (10–15 mg/10 mL) to the mixture for ~20–30 sec. The reaction was stopped by adding 50 μL of sodium bisulfate solution (30 mg/10 mL MilliQ water). The iodinated Ang peptide was separated from free Na[^{125}I] by passing the mixture through an activated Sep-Pak column. The Sep-Pak eluted iodinated Ang peptide was further purified with reverse-phase high performance liquid chromatography (HPLC) and only monoiodinated Ang peptides [^{125}I-Ang-(1-12), ^{125}I-Ang I, ^{125}I-Ang II and ^{125}I-Ang-(1-7)] were used in these studies. During each experiment, the purity of radiolabelled ^{125}I-Ang substrates was checked on the HPLC to verify that peptides are intact and not degraded before they were added to the PMs. Highly purified form of ^{125}I-Ang substrates (≥98% purity) were only used in our metabolism studies.

Plasma membrane preparation

Native and soluble plasma membranes (PMs) were prepared at 4° C as described previously (12;13). Briefly, frozen LV tissues (50–100 mg) were homogenized at 4° C in 1 mL reaction buffer (25 mM HEPES, 125 mM NaCl and 10 μM $ZnCl_2$, pH 7.4) using a Qiagen Tissue Lyzer (Valencia, CA) for 90 second at 25 Hz. The homogenate was centrifuged at low spin (200 g) for 1 min at 4° C to remove the connective tissues and cell debris. The supernatant was transferred into a new tube and centrifuged at 28,000 g for 20 min at 4° C. The pellet (native membranes) was washed twice by resuspending it in the reaction buffer, centrifuged, and stored at −80° C till its use for metabolism studies.

ACE and ACE2 activity were assayed in solubilized membrane. Native PMs were solubilized in a reaction buffer containing 0.5% triton X-100 on ice overnight. The soluble portion of the native membrane was separated from insoluble portion by centrifugation (28,000 g for 20 min at 4° C). The protein concentrations in each cardiac PMs (native and soluble) were measured by Bradford Reagent using bovine serum albumin as the standard, and the results were normalized in terms of per mg protein.

Metabolism Studies

The metabolism of human ^{125}I-Ang-(1-12) and other Ang peptides by LV PMs (native PMs for chymase and neprilysin and soluble PMs for ACE & ACE2) were studied under different combinations of RAS inhibitors as described previously (13). Briefly, the soluble or native

PMs (25–50 μg per reaction mixture) were preincubated for 10 min in a reaction buffer under various combinations of enzyme and peptidases inhibitors (see Table 2). After preincubation of PMs with different combinations of RAS inhibitors, highly purified human ^{125}I-Ang-(1-12), ^{125}I-Ang I or ^{125}I-Ang II [1 nmol/L each; ≥98% purity] was added to the reaction medium and incubated for specific time points (30 min for chymase, 60 min for ACE/NEP, and 120 min for ACE2) at 37° C. These time points were chosen on the basis of pilot studies showing full conversion of Ang-(1-12) into the products at each of the respective incubation periods. At the end of the incubation time, the reaction was stopped by adding equal volume of 1% phosphoric acid, mixed well, centrifuged (28,000 g for 20 min to remove the native PMs or 16, 000 g for 1 min in case soluble PMs) and stored at 4° C. On the day of Ang peptide analysis, the samples were filtered before separation by HPLC. We used a linear gradient from 10% to 50% mobile phase B at a flow rate of 0.35 mL/min at 32° C. The solvent system consisted of 0.1% phosphoric acid (mobile phase A) and 80% acetonitriles/0.1% phosphoric acid (mobile phase B). The eluted ^{125}I products were monitored by an in-line flow-through gamma detector (BioScan Inc., Washington, DC). Products were identified by comparison of retention time of synthetic [^{125}I] standard peptides and the data were analyzed with the Shimadzu LCSolution (Kyoto, Japan) acquisition software. Experiments were performed three or more times. The metabolic products are shown as percent of Ang peptides fraction generated from the parent substrate by specific RAS enzymes. The RAS enzyme activity was calculated based on the amount of parent ^{125}I-Ang substrate hydrolyzed into specific ^{125}I-Ang products by the LV PMs isolated from normal tissues at 37° C with or without the presence of specific RAS inhibitors. RAS enzyme activities are reported as fmoles of Ang product generated from the parent ^{125}I-Ang substrate (fmol × min^{-1} × mg^{-1}).

Western Blot Analysis and Immunohistochemistry

The expression of chymase protein in human normal LV PM samples (n=4) were assessed by immunoblot technique as previously described by us (13). Briefly, the LV PMs were separated by gel electrophoresis (10% gel) and transferred to polyvinylidene defluoridated membranes (PVDF). The PVDF membranes were probed with a primary monoclonal anti-human chymase antibody (CMA1 antibody from R&D System, Minneapolis, MN, Cat # MAB4099; 2 μg/mL). After incubation with the primary antibody, the membranes were probed with the horseradish peroxidase-conjugated secondary antibody (anti-mouse, 1:3000; Pierce Inc., Rockford, IL, USA). Immune complexes were visualized using ECL plus detection reagents (Thermo Scientific Pierce Protein Biology Products, Rockford, Illinois). The blots were probed for equal loading using RedAlert (14).

Statistical Analysis

Experiments were repeated three or more times. All values are reported as means ± SEM. The Student's *t*-test and repeated-measures ANOVA followed by a Turkey's post hoc test for multiple comparisons were used to determine significant differences at $P < 0.05$ using GraphPad Prism 5.0 software (San Diego, CA).

Results

^{125}I-Ang peptide hydrolysis

Under the conditions described in Methods, there was excellent separation of the radiolabeled products derived from the addition of either ^{125}I-Ang-(1-12) or ^{125}I-Ang I in both the presence and absence of the inhibitor cocktail.

In the presence of all RAS inhibitors a single peak corresponding to the Ang-(1-12) retention time was found on the chromatogram [≥ 98 ± 2% of ^{125}I-Ang-(1-12) remained

intact]. Figure 1 (panel A) shows Ang II as the only peptide peak detected from the hydrolysis of ^{125}I-Ang-(1-12) when chymostatin was excluded from the cocktail of inhibitors. Removal of either lisinopril (ACE inhibitor), SCH39370 (neprilysin inhibitor) or MLN-4760 (ACE2 inhibitor), results in the appearance of very minor peaks corresponding to the retention times for Ang I, Ang II, Ang-(1-7) (Figure 1, panels B, C and D, respectively). These data show that chymase is the preferred enzyme accounting for the formation of Ang II from Ang-(1-12) in normal human hearts.

Table 3 shows the average results of ^{125}I-Ang-(1-12) hydrolysis by LV tissue of normal hearts. Chymase constitutes the primary ^{125}I-Ang-(1-12) metabolizing enzyme in normal LV tissue while ACE, neprilysin and ACE2 showed essentially no hydrolytic activity on Ang-(1-12) as a substrate (Table 3).

Table 4 documents the products generated by PMs when ^{125}I-Ang I rather than ^{125}I-Ang-(1-12) is used as a substrate. Chymase-mediated Ang II formation from the ^{125}I-Ang I substrate (80 ± 5%, Table 4) was the predominant metabolite formed by the human LV samples. The fraction of Ang II produced from ^{125}I-Ang I metabolism is higher than that resulting from the incubation with ^{125}I-Ang-(1-12) (Table 3), a finding that corroborates prior evidence of chymase as a human Ang II forming enzyme from Ang I (8;15;16). In agreement with prior findings, Figure 2 shows the formation of ^{125}I-Ang II from ^{125}I-Ang-(1-12) by chymase in the presence of increasing concentration of unlabeled Ang I or human Ang-(1-12) peptide [0–250 μM]. In the presence of unlabeled Ang I or Ang-(1-12) ^{125}I-Ang II formation from ^{125}I-Ang-(1-12) by chymase was equally reduced. ACE-mediated Ang II formation from Ang I by normal PMs was higher (15 ± 3%) when compared to Ang-(1-12) (2 ± 1%). Similar to Ang-(1-12) (Table 3), Ang I was also poorly metabolized by neprilysin (Table 4).

The effect of using ^{125}I-Ang II as a substrate was evaluated by removal of the potent ACE2 inhibitor (MLN-4760) from the inhibitor cocktail. ACE2-mediated hydrolysis of ^{125}I-Ang II substrate into Ang-(1-7) product formation averaged 9.5 ± 1.8%. ACE2 mediated conversion of labeled Ang I was not investigated in these studies since the enzyme has a very low affinity for the decapeptide (5).

Relative Contributions of RAS enzymes in LV PMs

Figure 3 shows the relative expression of RAS enzymes activities in the LV PMs isolated from human samples. Enzymes activities were calculated from the specific Ang products generated from either Ang-(1-12) or Ang I (for chymase, ACE and neprilysin) or from Ang-(1-12) or Ang II (for ACE2) hydrolysis under different combinations of RAS inhibitors. In keeping with the metabolism studies, cardiac chymase activity was several folds higher as compared to ACE, neprilysin, and even ACE2. Chymase activity accounting for Ang II formation from both radiolabeled Ang-(1-12) and Ang I substrate hydrolysis averaged 30.2 ± 3.4 fmol × min^{-1} × mg^{-1} and 51.9 ± 3.4 fmol × min^{-1} × mg^{-1}, respectively (Figure 3). The generation of Ang II from Ang-(1-12) by ACE activity is much lower when compared to chymase in normal LV (8.0 ± 1.3 fmol × min^{-1} × mg^{-1}). On the other hand, higher ACE activity was detected in normal LV (16.2 ± 3.0 fmol × min^{-1} × mg^{-1}) when ^{125}I-Ang I was employed as a substrate instead of ^{125}I-Ang-(1-12). Low neprilysin activity was detected either using Ang-(1-12) or Ang I as substrate in normal subjects. Neprilysin activity detected in normal PMs averaged 2.4 ± 0.6 fmol × min^{-1} × mg^{-1} using ^{125}I-Ang-(1-12) substrate, and 2.7 ± 0.6 fmol × min^{-1} × mg^{-1} for ^{125}I-Ang I substrate. ACE2 activity was also low using ^{125}I-Ang-(1-12) as substrate in normal PMs (3.4 ± 0.6 fmol × min^{-1} × mg^{-1}) but significantly higher ACE2 activity was detected for ^{125}I-Ang II substrate [6.8 ± 1.1 fmol × min^{-1} × mg^{-1}as compared to ^{125}I-Ang-(1-12) substrate]. Chymase protein expression by Western blot technique is shown in Figure 4.

Discussion

This study provides the first characterization of the enzymatic pathways for Ang II and Ang-(1-7) production from Ang-(1-12) in LV tissue obtained from normal subjects. The results obtained in these experiments underscore the characteristics of the human cardiac membrane-bound enzymes accounting for the generation of angiotensin peptides from Ang-(1-12). Chymase, functioning as an ectoenzyme within the plasma membrane of LV cells, has a predominant activity in degrading either Ang-(1-12) or Ang I in normal heart tissue. ACE, neprilysin, or ACE2 do not contribute significantly to the formation of Ang II from Ang-(1-12) in normal hearts. In addition, we now show that chymase-mediated Ang II formation from Ang-(1-12) does not require an intermediate processing to Ang I. The existence of an alternate processing pathway for angiotensin peptide formation from Ang-(1-12) in human hearts is an important step in establishing whether direct production of Ang II from Ang-(1-12) contributes to adverse cardiac remodeling and failure (7;17) bypassing an intermedia processing into Ang I.

Our earlier demonstration of chymase expression and function in Ang-(1-12) metabolism in cardiac myocytes obtained from the atrial tissue of subjects with the diagnosis of atrial fibrillation (13) had posit the question of whether this metabolic pathway was active only in the diseased human heart. The current study now clearly demonstrates that normal human LV tissue, via a chymase pathway, has the capacity for direct generation of Ang II from Ang-(1-12). The past demonstration (7;13) that inhibition of Ang-(1-12) conversion into Ang II in the human atria by chymostatin was comparable to the results obtained with a highly selective orally active chymase inhibitor (TEI-F00806) (18;19) affirms that chymase is the cardiac enzyme accounting for the conversion of Ang-(1-12) into Ang II. In agreement with other studies we also showed that both human Ang I and human Ang-(1-12) competed equally for chymase to generate Ang II. Similarly, the chymase-mediated hydrolytic ^{125}I-Ang II formation from ^{125}I-Ang I, under same experimental conditions, was also equally reduced in the presence of increasing concentration of cold Ang-(1-12).

The identification of the dodecapeptide Ang-(1-12) as a source for angiotensin peptides generation was first reported by Nagata et al. (9) in the circulation and cardiovascular tissues of Wistar rats. These investigators further showed that administration of this extended form of Ang I elicited pressor and vasoconstrictor response that were blocked by either an ACE inhibitor or the Ang II receptor blocker candesartan. The catalytic activity of ACE in Ang-(1-12) metabolism was confirmed in additional studies in the circulation of WKY and SHR (20), isolated rat arteries (21), the rat brain (22), and in the serum and kidney of a congenic rat model of hypertension due to increased tissue renin expression (23). On the other hand, two other studies implicated chymase as a source for Ang II from Ang-(1-12) (12;24). In an isolated heart model of ischemia-reperfusion in the rat, inhibition of chymase with chymostatin significantly reduced Ang II production and attenuated Ang-(1-12)-induced vasoconstriction and myocardial damage following ischemia while ACE inhibition was without effect (24). In the other study we showed that while ACE and neprilysin accounted for Ang-(1-12) metabolism in the medium of neonatal myocytes from WKY rats, an additional role for chymase was found in SHR (12). This latter study suggested a chymase participation in conditions of acute or chronic cardiac stress.

It may be argued that studies of Ang-(1-12) metabolism in plasma membranes may differ from the conversion pathways existing in intact tissue. A comparative study in intact cardiac tissue from normal hearts is not practical or even possible as human fresh tissue will be impossible to recover. On the other hand, previous studies of Ang-(1-12) metabolism in the isolated perfused rat heart (11), rat neonatal cardiac myocytes (12), and those reported by Prosser et al. (21;24) in Sprague Dawley heart and the vasculature do not suggest differences

in Ang-(1-12) processing between intact cardiac tissue and isolated membranes. This interpretation agrees with the previous demonstration that both chymase and Ang-(1-12) were expressed endogenously in rat hypertensive neonatal cardiac myocytes (12) and diseased human atrial tissues (13).

Western blot findings in the human tissue confirmed the presence of the enzyme in cardiac tissue. It is known that chymase is stored in a macromolecular complex with heparin proteoglycan within mast cell granules and is more resistant to inhibit by macromolecular natural serine protease inhibitors such as a-antitrypsin (25;26). The chymase protein remains complexed even after degranulation from mast cells. The discrete cardiac chymase expression in the heart tissue suggest that chymase-dependent Ang-(1-12) processing may occur in the extracellular cardiac milieu although we also showed that Ang-(1-12) uptake by cardiac neonatal myocytes (12). Our studies also showed that levels of chymase expression differed from sample to sample, a finding that has been observed previously by others (27;28). Furthermore, the amount of Ang II formation from Ang-(1-12) by chymase activity was consistent with the chymase protein expression in these human samples. While this warrants further study, the variability of enzyme activity might be related to factors such age and gender. There was also some variability in the molecular weight of chymase protein expression in the studied tissues. More than one band for human cardiac chymase (major bands with molecular weight ~27–32 Kd and minor protein bands ~17–19Kd) have been reported by others (29;30) while Frith et al. (31) showed two chymase bands and a degraded product (32 Kd and 27 Kd) in human wounded cells crude lysates.

From the preceding it is now clear that there is a fundamental difference in the mechanisms of Ang II production and enzymatic pathways for cardiac Ang-(1-12) metabolism between rodents and humans, as both our current and past studies (13;15) and those reported by others (32–36) showed that chymase rather than ACE is the primary pathway for the production of Ang II in humans. In support for this interpretation, the alpha chymase gene is expressed in humans and baboons while both the alpha and beta forms of the chymase gene are expressed in rats and mouse (15). While the alpha chymase has highly specific and efficient Ang II-forming activity, the beta chymase shows broad substrate specificity and do not form Ang II (35). Differences in the processing enzymes accounting for Ang-(1-12) metabolism may be influenced also by the route of processing: i.e., intravascular versus the tissue interstitial compartments. Our data and those obtained elsewhere demonstrate that while Ang-(1-12) metabolism in the circulation is mediated by ACE (9;12;20;23), chymase is the primary enzyme accounting for the cleavage of this substrate in human heart tissue. We further showed that ACE2 does not contribute to Ang II formation from Ang-(1-12), although less efficiently, it is reported to convert Ang I to Ang-(1-9) (4;37).

While a robust clinical and experimental literature established the importance of the ACE-mediated Ang II production in the regulation of blood pressure and cardiorenal function, the role of this enzyme as a primary loci for Ang II formation from Ang I remains an open question as conversion of Ang I to Ang II in tissues can proceed despite complete ACE inhibition (35) and the effectiveness of these drugs in the prevention of cardiac arrhythmias, and the progression of cardiac dysfunction is not completely certain (16;17;38–40). Since the original observation by Urata et al. (8) of chymase as an Ang II-forming enzyme, evidence for its direct role in the pathogenesis of human cardiovascular disease continues to increase (15;19;27–29;34;36;41–47). The current studies and those reported by us previously (13) expand knowledge of the cardiac processing pathways accounting for Ang II formation via the actions of chymase rather than ACE or even neprilysin. The current findings further illustrate the complexity of alternate pathways for Ang II production and species differences. These data warrants reconsideration of current therapies for suppressing the pathological actions of Ang II in humans through the further evaluation of the potential

benefits of chymase inhibition in human cardiovascular disease. The discovery of a chymase-dependent primary pathway for the direct formation of Ang II from Ang-(1-12) by chymase in the human heart implies a distinct pathophysiological role of this Ang II-forming enzyme particularly in conditions in which the catalytic activities of renin or ACE are inhibited. In summary, in LV tissue obtained from normal hearts, the enzymatic pathway accounting for the production of Ang II entails the direct cleavage of Ang-(1-12) via chymase.

Acknowledgments

The studies reported here were supported by a grant (HL-051952) from the Heart, Lung, Blood Institute of the National Institutes of Health. We also acknowledge partial support provided by the Farley-Hudson Foundation, Jacksonville, NC.

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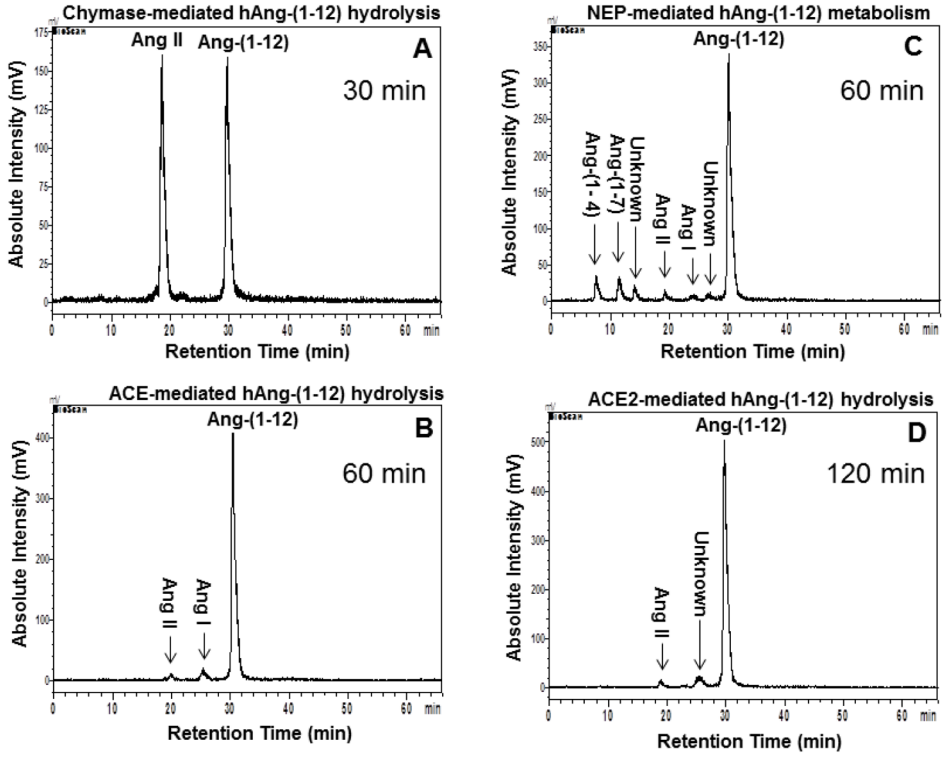

Figure 1.

^{125}I-Ang-(1-12) hydrolysis by Normal Left Ventricular Plasma Membranes. Chromatograms show the hydrolysis of ^{125}I-Ang-(1-12) in normal human left ventricular. *Panel A:* Without chymostatin (chymase-mediated hydrolysis, 30 min), *Panel B:* Without lisinopril (ACE-mediated hydrolysis, 60 min), *Panel C:* Without SCH39370 (NEP-mediated hydrolysis, 60 min), and *Panel D:* Without MLN-4760 (ACE2-mediated hydrolysis, 120 min). Results are representative of three or more separate metabolism experiments for each human sample.

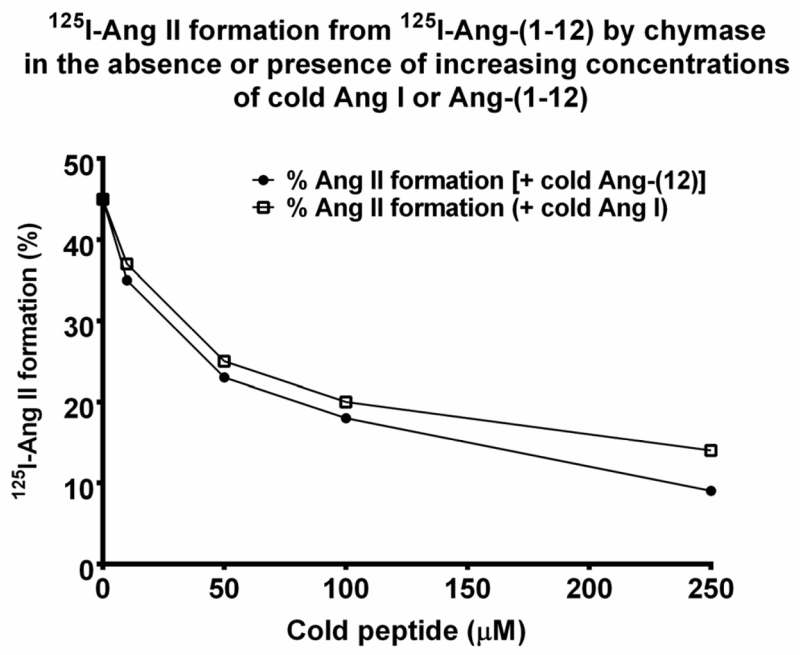

Figure 2.

HPLC analysis of ^{125}I-Ang II formation (%) from ^{125}I-Ang-(1-12) substrate by chymase in the absence or presence of cold Ang-(1-12) or Ang I. As described in Methods, the reaction mixture contained 50 μg of plasma membrane plus ALL RAS inhibitors (except chymostatin) plus substrate incubated in the absence or presence of increasing concentration of cold Ang-(1-12) or Ang I (range: 0–250 μM for 30 min at 37° C.

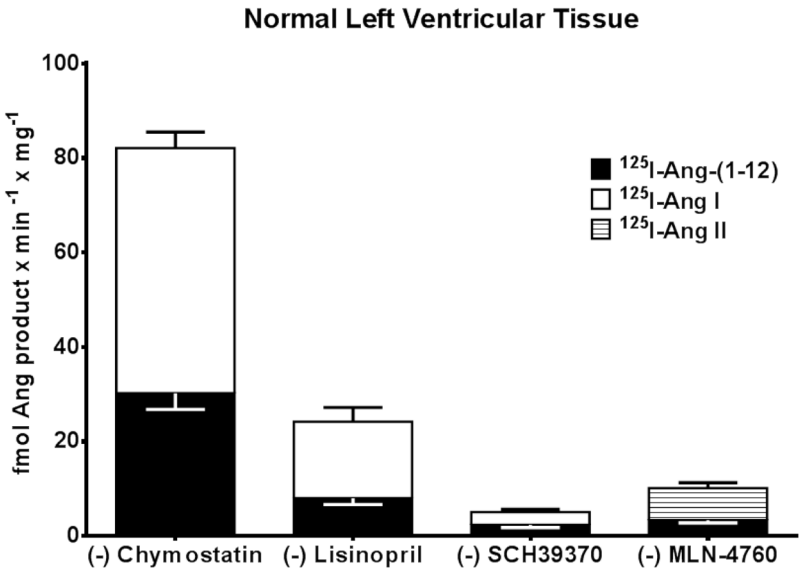

Figure 3.

RAS enzyme activities calculated based on the amount of parent ^{125}I-Ang substrate hydrolyzed into products by PMs prepared from human left ventricular tissue with or without the presence of specific RAS inhibitors. Values are Mean ± SEM from parent ^{125}I-Ang substrate. Each experiment was done at least three times and the results are the average of six normal human subjects.

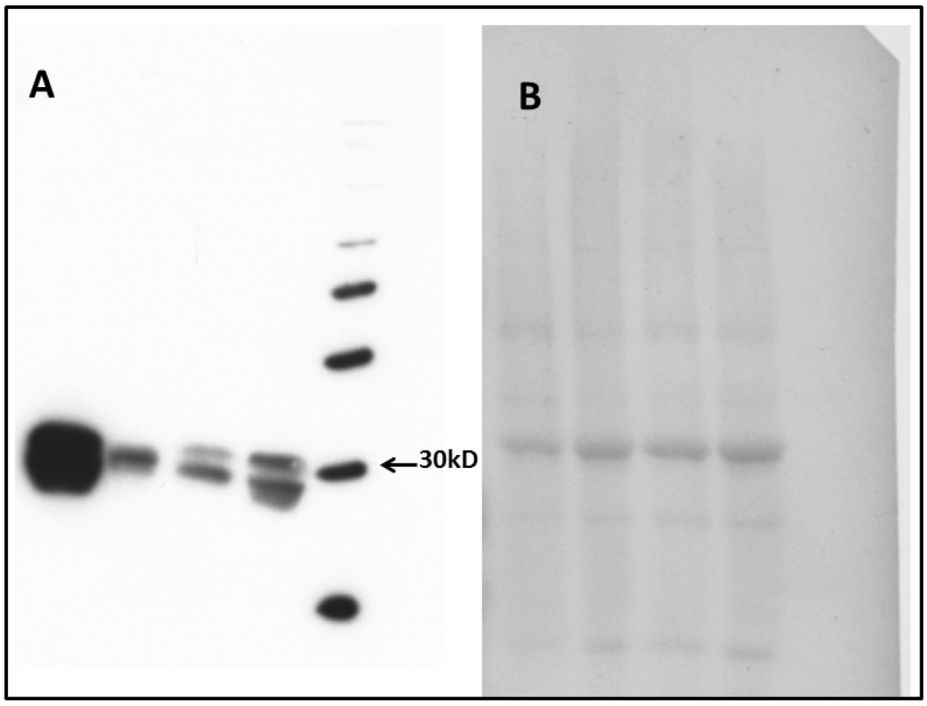

Figure 4.
Panel A, Chymase protein expression in human left ventricular tissue (n=4).

Table 1

Characteristics of Normal Human Heart Tissue

	Age	Gender	Cause of Death	Medications
Normal Tissue:				
Sample 1	49	male	MVA	None documented
Sample 2	38	female	MVA	None documented
Sample 3	61	male	MVA	None documented
Sample 4	42	male	MVA	None documented
Sample 5	42	male	MVA	None documented
Sample 6	25	male	MVA	None documented

Abbreviations: MVA, motor vehicle accident.

Table 2

Outline of Enzyme Inhibitors Used in the Experiments

Group	Inhibitors added (50 μM each)
All RAS inhibitors:	RAS inhibitors [chymostatin for chymase; lisinopril for angiotensin converting enzyme; SCH39370 for neprilysin; and MLN-4760 for angiotensin converting enzyme 2]; amastatin, bestatin, benzyl succinate, and P-chloromercuribenzoate (PCMB).
Minus RAS inhibitors:	All above inhibitors except one of the RAS inhibitors [chymostatin/lisinopril/SCH39370/MLN-4760] omitted at a time from the reaction mixture.

Table 3

Metabolism of ^{125}I-Ang-(1-12)* by RAS enzymes in Plasma Membranes Isolated from Left Ventricular Tissues

Enzyme/Peptide	Ang-(1-12)*	Ang I	Ang II	Ang-(1-7)	Ang-(1-4)	Unknown
Chymase Contribution: (chymostatin absent)						
	53 ± 7	ND	47 ± 7	ND	ND	ND
Angiotensin Converting Enzyme Contribution (lisinopril absent)						
	93 ± 1	5 ± 1	2 ± 1	ND	ND	ND
Neprilysin Contribution: (SCH39370 absent)						
	87 ± 2	1.0 ± 0.2	2.0 ± 0.3	3 ± 1	3.0 ± 0.7	4 ± 1
Angiotensin Converting Enzyme 2 Contribution: (MLN-4760 absent)						
	95 ± 1	ND	1.0 ± 0.2	ND	ND	4 ± 1

HPLC of human ^{125}I-Ang-(1-12) metabolic products generated by PMs prepared from human LV tissue incubated with or without the presence of RAS inhibitors. The ^{125}I-Ang-(1-12) substrate was ≥98% intact before adding to the PM for metabolism reaction. Values are expressed as % (Mean ± SEM) of Ang peptides generated from parent ^{125}I-Ang-(1-12) substrate.

*Represents amount of parent ^{125}I-Ang-(1-12) substrate that remained unmetabolized at the end of reaction.

ND = not detected.

Table 4

Metabolism of ^{125}I-Ang I by Plasma Membranes Isolated from Left Ventricular Tissue

Enzyme/Peptide	Ang I	Ang II	Ang-(1-7)	Ang-(1-4)	Unknown
Chymase Contribution: (chymostatin absent)					
	20 ± 5	80 ± 5	ND	ND	ND
Angiotensin Converting Enzyme Contribution: (lisinopril absent)					
	85 ± 3	15 ± 3	ND	ND	ND
Neprilysin Contribution: (SCH39370 absent)					
	82 ± 2	6 ± 1	7 ± 1	4 ± 1	1 ± 0.2

Values are expressed as % (Mean ± SEM) of Ang peptides generated from parent ^{125}I-Ang substrate (purity ≥98%).

*Percent of corresponding ^{125}I-Ang I substrate that remained un-metabolized at the end of reaction. Each experiment was done at least three times and the results are the average of six normal human subjects.

ND = not detected.