Chymase-Dependent Angiotensin II Formation in Human Vascular Tissue

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Background—Some reports have suggested, that, in vitro, human heart chymase in homogenates contributes little to angiotensin (Ang) II formation in the presence of natural protease inhibitors such as α-antitrypsin. We studied whether chymase bound to heparin, resembling an in vivo form, could contribute to Ang II formation in the presence of natural protease inhibitors.

Methods and Results—The Ang II formation was increased time-dependently after incubation in an extract (1 mg of protein/mL) of human vascular tissues containing Ang I. The concentration of Ang II in the extract after incubation for 30 minutes was 1.67±0.06 nmol/mL, and we regarded this quantity of Ang II as 100%. The Ang II formation was inhibited 10%, 95%, and 96% by 1 μmol/L lisinopril, 100 μmol/L chymostatin, and 0.1 g/L α-antitrypsin, respectively. The extract was applied to a heparin affinity column. After the column was washed with PBS, the eluted PBS contained a weak Ang II-forming activity, which was completely inhibited by lisinopril. The eluted PBS, to which >0.8 mol/L NaCl had been added, showed a strong Ang II-forming activity which was inhibited by chymostatin and α-antitrypsin. After the application of the extract, the column was washed with PBS and then an Ang I solution in PBS was applied to the column. The Ang II formation in the PBS eluted from the incubated column was increased time-dependently. The concentration of Ang II in the PBS (1 mL) eluted from the column after incubation for 30 minutes was 2.56±0.28 nmol/mL, and we regarded this quantity of Ang II as 100%. To study the effects of inhibitors, the extract (1 mg of protein/mL) was applied to a heparin affinity column (1 mL) which was preequilibrated with PBS (3 mL); 100 μmol/L chymostatin or 0.1 g/L α-antitrypsin in PBS (1 mL) was then applied to the column. After the column was washed with PBS (3 mL), Ang I solution (1 mg/mL) in PBS was applied to the column, and the column was incubated for 30 minutes. The Ang II formation in the PBS eluted from the column was suppressed up to 5% by application of chymostatin, although this was not affected by application of α-antitrypsin.

Conclusions—These findings suggest that human chymase bound to heparin plays a functional role in Ang II formation in the presence of natural protease inhibitors such as α-antitrypsin. (Circulation. 1999;100:654-658.)

Key Words: chymase ■ angiotensin ■ heparin ■ vascular tissue

Chymase is a chymotrypsin-like serine protease contained in the secretory granules of mast cells. Chymases have been isolated and their enzymatic characteristics have been studied in rats,1–3 dogs,4,5 hamsters,6,7 monkeys,8 and humans.9–12 In general, these chymases hydrolyze the C-terminal side of proteins after aromatic amino acids such as Phe, Tyr, and Trp. Using the substrate angiotensin (Ang) I, hamster,6 monkey,8 and human9–12 chymases cleave the Phe8–His9 bond of Ang I to yield Ang II, whereas rat chymase10 cleaves the Tyr4–Ile5 bond to form inactive fragments. Chymase is also involved in processing various hormonal peptides,13,14 but it is unclear whether chymase plays a major role in physiological and pathophysiological functions in vivo.

Recently, Kokkonen et al15 demonstrated that chymase was responsible for most Ang II formation in human heart homogenate in the absence of natural protease inhibitors, whereas in the presence of these inhibitors, chymase-dependent Ang II formation was not detected. They therefore concluded that the chymase-dependent Ang II-forming pathway is emphasized in the absence of these inhibitors in human heart homogenate and in the membrane extract but that chymase in human heart may only play a small role in Ang II formation in vivo. Zisman et al16 reported that an ACE inhibitor reduced Ang II formation by 89% after intracoronary infusion of Ang I, and they also concluded that the ACE-dependent Ang II pathway was the predominant pathway for Ang II formation in human heart. However, we demonstrated that 30% of Ang I-dependent vasocontractile responses in isolated human gastroepiploic arteries were suppressed by captopril, an ACE inhibitor; the remainder was blocked by chymostatin, a chymase inhibitor.17 These findings suggested that this chymase-like enzyme has a functional role in Ang II formation in human vascular tissue. Recently, we isolated and identified this enzyme as chymase.12

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Human chymase is stored in a macromolecular complex with heparin proteoglycan in the cytoplasmic secretory granules in mast cells, and the complexed enzyme has maximal activity immediately on release into the extracellular matrix in vascular tissues after degranulation. The complexed enzyme is more resistant to inhibition by macromolecular protease inhibitors such as α-antitrypsin than the purified enzyme in vitro.

In the present study, we investigated whether or not chymase bound to heparin has an Ang II-forming activity in the presence of natural protease inhibitors such as α-antitrypsin in the extract of human vascular tissue.

Materials
Ang I, Ang II, and chymostatin were obtained from the Peptide Institute (Mino, Japan). Aprotinin and α-antitrypsin were purchased from Sigma Chemical Co (St. Louis, Mo). Heparin (1 mg/mL) was obtained from Gibco (Rockville, Md).

Human Vascular Tissues
Human gastroepiploic arteries were obtained from the surgically resected stomachs of 8 patients (3 men and 2 women, mean age of 48.8±2.9 years) who underwent total gastrectomy because of gastric cancer. All patients had no apparent vascular complications (eg, hypertension, atherosclerosis, and diabetic vasculopathy). Their arteries were transported in ice-cooled Tyrode’s solution consisting of 137 mmol/L NaCl, 2.7 mmol/L KCl, 1.8 mmol/L CaCl₂, 1.1 mmol/L MgCl₂, 0.42 mmol/L NaHPO₄, 12 mmol/L NaHCO₃, and 5.7 mmol/L glucose, pH 7.4, and were stored at 4°C before use in experiments.

Preparation of Extract from Human Vascular Tissue
The arteries were minced and homogenized in 10 volumes (wt/vol) of Tyrode’s solution consisting of 137 mmol/L NaCl, 2.7 mmol/L KCl, 1.8 mmol/L CaCl₂, 1.1 mmol/L MgCl₂, 0.42 mmol/L NaHPO₄, 12 mmol/L NaHCO₃, and 5.7 mmol/L glucose, pH 7.4. The homogenate was centrifuged at 20,000 g for 30 minutes at 4°C. The supernatant was discarded, and the pellets were resuspended and homogenized in 10 volumes (wt/vol) of 10 mmol/L phosphate buffer, pH 7.4, containing 2 mol/L potassium chloride and 0.1% (v/v) Nonidet P-40. The homogenate was stored overnight at 4°C and centrifuged at 20,000 g for 30 minutes at 4°C. The supernatant was collected for measurement of the Ang II formation.

Ang I Conversion in Extract
Aliquots of the extract (1 mg of protein/mL) were incubated for 0 to 60 minutes at 37°C with 1 mg/mL of Ang I in PBS (final incubation volume of 0.1 mL). For the studies of inhibitor effects, 1 μmol/L lisinopril, 100 μmol/L chymostatin, 1 μmol/L aprotinin, or 0.1 g/L α-antitrypsin was added and preincubation was conducted for 10 minutes at 37°C, followed by incubation for 30 minutes at 37°C with 1 mg/mL of Ang I in PBS (final incubation volume of 0.1 mL). The reaction was terminated by addition of 0.15 mL of 150 g/L trichloroacetic acid, followed by centrifuging at 20,000 g for 10 minutes. The supernatant was collected for measurement of the Ang II formation.

Heparin Column
The extract (0.1 mg of protein/mL) was applied to a heparin affinity column (1 mL) which was preequilibrated with PBS. The column was eluted with PBS (1 mL), to which had been added 0.0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, or 1.8 mol/L NaCl. Each of these media eluting from the column was collected for measurement of the Ang II formation. To study the effects of enzyme inhibitors on the Ang II formation, aliquots of the media eluted from the column were preincubated for 10 minutes at 37°C with 1 μmol/L lisinopril, 100 μmol/L chymostatin, or 0.1 g/L α-antitrypsin, followed by incubation for 30 minutes at 37°C with 1 mg/mL Ang I in PBS (final incubation volume of 0.1 mL).

The extract (1 mg of protein/mL) was applied to a heparin affinity column (1 mL) which was preequilibrated at 37°C and was preequilibrated with PBS. Ang I solution (1 mg/mL) in PBS was applied to the column, and the column was incubated at 37°C for 0, 5, 10, 30, or 60 minutes. After the incubation, the column was eluted with PBS (1 mL), and the medium was collected for measurement of the Ang II formation. To study the effects of inhibitors, the extract (1 mg of protein/mL) was applied to a heparin affinity column (1 mL) which was preequilibrated with PBS (3 mL); 100 μmol/L chymostatin or 0.1 g/L α-antitrypsin in PBS (1 mL) was then applied to the column. After the column was washed with PBS (3 mL), Ang I solution (1 mg/mL) in PBS was applied to the column, and the column was incubated at 37°C for 30 minutes. After the incubation, the column was eluted by PBS (1 mL), and the PBS eluted from the column was collected for measurement of the Ang II formation.

Determination of Ang I Fragments and Protein Concentration
The media from the homogenate, the extract, or the slices were applied to a TSKgel ODS-80 TM column (4.6 mm×250 mm ID, Tosoh), which was eluted with a linear gradient of methanol (10% to 90%) in 10 mmol/L phosphoric acid, pH 3.8, at a flow rate of 0.5 mL/min. The protein concentration of the extract was measured by bicinchoninic acid protein assay reagent (Pierce Chemical) using bovine serum albumin as a standard.

Statistical Analysis
All values are expressed as mean±SEM. Two-way ANOVA and Student’s t test were used for statistical comparisons. P<0.05 was considered statistically significant.

Results
Ang I Conversion Extract from Human Vascular Tissue
Ang II formation was observed after incubation of the extract (1 mg of protein/mL) from human vascular tissues with Ang I (1 mg/mL) and Ang II was found to be linearly generated up to 60 minutes. The effects of the enzyme inhibitors were investigated with Ang I conversion for 30 minutes during the phase of linear formation of this product. The concentration of Ang II in the extract after incubation for 30 minutes was 1.67±0.06 nmol/mL, and we regarded this quantity of Ang II as 100%. The Ang II formation was not inhibited by aprotinin (1 mmol/L). The Ang II formation was inhibited 10% by lisinopril (1 μmol/L) and was inhibited 95% by chymostatin (100 μmol/L) and 96% by α-antitrypsin (0.1 g/L) (Figure 1).

Binding Affinity of Chymase to Heparin
The Ang II formation in PBS (1 mL) eluted from the column after incubation with Ang I (1 mg/mL) for 30 minutes was 0.25±0.01 nmol/mL, although it was not detectable in the eluted PBS that contained up to 0.6 mol/L NaCl (Figure 2). In the eluted PBS, to which had been added 0.8, 1.0, and 1.2 mol/L NaCl, the Ang II formation was 0.08±0.02, 1.02±0.09, and 0.12±0.02 nmol/mL, respectively (Figure 2). Ang II was not detected in the buffer containing over 1.4 mol/L NaCl (Figure 2). The Ang II formation in the PBS...
eluted from the column was completely inhibited by lisinopril (1 \( \mu \)mol/L) but not by chymostatin (100 \( \mu \)mol/L) or \( \alpha \)-antitrypsin (0.1 g/L), whereas that in the eluted PBS to which had been added between 0.8 and 1.2 mol/L NaCl was completely suppressed by chymostatin (100 \( \mu \)mol/L) or \( \alpha \)-antitrypsin (0.1 g/L), but not by lisinopril (data not shown).

**Effects of Inhibitors on Chymase Bound to Heparin**

After application of the extract (1 mg of protein/mL), Ang I (1 mg/mL) was applied to the column and then the column was incubated for 0, 5, 10, 30, or 60 minutes. The concentrations of Ang II in the PBS (1 mL) eluted from the column were 0, 0.34 \( \pm \)0.03, 0.78 \( \pm \)0.08, 2.56 \( \pm \)0.28, and 5.12 \( \pm \)0.67 nmol/mL, respectively (Figure 3). We regarded the quantity of Ang II after incubation for 30 minutes as 100% and studied the effects of inhibitors on this Ang II formation. After application of the extract (1 mg of protein/mL), chymostatin (100 \( \mu \)mol/L) and \( \alpha \)-antitrypsin (0.1 g/L) were applied to the column; Ang I was then applied to the column. The Ang II formation in the PBS eluted from the column was suppressed up to 5% by application of chymostatin, although this was not affected by application of \( \alpha \)-antitrypsin (Figure 4).

**Discussion**

The study results showed that Ang I was converted to Ang II time-dependently in the extract of human vascular tissue. The Ang II formation was suppressed by 90%, 5%, and 4% with lisinopril, chymostatin, and \( \alpha \)-antitrypsin, respectively. These results suggested that chymase rather than ACE predominantly converted Ang I to Ang II in the extract of human vascular tissue. Results similar to these were reported by Urata et al\(^{11}\) in the extract of human heart membrane. In contrast, Zisman et al\(^{16}\) showed that Ang II formation after intracoronary administration of Ang I in the human heart was reduced by 89% with an ACE inhibitor. These authors suggested that the predominant pathway for Ang II formation by chymase in human heart tissue extract, as reported by Urata et al,\(^{11}\) could be observed only in the absence of internal inhibitors. Kokkonen et al\(^{15}\) suggested that most Ang II formation in the heart homogenate was a chymase-dependent pathway. However, this pathway is inhibited by human interstitial fluid containing \( \alpha \)-antitrypsin, and these authors concluded that human chymase was not responsible for Ang...
II formation in vivo. In this study, we also observed that the Ang II formation in the extract from human vascular tissue in the absence of the inhibitors was predominantly a chymase-dependent pathway, but Ang II formation was not detected in the presence of α-antitrypsin. Therefore, chymase-dependent Ang II formation in the extract was suppressed in the presence of the natural protease inhibitors.

In vivo, chymase is stored in a macromolecular complex with heparin proteoglycan within mast cell granules; the enzyme remains complexed after degranulation.21 The complexed enzyme is more resistant to inhibition by macromolecular protease inhibitors, such as α-antitrypsin, than the purified enzyme.20 In our study, the Ang II formation in the PBS eluted from the heparin column after application of the extract from human vascular tissues was inhibited by lisinopril only, suggesting that the Ang II formation was dependent on ACE. On the other hand, in the eluted PBS containing 0.8, 1.0, and 1.2 mol/L NaCl, all of the Ang II formation was inhibited by chymostatin or α-antitrypsin but not by lisinopril, suggesting that the Ang II formation was dependent on chymase. These findings clearly showed that chymase, but not ACE, was bound to the heparin column in PBS. Furthermore, we demonstrated, for the first time, that the chymase bound to the heparin gel converted Ang I to Ang II and the conversion of Ang I to Ang II was completely suppressed by chymostatin but not by α-antitrypsin. The difference in the inhibitory effects of chymostatin (M<sub>i</sub> = 604) and α-antitrypsin (M<sub>i</sub> = 50 000) may be due to the different sizes of the molecules. Therefore, natural serine protease inhibitors such as α-antitrypsin hardly inhibit the chymase bound to heparin as in the in vivo condition.

The chymase substrate in vivo has not yet been elucidated. For hormonal peptides, human chymase converts Ang I to Ang II but hardly hydrolyzes other peptides.11,12 Ang II is known to play a role in maintaining blood pressure. The chymase-dependent Ang II formation may be irrelevant to blood pressure because ACE inhibitors are clinically effective for various types of hypertension. Recent reports suggest that Ang II plays crucial roles in the migration and proliferation of vascular tissues.24,25 For example, an ACE inhibitor was effective in preventing the proliferation of vascular tissue after balloon injury of vessels in rats.26 Rat vascular tissues contain only ACE as the Ang II-forming enzyme,17 and these results suggest that vascular Ang II formation plays a crucial role in tissue proliferation. On the basis of these reports, we asked whether an ACE inhibitor suppresses human vascular restenosis after percutaneous transluminal coronary angioplasty in humans: the result was negative.27,28 Such species’ differences in the effects of ACE inhibitors on neointimal formation after injury may depend on whether or not a given species possesses Ang II-forming chymase in vascular tissue. Previously, we reported that dogs had a chymase-like Ang II-forming enzyme in vascular tissues,29 and chymase-like activities in the injured vessels were significantly increased in comparison with those in noninjured vessels.30 In this model, an Ang II receptor antagonist was effective in preventing neointimal formation after balloon injury of vessels in dogs but an ACE inhibitor was ineffective.31 Therefore, the chymase-dependent Ang II formation in vascular tissue may be closely related to promoting growth. However, this role of chymase in vivo has yet to be demonstrated by antiproliferative effects of a chymase inhibitor in this model.

In conclusion, natural internal inhibitors such as α-antitrypsin strongly inhibited the chymase-dependent Ang II formation in the extract, but chymase bound to heparin, as in the in vivo condition, converted Ang I to Ang II in the presence of natural protease inhibitors in plasma and vascular tissues. These findings suggest that chymase plays a functional role in Ang II formation in human vascular tissues in vivo.

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References


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