Primacy of cardiac chymase over angiotensin converting enzyme as an angiotensin-(1-12) metabolizing enzyme

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Abstract

We showed previously that rat angiotensin-(1-12) [Ang-(1-12)] is metabolized by chymase and angiotensin converting enzyme (ACE) to generate Angiotensin II (Ang II). Here, we investigated the affinity of cardiac chymase and ACE enzymes for Ang-(1-12) and Angiotensin I (Ang I) substrates. Native plasma membranes (PMs) isolated from heart and lung tissues of adult spontaneously hypertensive rats (SHR) were incubated with radiolabeled ^{125}I-Ang-(1-12) or ^{125}I-Ang I, in the absence or presence of a chymase or ACE inhibitor (chymostatin and lisinopril, respectively). Products were quantitated by HPLC connected to an in-line flow-through gamma detector. The rate of ^{125}I-Ang II formation from ^{125}I-Ang-(1-12) by chymase was significantly higher (heart: 7.0 ± 0.6 fmol/min/mg; lung: 33 ± 1.2 fmol/min/mg, $P < 0.001$) when compared to ^{125}I-Ang I substrate (heart: 0.8 ± 0.1 fmol/min/mg; lung: 2.1 ± 0.1 fmol/min/mg). Substrate affinity of ^{125}I-Ang-(1-12) for rat cardiac chymase was also confirmed using excess unlabeled

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Ang-(1-12) or Ang I (0–250 μM). The rate of ^{125}I-Ang II formation was significantly lower using unlabeled Ang-(1-12) compared to unlabeled Ang I substrate. Kinetic data showed that rat chymase has a lower K_m (64 ± 6.3 μM *vs* 142 ± 17 μM), higher V_{max} (13.2 ± 1.3 μM/min/mg *vs* 1.9 ± 0.2 μM/min/mg) and more than 15-fold higher catalytic efficiency (ratio of V_{max}/K_m) for Ang-(1-12) compared to Ang I substrate, respectively. We also investigated ACE mediated hydrolysis of ^{125}I-Ang-(1-12) and ^{125}I-Ang I in solubilized membrane fractions of the SHR heart and lung. Interestingly, no significant difference in ^{125}I-Ang II formation by ACE was detected using either substrate, ^{125}I-Ang-(1-12) or ^{125}I-Ang I, both in the heart (1.8 ± 0.2 fmol/min/mg and 1.8 ± 0.3 fmol/min/mg, respectively) and in the lungs (239 ± 25 fmol/min/mg and 248 ± 34 fmol/min/mg, respectively). Compared to chymase, ACE-mediated Ang-(1-12) metabolism in the heart was several fold lower. Overall our findings suggest that Ang-(1-12), not Ang I, is the better substrate for Ang II formation by chymase in adult rats. In addition, this confirms our previous observation that chymase (rather than ACE) is the main hydrolyzing enzyme responsible for Ang II generation from Ang-(1-12) in the adult rat heart.

Keywords

Rat cardiac chymase; Angiotensin-converting enzyme; Angiotensin-(1-12); Angiotensin I; Angiotensin II; Renin-angiotensin system

1. Introduction

The renin-angiotensin system (RAS), as a major mechanism contributing to the regulation of blood pressure, body fluid, and tissue remodeling, has a critical role in the maintenance of tissue perfusion and homeostasis [1–3]. Seminal studies demonstrating the existence of an inhibitory arm of the RAS through the expression of angiotensin-(1-7) and angiotensin converting enzyme 2 (ACE2) [4] are now expanded with the discovery of an extended form of angiotensin I (Ang I) [5] which our laboratory characterized as an intracrine tissue precursor for direct angiotensin II (Ang II) production [6–8]. The ubiquitous distribution of the dodecapeptide angiotensin-(1-12) [Ang-(1-12)] in rodent tissue [5] was shown by us to be augmented in the heart of spontaneously hypertensive rats (SHR) [9] and also in the left atrial appendage tissue of patients with left-sided heart disease [10].

In pursuing the hypothesis that Ang-(1-12) serves as an intracrine substrate for direct Ang II formation by chymase in the heart [11,12], the question was raised as to whether ACE plays any role in the hydrolysis of Ang-(1-12) into Ang II either directly or through the intermediate production of Ang I. Previous studies demonstrating that both ACE and chymase could be involved in degrading Ang-(1-12) into Ang I and Ang II [6,13,14] prompted us to study the comparative specific enzymatic activities and affinities of ACE and chymase for Ang II formation. Although chymase, as an Ang II-forming enzyme from Ang I, has a long-standing history [15–19], the clinical importance of this alternate pathway continues to remain unappreciated. Recent research [20], however, clearly suggests that the documented relative therapeutic efficacy of ACE inhibitors and Ang II receptor blockers (ARBs) may be due to activation of an alternate pathway for Ang II formation, the inability of these drugs to access the intracellular sites at which Ang II is generated, or both

mechanisms. While the existence of an intrinsic mechanism for the intracellular generation of Ang II in cardiac myocytes has been demonstrated by others [21–27], no data exist as to whether Ang-(1-12) or Ang I is the preferred substrate for cellular Ang II formation via chymase or ACE.

2. Materials and methods

2.1. Reagents

Rat angiotensin-(1-12) (>99% purity) was purchased from GenScript USA Inc. (Piscataway, NJ). MLN-4760 (ACE2 inhibitor) was obtained from Millennium Pharmaceuticals (Cambridge, MA). Lisinopril (ACE inhibitor), SCH39370 (neprilysin inhibitor), chymostatin (chymase inhibitor), amastatin, bestatin and benzyl succinate and 4-Chloromercuribenzoic acid (PCMB) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Radioactive ^{125}I was purchased from PerkinElmer Life and Analytical Sciences, Inc. (Waltham, Massachusetts). All other chemicals used in this study were of analytical grade and were obtained from Sigma (St. Louis, MO) and Fisher Scientific (Atlanta, GA).

2.2. Plasma membrane preparation

Native and soluble plasma membranes (PMs) were prepared from the heart and lungs of adult male SHR rats at 4 °C as described previously [7,8,28]. Briefly, frozen cardiac left ventricular or lung tissues (50–100 mg) were homogenized in 1 mL reaction buffer containing 25 mM HEPES, 125 mM NaCl and 10 μM $ZnCl_2$ (pH 7.4) using a Qiagen Tissue Lyzer (Valencia, CA) for 60 s at 20 Hz. The homogenates were centrifuged at low spin (200 g) for 1 min to remove the connective tissues and cell debris. The supernatants were transferred into clean tubes and centrifuged at 28,000 g for 20 min to collect the native PMs. The resultant pellets (native PMs) were washed twice by re-suspending them in the reaction buffer, centrifuged, and stored at −80 °C until their use for evaluation of angiotensin peptide metabolism and measure of chymase and ACE enzyme activities.

ACE activity was measured in the solubilized PMs. For this, the native PMs were solubilized in a reaction buffer containing 0.5% triton X-100 on ice overnight. The soluble portion of the native PMs was separated from insoluble material by centrifugation at 28,000 g for 20 min. The protein concentrations (mg protein/mL) of PMs (native and soluble) were measured by BCA protein assay kit (Pierce, Thermo Scientific, Rockford, IL).

2.3. HPLC analysis of ^{125}I-Ang metabolic products

Metabolic products of ^{125}I-Ang-(1-12) and ^{125}I-Ang I by PMs were analyzed by reverse-phase high-performance liquid chromatography (HPLC) under different combinations of RAS inhibitor cocktails as described in Table 1. Briefly, the PMs (50–100 μg per reaction mixture) were pre-incubated with various combinations of RAS and peptidase inhibitors (50 μM each) for 15 min, then a highly purified ^{125}I-Ang-(1-12) or ^{125}I-Ang I (1 nmol/l) substrate was added to the reaction mixtures and incubated for an additional 60 min (for chymase) or 120 min (for ACE) at 37° C. The enzymatic reactions were stopped by adding an equal volume of 1% phosphoric acid, mixing well and centrifuging at 28,000 g for 20 min to remove the PMs. The clear supernatants were filtered and ^{125}I-Ang products were

separated by HPLC on a C-18 column. A linear gradient from 10% to 50% mobile phase B at a flow rate of 0.35 mL/min at 32° C was used for the HPLC analyses. The solvent system consisted of 0.1% phosphoric acid (mobile phase A) and 80% acetonitrile/0.1% phosphoric acid (mobile phase B). The eluted [125]IAng products were monitored by an in-line flow-through gamma detector (BioScan Inc., Washington, DC). Products were identified by comparison of retention times of synthetic [^{125}I] standard Ang peptides and the data were analyzed with Shimadzu LCSolution (Kyoto, Japan) acquisition software.

2.4. ^{125}I-Ang-(1-12) and ^{125}I-Ang I metabolism by chymase and ACE

The affinities of cardiac and lung chymase and ACE to hydrolyze the ^{125}I-Ang-(1-12) or ^{125}I-Ang I substrate were analyzed by measuring the amount of ^{125}I-Ang products in the presence of a cocktail containing all RAS inhibitors and after subtracting from the cocktail either chymostatin or lisinopril. Supernatants were collected after exposing the ^{125}I-Ang-(1-12) or ^{125}I-Ang I substrate to native PMs for 60 min (cardiac chymase) and soluble PMs for 120 min (cardiac ACE) at 37 °C; the generated products were analyzed by HPLC. For lung chymase and ACE, the PMs were incubated for only 30 min at 37° C. The enzyme activities were calculated based on the amount of ^{125}I-Ang products generated from ^{125}I-Ang-(1-12) or ^{125}I-Ang I by chymase and ACE. Enzyme activities were reported as fmoles of ^{125}I-Ang II formation from parent substrate [^{125}I-Ang-(1-12) or ^{125}I-Ang I] per min per mg protein (fmol/ min/mg protein).

The affinities of chymase for Ang-(1-12) or Ang I substrates were investigated by measuring the amount of radiolabeled ^{125}I-Ang II generated from ^{125}I-Ang-(1-12) substrate in the presence of increasing concentrations of non-radiolabeled Ang-(1-12) or Ang I. Briefly, native PMs (50–100 μg) were incubated with radiolabeled ^{125}I-Ang-(1-12) with or without increasing concentrations of non-radiolabeled Ang-(1-12) or Ang I substrate (range: 0–250 μM Ang peptides) added into the reaction mixture in the presence of all RAS inhibitors cocktail or absence of chymostatin only for 120 min at 37 °C. At the end of the incubation time, the reaction mixtures were stopped by adding an equal volume of 1% phosphoric acid and the amount of ^{125}I-Ang II formation from ^{125}I-Ang-(1-12) was analyzed by HPLC as described above.

2.5. K_m and V_{max} of cardiac chymase for Ang-(1-12) and Ang I substrates

To determine the K_m and V_{max} of cardiac chymase, native PMs (100 μg) were incubated with increasing concentrations (5–300 μM) of either Ang-(1-12) or Ang I substrate in the presence of lisinopril (200 μM; ACE inhibitor) at 37 °C for 30 min. The Ang II product formation from Ang-(1-12) or Ang I substrate was separated by HPLC and fraction elution was monitored as the absorbance at 215 nm. The concentration of Ang II was determined using a standard curve of Ang II synthetic peptide. The K_m and V_{max} of cardiac chymase for Ang-(1-12) and Ang I substrates were calculated using a Michaelis-Menten equation.

2.6. Statistical analysis

Experiments were repeated three or more times. All values are reported as mean ± SE. The Student's *t*-test and repeated-measures ANOVA followed by a Tukey's post hoc test for

multiple comparisons were used to determine the significant differences at a probability of 0.05 using GraphPad Prism 5.0 software (San Diego, CA).

3. Results

Fig. 1(A–F) illustrates the HPLC chromatograms of peptides generated during incubation of ^{125}I-Ang-(1-12) and ^{125}I-Ang I when either chymostatin (Panels 1C – 1D) or lisinopril (Panels 1E – 1F) were subtracted from the inhibitor cocktail (panels 1A – 1B) under the conditions described in the Methods. As shown in panels A and B of Fig. 1, no ^{125}I-Ang II metabolic product formation was detected in the presence of all inhibitors either, from ^{125}I-Ang-(1-12) or ^{125}IAng I. In the absence of chymostatin only, a large peak of ^{125}I-Ang II product formation by chymase was detected from the ^{125}I-Ang-(1-12) parent substrate (Fig. 1C). Using ^{125}I-Ang I as a substrate, however, only a small peak of ^{125}I-Ang II was detected in the absence of chymostatin (Fig. 1D). On the other hand, a small ^{125}IAng II peak was detected from either ^{125}I-Ang-(1-12) or ^{125}I-Ang I substrate in the absence of lisinopril only (Fig. 1E and F). Further analysis revealed that lung chymase (native PMs) also generates a large peak of ^{125}I-Ang II product from ^{125}I-Ang-(1-12), but not from the ^{125}I-Ang I parent substrate (Fig. 2A and B).

The substrate specificity of chymase for ^{125}I-Ang-(1-12) was further confirmed using increasing concentrations of non-radiolabeled Ang-(1-12) or Ang I. This analysis indicated that radiolabeled ^{125}I-Ang II formation from ^{125}I-Ang-(1-12) substrate by cardiac chymase was significantly reduced with increasing concentrations of non-radiolabeled Ang-(1-12) compared to non-radiolabeled Ang I (Fig. 3). Kinetic analysis (K_m, V_{max} and catalytic efficiency) of cardiac chymase was also determined for Ang-(1-12) and Ang I substrates. A representative curve showing Ang II formation by cardiac chymase with increasing concentrations of Ang-(1-12) and Ang I substrates is shown in (Fig. 4). The K_m and V_{max} were 64 ± 6.3 μM and 13.2 ± 1.3 μM/min/mg of chymase/Ang-(1-12) and 142 ± 17 μM and 1.9 ± 0.2 μM/min/mg of chymase/Ang I reactions, respectively. The catalytic efficiency (the ratio of V_{max}/K_m ratio) was more than 15-fold higher for chymase/Ang-(1-12) compared to chymase/Ang I. These findings further support the previous demonstration that cardiac chymase has much higher substrate specificity and catalytic efficiency for Ang-(1-12) compared to Ang I substrates.

The measurements of cardiac and lung chymase and ACE activities based on the amounts of ^{125}I-Ang II production from either ^{125}I-Ang-(1-12) or ^{125}I-Ang I substrate are illustrated in Table 2. The results indicate that the chymase activity in heart (7.0 ± 0.6 fmol/ min/mg, P < 0.001) and lung (33 ± 1.2 fmol/min/mg, P < 0.001) PMs are several fold higher (8.75 and 15.7-fold, respectively) using ^{125}IAng-(1-12) substrate compared to that obtained using ^{125}I-Ang I substrate (0.8 ± 0.1 fmol/min/mg and 2.1 ± 0.1 fmol/min/mg, respectively). On the other hand, no significant differences in ACE activity were found in heart (1.8 ± 0.2 fmol/min/mg and 1.8 ± 0.3 fmol/min/mg) and lung (239 ± 25 fmol/min/mg and 248 ± 34 fmol/min/mg) using either ^{125}I-Ang-(1-12) or ^{125}I-Ang I substrates, respectively.

4. Discussion

Although an ACE-independent pathway for Ang II production from Ang I is well established [20], we now show for the first time that the hydrolytic activity of the chymase-mediated alternate pathway for Ang-(1-12) is at least 8–15-fold higher than the comparative activity for the Ang I substrate in the heart and lung tissue of SHR. Furthermore, our results demonstrate that with increasing concentrations of non-radiolabeled substrate, the [125]IAng II formation from ^{125}I-Ang-(1-12) by chymase was significantly lower in the presence of unlabeled Ang-(1-12) than unlabeled Ang I. Cardiac chymase kinetics also showed a 2-fold lower K_m (64 ± 6.3 vs 142 ± 17 μM), a 7-fold higher V_{max} (13.2 ± 1.3 vs 1.9 ± 0.2 μM/ min/ mg), and more than a 15-fold higher catalytic efficiency (the ratio of V_{max}/K_m) for Ang-(1-12) compared to Ang I substrate. These data document for the first time that Ang-(1-12) rather than Ang I is a much better substrate for rat chymase.

Chymases are a family of mast cell serine proteases involved in angiotensin peptide hormone processing [29–32]. In non-rodent mammals only the α-form of the chymase gene selectively hydrolyzes the Phe^8-His^9 bond of Ang I to generate Ang II. Rodents have several isoforms of β-chymase in addition to the α-chymase [29,33]. One of the forms of the β-chymase (called rat mast cell protease-1; RMCP1) shows a strong preference to hydrolyze the Tyr^4-Ile^5 bond of angiotensin peptides, thus preventing Ang II formation from Ang I [31]. Although abundant expression of the RMCP1 form is found in connective tissue mast cells [34], the relative expressions of these chymase isoforms in the rodent heart are not well documented. Our findings suggest that a very high content of Ang II-forming chymase was present in the rat heart tissue compared to Ang II-destroying β-chymase (RMCP1). A very small peak corresponding to the ^{125}I-Ang-(1-4) fragment [Asp^1-Arg^2-Val^3-Tyr^4-product of RMCP1] was detected from the Ang-(1-12) hydrolysis in cardiac membrane preparations. This finding suggests that the expression of the Ang II-destroying β-chymase isoform (RMCP1) was very low in the SHR heart. On the other hand, our findings do not exclude the possibility that RMCP1 may have a much higher specificity to cleave the Tyr^4-Ile^5 bond for bigger angiotensin peptides (Ang-12 and Ang I) rather than the smaller Ang II sequence. While our data clearly show that cardiac chymase rapidly hydrolyzes ^{125}I-Ang-(1-12) into ^{125}I-Ang II, further studies are necessary to determine the hydrolytic ability of β-chymase isoforms for Ang II, Ang I and Ang-(1-12) peptides to demonstrate the substrate preference between Ang II-forming and destroying isoforms of cardiac chymases.

Ang-(1-12) was first identified by Nagata and colleagues [5] in tissue and plasma of a Japanese strain of Wistar rats. Our later studies in neonatal WKY and SHR cardiac myocytes [6], anephric normotensive WKY rats [35], SHR [9], and human atrial tissues [7] demonstrated that both Ang-(1-12) and chymase were predominantly expressed intracellularly in cardiac tissue. Our recent translational studies in human and rodent cardiac tissue showed that Ang-(1-12) was the preferred substrate for intracellular Ang II formation via chymase rather than ACE [7,8]. As summarized in recent publications from our laboratory [12,20], these findings are in keeping with the limited efficacy of ACE inhibitors and ARBs to suppress the pathological actions of Ang II in cardiovascular disease [36] since these drugs act on the cell surface rather than in the intracellular sites where the biotransformation of angiotensinogen or Ang-(1-12) [Ang-(1-12)] occurs.

While ACE plays a major role in the circulation to generate Ang II, it has been convincingly shown that chymase plays a major role in rodent and human heart [7,8,10,11,37]. Since chymase activity is only detected in tissues and not in the circulation, the biochemical mechanisms forming Ang II within the cellular microenvironment are accounted for by alternative enzymatic pathways that are different than those in the circulation and that can't be blocked by either ACE inhibitors or ARBs [36,38]. The inability to block Ang II formation through these approaches suggests that these agents are not cell permeable, they are inhibited intracellularly, or they fail to reach the intracellular compartment containing Ang-(1-12) or Ang I. The intracellular formation of Ang II via the chymase pathway is independent of and unaffected by inhibition of Ang II production by ACE inhibitors or AT_1R blockade [39,40]. A number of factors suggest that the Ang II formation pathway is mediated primarily via chymase and Ang-(1-12). First, a robust literature documents a primary role for chymase in Ang II formation [6,8,10,11]. Wei et al. [41] showed that chronic ACE inhibition did not change the concentration of Ang II in the interstitial fluid of the left ventricle of conscious mice. Ferrario et al. [36] found no changes in cardiac content of Ang II in Lewis rats that were medicated for a fortnight with either lisinopril or losartan alone or in combination. Second, the catalytic activity of chymase is 20-fold higher than ACE [32], and renin is not involved in Ang-(1-12) metabolism [35,42]. And finally, our present findings demonstrate that rat chymase metabolizes Ang-(1-12) in cardiac tissue with an affinity many fold higher than for Ang I.

Our present findings clearly demonstrate that Ang-(1-12) is a much better substrate in cardiac tissue to generate biologically active Ang II peptide via chymase. Compared to Ang I, high levels of Ang-(1-12) were found in several tissues including the heart [5]. We have shown increased expression of Ang-(1-12) and chymase in neonatal cultured cardiac myocytes of SHR compared to WKY which may serve as the preferred pathway to generate Ang II in the heart [6].

In summary, the results of the present study clearly suggest that rat chymase has very high substrate specificity and catalytic efficiency for Ang-(1-12) and that it is mainly responsible for generating cellular Ang II. Although renin shows high specificity for the cleavage of the Leu^{10}-Leu^{11} bond at the N-terminus of rat angiotensinogen, our studies in the isolated heart preparations and anephric rats show that Ang II formation from Ang-(1-12) was not mediated by renin [35,42]. This working paradigm may resolve previous observations that Ang II formation in tissues is not prevented by ACE inhibitors and Ang II receptor blockers (ARBs) [24–26,36,38,40]. Our studies suggest that selective chymase inhibitors may provide greater benefit in the management of adverse cardiac remodeling than the previous therapeutic approaches.

Acknowledgments

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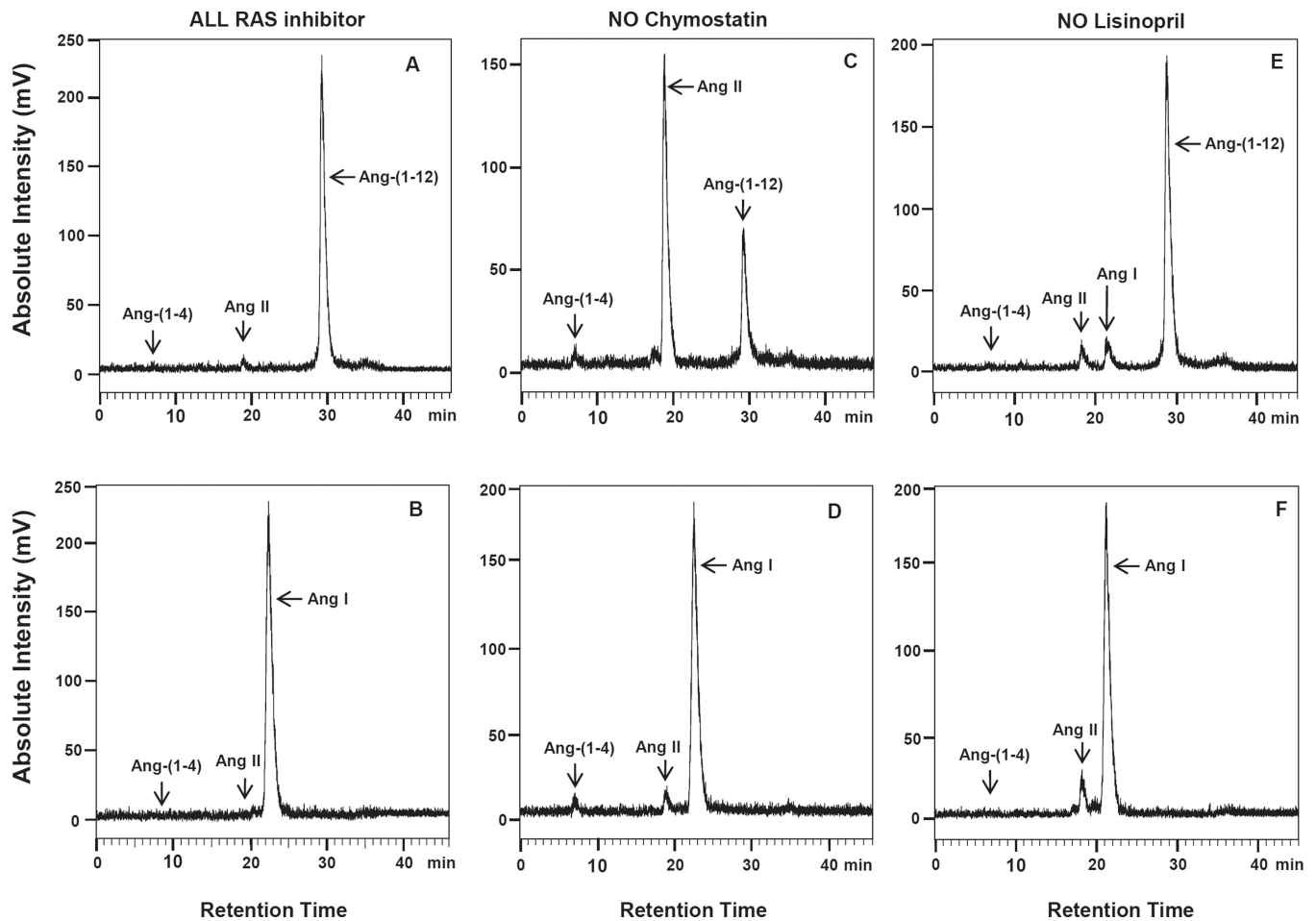
Fig. 1. Rat ^{125}I-Ang-(1-2) and ^{125}I-Ang I substrate hydrolysis by PMs isolated from SHR heart
Chromatograms show the ^{125}I-Ang II product generation from ^{125}I-Ang-(1-12) and ^{125}I-Ang I by PMs isolated from the SHR heart. Panel A and B: In the presence of all RAS inhibitors (including chymostatin and lisinopril). Panel C and D: In the absence of chymostatin (chymase inhibitor) only. Panel E and F: In the absence of lisinopril (ACE inhibitor) only. Results are representative of three or more separate metabolism experiments for each group.
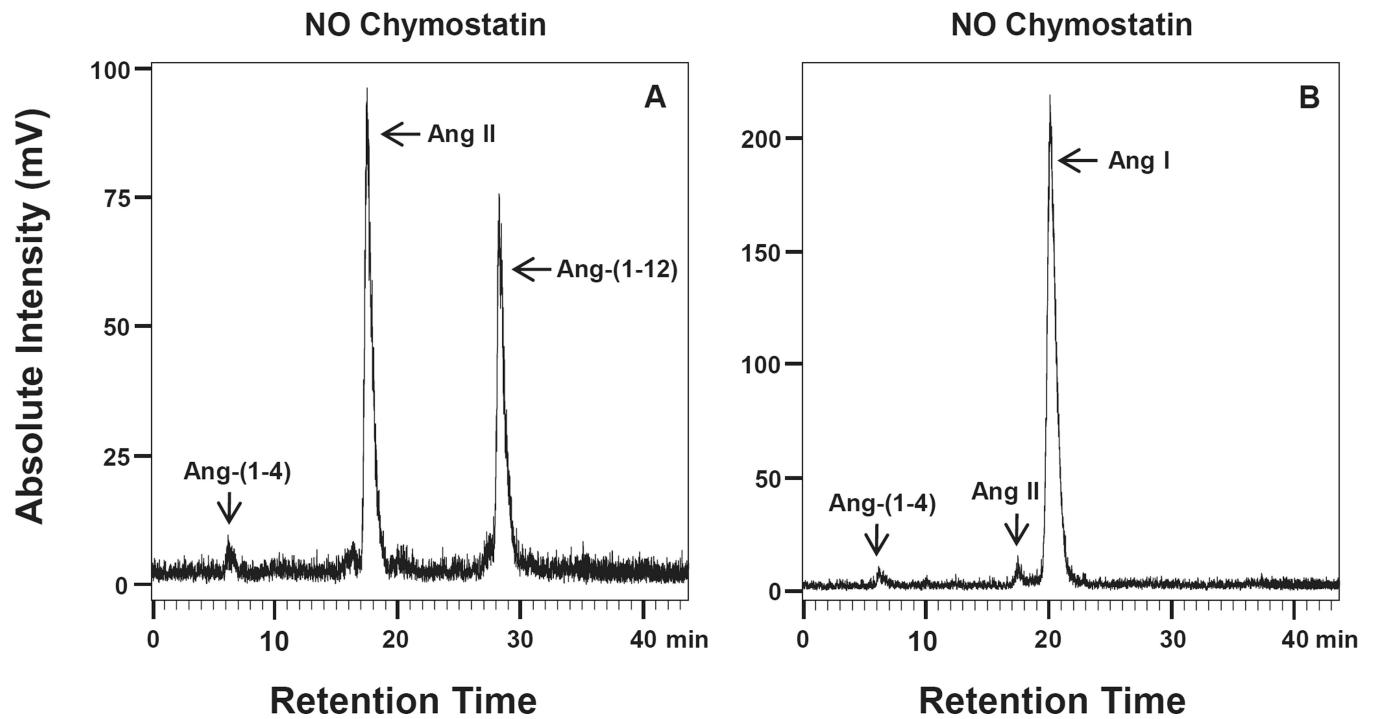
Fig. 2. ^{125}I-Ang-(1-12) and ^{125}I-Ang I substrate hydrolysis by PMs isolated from SHR lung
Chromatograms show the ^{125}I-Ang II product generation in the absence of chymostatin (chymase inhibitor) only from (A) ^{125}I-Ang-(1-12), and (B) ^{125}I-Ang I substrate. Results are representative of three or more separate metabolism experiments for each group.
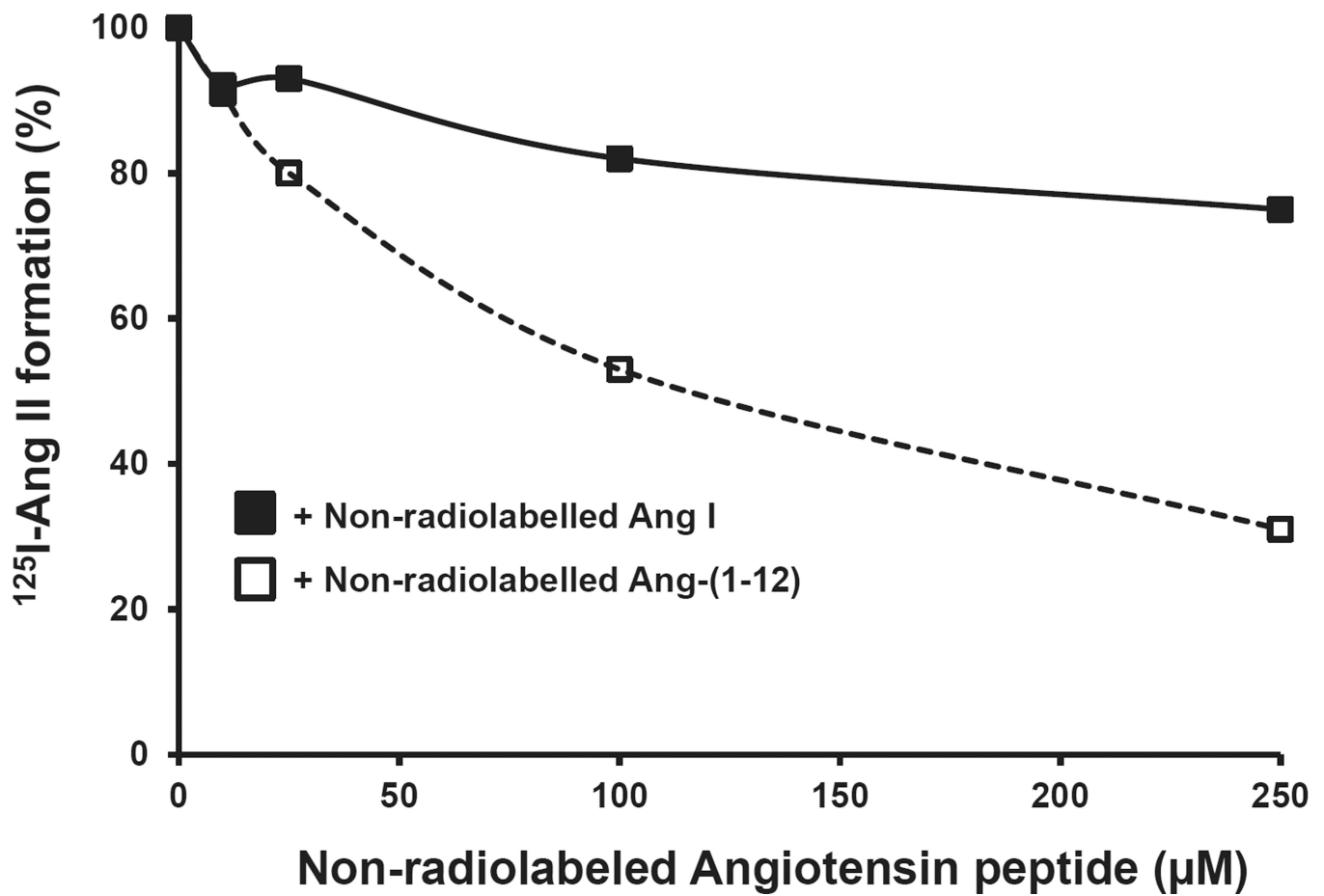
Fig. 3. Affinity of rat cardiac chymase for Ang-(1-12) and Ang I substrates
The decrease in ^{125}I-Ang product formation (%) from rat ^{125}I-Ang-(1-12) substrate by PMs incubated in the absence of chymostatin only (chymase inhibitor) with increasing concentrations (0–250 μM) of non-radiolabeled substrates (□) Ang-(1-12) or (■) Ang I. Results are representative of three or more separate experiments for each group.
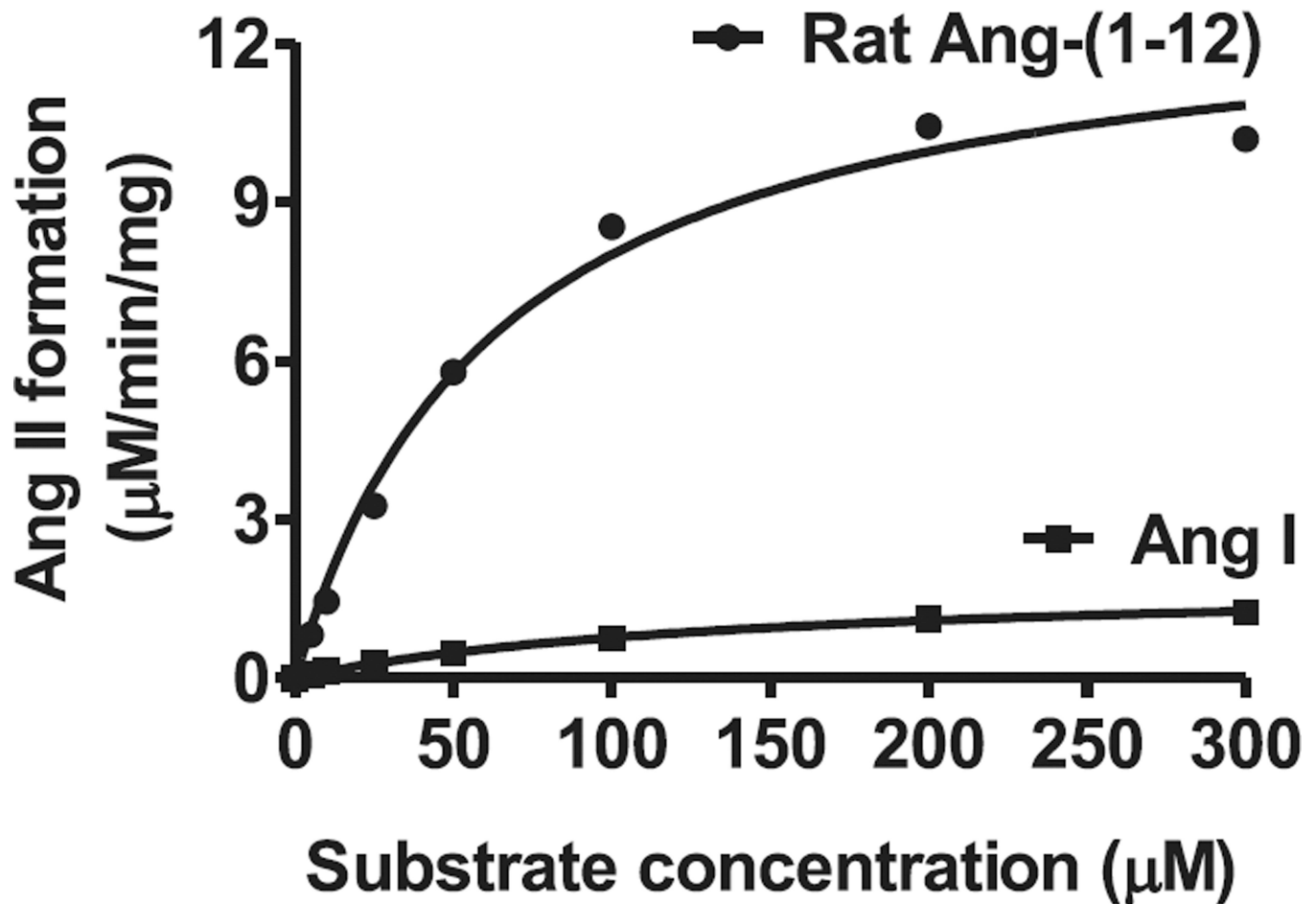
Rat chymase

Fig. 4. Kinetics (K_m and V_{max}) of rat cardiac chymase for Ang-(1-12) and Ang I substrates
PMs (100 μg) were incubated with increasing concentrations (5–300 μM) of Ang-(1-12) or Ang I substrate in the presence of lisinopril (200 μM, ACE inhibitor) at 37° C for 30 min. The Ang II generated was analyzed by HPLC connected to UV-detector at 215 nm. The K_m and V_{max} of cardiac chymase for Ang-(1-12) and Ang I substrates were calculated using the Michaelis-Menten equation.

Table 1

Outline of enzyme inhibitors used in this study.

Group	Inhibitors added (50 μM each)
All Inhibitors	RAS inhibitors (chymostatin for chymase; lisinopril for ACE; MLN-4760 for ACE2; and SCH39370 for neprilysin), aminopeptidase inhibitors (amastatin and bestatin), carboxypeptidase inhibitor (benzyl succinate) and PCMB.
No chymostatin	All above inhibitors except chymostatin.
No lisinopril	All above inhibitors except lisinopril.

Table 2

Metabolism of ^{125}I-Ang-(1-12) and ^{125}I-Ang I by chymase and ACE enzymes in SHR heart and lung (PMs).

Tissue/Substrate	Chymase activity	ACE activity
Heart (PMs)		
^{125}I-Ang-(1-12) substrate	$7.0 \pm 0.6^{a,b}$	$1.8 \pm 0.2^{c,d}$
^{125}I-Ang I substrate	0.8 ± 0.1^{b}	1.8 ± 0.3
Lungs (PMs)		
^{125}I-Ang-(1-12) substrate	$33 \pm 1.2^{a,b}$	$239 \pm 25^{c,d}$
^{125}I-Ang I substrate	2.1 ± 0.1^{b}	248 ± 34

Chymase and ACE mediated generation of ^{125}I-Ang II product from the metabolism of either ^{125}I-rAng-(1-12) or ^{125}I-Ang I substrate (1 nmol/L) were analyzed in SHR rat heart and lung native and soluble PMs preparations as described in the Methods. The contribution of chymase and ACE activity (fmol Ang II formation/min/mg) was calculated based on ^{125}I-Ang II product analysis by HPLC in each sample incubated with or without specific chymase or ACE inhibitor as described in Table 1. Data are expressed as mean ± SEM. Each experiment was done at least three times.

[a] Significantly different ($P < 0.001$) vs. corresponding ^{125}I-Ang I substrate.

[b] Significantly different ($P < 0.001$) vs. corresponding ACE activity.

[c] NS = Not significantly different vs. corresponding ^{125}I-Ang I substrate

[d] NS = Not significantly different vs. corresponding ACE activity.