Differences in Tissue Angiotensin II–Forming Pathways by Species and Organs In Vitro

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Abstract—Angiotensin (Ang) II plays an important role in cardiovascular homeostasis, not only in the systemic circulation but also at the tissue level, and is involved in remodeling of the heart and vasculature under pathological conditions. Although alternative Ang II–forming pathways are known to exist in various tissues, the details of such pathways remain unclear. The aim of this study was to examine tissue Ang II–forming activities and to identify the responsible enzyme in several organs (lung, heart, and aorta) in various species (human, hamster, rat, rabbit, dog, pig, and marmoset). Among the organs examined, the lung contained the highest Ang II–forming activity. The responsible enzyme for pulmonary Ang II formation was angiotensin I-converting enzyme (ACE) in all of the species except the human lung, in which a chymase-like enzyme was dominant. In the heart, the highest total Ang II–forming activity was observed in humans, and a chymase-like enzyme was dominant in all of the species except rabbit and pig. Aorta exhibited a relatively high total Ang II–forming activity, with a predominance of chymase-like activity in all of the species except rabbit and pig, in which ACE was dominant. Our results indicate that there were remarkable differences in Ang II–forming pathways among the species and organs we examined. To study the pathophysiological roles of ACE-independent Ang II formation, one should choose species and/or organs that have Ang II–forming pathways similar to those in humans.

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Key Words: angiotensin-converting enzyme ■ chymase ■ kallikrein ■ heart ■ lung ■ aorta

Angiotensin (Ang) II plays an important role in cardiovascular homeostasis, not only as an endocrine hormone but also as a tissue autocrine and paracrine factor, and is involved in remodeling of the heart and vasculature in pathological conditions. The beneficial effects of blocking the renin-angiotensin system with angiotensin-converting enzyme (ACE) inhibitors have been demonstrated by improvement of the morbidity and mortality of patients with cardiovascular diseases; these drugs are widely used for the treatment of hypertension and congestive heart failure. ACE inhibitors have also helped to elucidate the existence of alternative Ang II–forming pathways, since Ang II is formed by several enzymes (angiotensinogen or Ang I). Among the organs examined, the lung contained the highest Ang II–forming activity. The responsible enzyme for pulmonary Ang II formation was angiotensin I-converting enzyme (ACE) in all of the species except for the human lung, in which a chymase-like enzyme was dominant. In the heart, the highest total Ang II–forming activity was observed in humans, and a chymase-like enzyme was dominant in all of the species except rabbit and pig. Aorta exhibited a relatively high total Ang II–forming activity, with a predominance of chymase-like activity in all of the species except rabbit and pig, in which ACE was dominant. Our results indicate that there were remarkable differences in Ang II–forming pathways among the species and organs we examined. To study the pathophysiological roles of ACE-independent Ang II formation, one should choose species and/or organs that have Ang II–forming pathways similar to those in humans.

Although many animals possess chymase, its biochemical and physiological roles seem to vary by species. For example, rat chymase I, which was identified in rat peritoneal mast cells, is an Ang II–degrading enzyme rather than an Ang II–forming enzyme. The biochemical heterogeneity of mammalian chymases poses a problem in choosing an appropriate animal model for study of the pathophysiological significance of chymase. Human chymase appears to be involved in clinical disorders such as the development of atherosclerotic lesion, acute coronary syndrome, metabolism of apolipoprotein, and degradation of extracellular matrix. Therefore, to clarify its roles(s), it was necessary to obtain more information about the differences in the mechanism of tissue Ang II formation among various species and organs and to compare these...
results with those in humans. In the present study, tissue Ang II–forming activities were determined in several organs of various species (human, hamster, rat, rabbit, dog, pig, and marmoset). We found remarkable differences in Ang II–forming pathways among species and organs.

Methods

Human Samples

Human tissues including hearts (mean age, 66.0 ± 3.6 years old; M:F = 3:1; n = 4), aorta (mean age, 56.4 ± 9.4 years; M:F = 4:1; n = 5), and lungs (mean age, 70.5 ± 4.9 years; M:F = 4:2; n = 6) were obtained from routine autopsy at the Department of Pathology at Fukuoka University Hospital. The causes of death in these patients were malignancy (n = 7), acute myocardial infarction (n = 2), and chronic renal failure (n = 1). The patients were not treated with ACE inhibitors or digitalis. Tissues without apparent pathological changes were selected and used for the study, and this was confirmed by microscopic examination (data not shown). Heart samples from the patients who died of acute myocardial infarction were excluded. Heart specimens were obtained from the middle portion of the left ventricle free wall, aortic samples were from the abdominal aorta, and pulmonary samples were from the left lower lobe. All samples were obtained within 6 hours after death. After the tissues were excised, each tissue was dissected into 0.5-g pieces, rinsed in saline, and immediately frozen for storage at −80°C. Our previous study confirmed that the tissue Ang II–forming activity in vitro remains unchanged until 9 to 10 hours after death.27

Animal Samples

To identify an appropriate animal model for studying human chymase, animal samples were obtained from hamsters (Syrian hamster, 130 to 150 g, male, n = 5; Kyudo Co, Tosu, Japan), rats (Sprague-Dawley rat, 200 to 230 g, male, n = 5; Kyudo Co, Tosu, Japan), rabbits (Japanese White rabbit, 2 kg, male, n = 11; Biotech Co, Tosu, Japan), dogs (adult mongrel dog, 12 kg, male, n = 5; Animal Management Center, Fukuoka, Japan), pigs (Yorkshire pig, BW, 20 to 30 kg, male, n = 5), and monkeys (marmoset, 350 to 430 g, male, n = 3). These animals are normally used in cardiovascular experiments. To compare the tissue Ang II–forming activities in vitro in systemic and pulmonary circulation, the same organs (heart, aorta, and lung) were studied in each species. Samples were obtained from hamsters, rats, rabbits, and dogs after death by bleeding under amobarbital (Isomital, Nihonshinyaku) anesthesia. Tissues of pigs were kind gifts from Dr Shimokawa of the Research Institute of Angiocardiology, School of Medicine, Kyushu University, and those of monkeys were from Dr Inada at Takeda Chemical Industries, Ltd. Samples of each tissue were dissected into 0.5-g pieces, rinsed in saline, and immediately frozen for storage at −80°C. The use of human and animal tissues was approved by the Internal Review Committee of Fukuoka University.

Preparation of Tissue Particulate Fraction

After being thawed and weighed, tissues were dissected into small pieces. They were placed in 50 mmol/L NaH2PO4 buffer (0.5 g/5 mL), pH 7.4, at 4°C and homogenized using a Polytron homogenizer (Kinematica GMBH) at 9000 rpm for 15 seconds at 4°C. The homogenates were centrifuged at 30,000g for 20 minutes at 4°C. The supernatants were discarded, the pellets were resuspended in the same buffer, and homogenization and centrifugation were repeated once. The final tissue pellets were resuspended in 50 mmol/L NaH2PO4 buffer, pH 7.4, containing 100 mmol/L NaCl and 10 mmol/L MgCl2 using a hand-driven glass/glass homogenizer. The protein concentration of the particulate fraction was measured using Protein Assay Reagent (Pierce).

Assessment of Ang II Formation in Particulate Fraction

Ang II–forming activity from Ang I (Peptide Institute Inc) was determined as described elsewhere with some modification.18 The particulate fraction prepared as above was incubated with synthetic Ang I (200 μmol/L) with or without inhibitors at 37°C for the appropriate time period. The detailed information for the protein doses used, incubation time, linearity of Ang II production, and the inhibitory actions of each inhibitor are provided in “Results.” The formed Ang II was analyzed by reverse-phase high-performance liquid chromatography (HPLC; Shimazu) using a C18 reverse-phase HPLC column (2.2 x 25 cm; Vydac) with a 15-minute linear acetonitrile gradient (3% to 13%) in 25 mmol/L triethylamine-phosphate buffer, pH 3, at a flow rate of 2 mL/min. Retention times of synthetic His-Leu, Ang I(5-10), Ang II, and Ang I were 1.7, 8.8, 9.4, and 12.3 minutes, respectively. Peaks at other retention times (2.0, 8.4, 11.0, 11.5, 14.6, and 15.4 minutes, respectively) were not identified. Ang II–forming activities were expressed as nanomoles of Ang II formed per minute per milligram of protein. Captopril- or chymostatin-inhibitable (both from Sigma Chemical Co) and aprotinin (Bayer)–insensitive Ang II formation were expressed as ACE or chymase-like activities, and the resultant captopril- or chymostatin-insensitive activity was presented as non-ACE or non–chymase-dependent Ang II–forming activity, respectively. All analyses for each sample were performed in duplicate, and the reproducibility and quality of all data were confirmed before the statistical analysis. The interassay and intra-assay coefficients of variation of this assay were 8.6% (n = 12) and 5.1% (n = 10), respectively. Under our assay conditions, the effects of endogenous ACE inhibitor reported in rat heart can be ignored, since this inhibitor exists in the supernatant of heart homogenate but not in the pellet.29 Therefore, it should have been removed in our tissue preparation.

Statistical Methods

All data are presented as mean ± SE. Statistical analyses were performed using Scheffé’s F test and 1-way ANOVA to compare the levels of total, captopril-, chymostatin-, and aprotinin-inhibitable Ang II–forming activities among the different species and organs in the same species. A paired Student’s t test was used to compare the Ang II–forming activity in the same organ from the same species. A value of P < 0.05 was considered statistically significant.

Results

Preliminary Experiments

Preliminary experiments were performed in each organ and species except human to determine the global levels of Ang II–forming activity and its time dependency with various incubation times (15, 30, 45, and 60 minutes). Ang II and other metabolite formation in cardiac tissues from several species was almost linear with incubation for up to 60 minutes, except in marmoset (Figure 1). Other tissues, such as aorta and lungs, also showed linear Ang II formation (data not shown). On the basis of these results, the incubation time and protein concentrations of the particulate fractions were selected for actual incubation (Table), which was then carried out at 37°C. There were no significant differences in protein doses between species and organs except for dog heart and aorta, which contained the lowest levels of Ang II–forming activity, necessitating higher levels of protein for the assay. The averaged residual amounts of Ang I after the incubation (percentage of molar ratio versus added Ang I) for each sample are also listed in the Table. The amount of Ang I used in our assay condition was at most <40% except in hamster aorta (41.8%). Because the production of other metabolites was not overwhelming, the substrate Ang I concentration was still high enough during incubation.

Before the experiments with various inhibitors, the inhibitory actions of captopril and chymostatin were tested in all of the organs from all of the species. Dose-dependent inhibition with
the ACE inhibitor captopril (1, 10, 100, and 1000 µmol/L) and chymostatin (0.1, 1, 10, 100, and 1000 µmol/L) was determined in a representative sample of each organ from each species. Both captopril and chymostatin produced saturable inhibition in cardiac tissues (Figure 2), as well as in other tissues (data not shown). These results indicate that 100 µmol/L captopril and 10 µmol/L chymostatin were the lowest concentrations for obtaining saturable inhibitory effects, respectively. The aprotinin concentration (10 µmol/L) was determined based on a previous study, since most of the samples used in the present study did not show constant aprotinin-inhibitable Ang II–forming activity (data not shown). Therefore, the dose-dependent inhibitory effect of aprotinin could not be tested adequately. On the basis of these preliminary experiments, actual incubations for each tissue and species were performed with or without 100 µmol/L captopril, 10 µmol/L chymostatin, or 10 µmol/L aprotinin, respectively.

Ang II–Forming Activities in Several Organs From Several Species

Tissue Ang II–forming activities in vitro were determined in several organs in different species. Among the organs examined in the present study, the lung contained the highest Ang II–forming activity in all species, followed by aorta and heart in the animal (Figure 3). However, in human tissue, Ang II–forming activity in the heart was higher than that of aorta. In addition, human heart contained the highest total Ang II–forming activity, with a predominance of chymase-like Ang II–forming activity, of all of the species examined (Figure 3). In heart, the main enzyme responsible for Ang II formation was chymase-like enzyme in all cases except rabbit and pig heart (Figure 4A). Aorta contained relatively higher total Ang II–forming activities, with a predominance of chymase-like Ang II–forming activity, in all species except rabbit and pig (Figure 4B).

In the lung, the predominant Ang II–forming enzyme was captopril-sensitive enzyme, ACE, in all of the species except for human lung, in which chymase-like enzyme was dominant. Among the various species, hamster lung contained the highest total Ang II–forming activity. The Ang II–forming activity in rabbit lung was inhibited with both captopril and chymostatin by 80% and 85%, respectively, resulting in equivalent levels of ACE and chymase-like enzyme activities (Figure 4C). Similar phenomena were also observed in the dog and marmoset lungs.

Another interesting result was that treatment with aprotinin significantly increased Ang II–forming activity in some tissues in several species, including pig heart (0.90±0.14 to 0.95±0.14 nmol · min⁻¹ · mg protein⁻¹ [U]; P<0.05) and aorta (0.85±0.21 to 1.20±0.16 U, P<0.05), marmoset heart (0.23±0.03 to 0.39±0.06 U, P<0.05) and aorta (4.23±1.17 to 9.44±2.23 U, P<0.05), and rabbit aorta (0.23±0.04 to 0.26±0.15 U, P<0.05) and lung (8.63±1.00 to 16.2±1.64 U, P<0.05). On the other hand, there were slight decreases in Ang II–forming activities with aprotinin treatment in some tissues in several species, including human heart (2.40±0.54

Figure 1. Time-dependent Ang II and other metabolite formation in cardiac tissues from several species. Particulate fractions of cardiac tissues were incubated for different periods of time (15, 30, 45, and 60 minutes) with synthetic Ang I to study the time dependency of Ang II formation. The formed Ang II and other metabolites were analyzed by reverse-phase HPLC. See details in “Methods.” ■ indicates peak (p) at retention time 9.4 minutes (Ang II); □, p 1.7 minutes (His-Leu); ▲, p 2.0 minutes (unidentified); ◇, p 3.9 minutes (unidentified); ○, p 8.4 minutes (unidentified); ●, p 8.8 minutes (Ang I [5–10]); Δ, p 11.0 minutes (unidentified); ○, p 11.5 minutes (unidentified); ◯, p 14.6 minutes (unidentified); △, p 15.4 minutes (unidentified); and area, arbitrary units of integrated area of each peak from HPLC analyses.
enzyme in the lung may have a greater physiological role in humans than in other species. Interesting findings in the aorta and heart were that chymase-like enzymatic activity was predominantly responsible for Ang II formation except in rabbit and pig aorta. This biochemical predominance of chymase-like enzymatic activity over ACE has been repeatedly observed in several human tissues, including vessels \(^9,^{30}\) and heart.\(^{10,31}\) However, no report was available regarding organ differences in Ang II–forming activities. The present study was the first to compare total and chymase-like enzyme–dependent Ang II formation in lung, heart, and aorta simultaneously and to show a definitive difference in Ang II–forming activity and the responsible enzymes.

There are several possible reasons for the differences in tissue Ang II–forming activity among tissue and species: ie, differences in the origin and subclass, density, and heterogeneity of each cell must be considered. Chymase mRNA and immunoreactivity in the human heart can be detected in mast cells, endothelial cells, and mesenchymal cells.\(^{32}\) However, there has been no systematic study of the tissue distribution of chymase mRNA and immunoreactivity in species other than humans.\(^{32,33}\) In rodents, chymase immunoreactivity was detected in mast cells but not in other cell types.\(^{34}\) These results led us to consider that the increased chymase activity in animal tissue is due mainly to the increased distribution of

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### Table: Rate of Ang I Degradation and Production of Ang II and Other Metabolites in All Organs

<table>
<thead>
<tr>
<th>Organ/Species</th>
<th>n</th>
<th>Incubation Time, min</th>
<th>Protein Dose, mg</th>
<th>Residual Ang I After Incubation, %</th>
<th>Formed Ang II, %</th>
<th>Other Metabolites, %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Heart</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Human</td>
<td>4</td>
<td>45</td>
<td>39.7±9.2</td>
<td>91.7±1.1</td>
<td>3.5±0.6</td>
<td>4.8±0.9</td>
</tr>
<tr>
<td>Hamster</td>
<td>5</td>
<td>45</td>
<td>43.8±1.0</td>
<td>83.2±1.0</td>
<td>5.1±0.4</td>
<td>11.6±0.7</td>
</tr>
<tr>
<td>Rat</td>
<td>5</td>
<td>45</td>
<td>33.0±1.1</td>
<td>76.5±2.3</td>
<td>2.1±0.1</td>
<td>21.3±2.2</td>
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<tr>
<td>Rabbit</td>
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<td>45</td>
<td>27.8±1.1</td>
<td>77.1±0.6</td>
<td>4.0±0.4</td>
<td>18.9±0.5</td>
</tr>
<tr>
<td>Dog</td>
<td>5</td>
<td>45</td>
<td>399.9±22.88</td>
<td>73.3±1.3</td>
<td>3.8±1.1</td>
<td>22.9±1.7</td>
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<tr>
<td>Pig</td>
<td>6</td>
<td>45</td>
<td>31.0±1.0</td>
<td>82.3±1.4</td>
<td>2.3±0.2</td>
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<td>30</td>
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<td>94.8±1.1</td>
<td>5.2±1.2</td>
<td>1.0±0.1</td>
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<td><strong>Aorta</strong></td>
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<tr>
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<td>45</td>
<td>10.6±0.4</td>
<td>83.3±1.3</td>
<td>0.6±0.1</td>
<td>17.2±0.8</td>
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<td>65.4±2.0</td>
<td>10.3±1.7</td>
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</table>

* Molar ratio of residual Ang I vs total Ang I added; †molar ratio of formed Ang II vs total Ang I added; ‡molar ratio of total Ang I added minus residual Ang I minus formed Ang II vs total Ang I added.

§P<0.05 vs others.

to 1.93±0.43 U, P<0.05), hamster aorta (6.88±0.50 to 5.90±0.73 U, P<0.05), rat aorta (1.08±0.09 to 0.95±0.10 U, P<0.05) and lung (18.4±0.98 to 17.2±0.38 U, P<0.05), dog aorta (0.16±0.04 to 0.13±0.04 U, P<0.05) and lung (20.4±1.71 to 11.2±0.74 U, P<0.05), and pig lung (7.12±0.98 to 5.23±0.88 U, P<0.05).

### Discussion

In this study, we examined total Ang II–forming activities by analyzing the responsible enzymes in several organs from several species and compared the results with those in humans. Our results suggest that each organ in each species has an unique profile with regard to tissue Ang II formation. The present results suggest that it is difficult to specify an appropriate animal model for studying tissue Ang II formation in humans. None of the organs from any of the species examined had a profile that was identical to those for human tissues. However, chymase-like enzyme predominance in cardiac and aortic Ang II formation was seen not only in human tissue but also in that of most of the other species, except for rabbits and pigs.

In addition, pulmonary chymase-like enzymatic activity was highest in human tissues, indicating that chymase-like enzyme in the lung may have a greater physiological role in

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mast cells. In fact, there are considerable differences in the density and subclass of mast cells in the lung, heart, and alimentary tract. Mast cells were found in normal surface airway in human, monkey, dog, and pig lungs but not in rodent, cat, cow, or bird lungs. Schwartz et al reported that chymase-containing mast cells (CMCs) are rare in the peripheral region of normal human lungs. In contrast, the proportion of CMCs in the bronchial epithelium is considerably higher (≈23% of all mast cells).

In certain pathological conditions, the proportion of CMCs may be increased. For example, the mean ratio of CMCs to mast cells containing only tryptase (TMCs) in bronchoalveolar lavage was 0 to 0.2 in normal control and 7.3 in cystic fibrosis, whereas levels of tryptase did not differ between the 2 groups, indicating a marked shift in the distribution of CMCs and TMCs.

Heterogeneity in mast cell response was also reported by Patella et al. Mast cells isolated from skin, lung, and heart were compared in their secretary response to IgE receptor stimulation by its antibody. Human heart mast cells responded well to anti-IgE antibody, but human skin mast cells did not. Substance P and morphine induced the release of histamine from skin mast cells but not from heart and lung mast cells. These results indicate that not only the densities and its subclass but also the characters of mast cells are important for their pathophysiological roles in each tissue. It is not confirmed, however, which factor contributed most to the species or organ differences in the Ang II–forming activity.

Recent physiopharmacological studies have emphasized the clinical significance of an alternative ACE-independent, Ang II–forming pathway in the systemic circulation and tissues. Our recent results in human heart and aorta suggested that chymase immunoreactivity increased in the ischemic region after myocardial infarction and that the enzymatic and immunohistochemical activities of human chymase were significantly higher in the atherosclerotic region than in the normal aorta. In addition, a recent clinical trial, ELITE, showed that the angiotensin subtype 1 (AT1) receptor antagonist losartan was superior to the ACE inhibitor captopril in reducing mortality in elderly patients with congestive heart failure. One possible explanation for the difference in beneficial outcome between ACE inhibitor and AT1 receptor antagonist is that all of the Ang II derived from both ACE and non-ACE enzymes could be blocked by AT1 receptor antagonist but not by ACE inhibitor. These clinical

Figure 2. Dose-dependent inhibition of cardiac Ang II formation by captopril and chymostatin. Particulate fractions of cardiac tissues were incubated with synthetic Ang I in the presence of different doses of captopril (1, 10, 100, and 1000 μmol/L, □) or chymostatin (0.1, 1, 10, 100, and 1000 μmol/L, □). The Ang II formed in the presence of inhibitor was presented as the percentile versus that formed without inhibitors. Each data point represents the average of duplicate analyses. Please note that saturable inhibition was observed with both captopril and chymostatin.

Figure 3. Comparison of total Ang II–forming activities in all species in heart (open columns), aorta (hatched columns), and lung (solid columns); *P<0.05 vs pulmonary total Ang II–forming activities; †P<0.05 vs aortic total Ang II–forming activities; ‡, §, ||, P<0.05 vs total Ang II–forming activities in human heart, aorta, and lung, respectively.
data potentially suggest that non-ACE (probably due to chymase)-dependent Ang II formation in humans plays a significant role in pathological processes of cardiovascular diseases.

Although there were significant differences in chymase-like activities among tissues or species or between intact and diseased tissues, our biochemical results including the like activities among tissues or species or between intact and diseased tissues. This result potentially suggests that the lung in particular species may contain an unknown substance inducing nonspecific inhibition of each inhibitor or both. Obviously, further study will be necessary to clarify the mechanism of this phenomenon.

The present study revealed that a broad serine proteinase inhibitor, aprotinin, did not show a consistent inhibitory effect on Ang II–forming activity in vitro and its inhibitory effects differ even in the same tissue or species. Specifically, in aorta, aprotinin decreased Ang II–forming activity in hamster, rat, and dog, while it increased activity in the dog, pig, and marmoset; in the pig, aprotinin decreased Ang II–forming activity in the lung but increased it in the heart and aorta. These inconsistent results indicate that each tissue from each species may have a different sensitivity to aprotinin in Ang II–forming activities. The tissues in which Ang II–forming activities are decreased with aprotinin appear to contain aprotinin-sensitive Ang II–forming enzymes such as kallikrein or cathepsin G. On the other hand, it is unlikely that the tissues in which Ang II–forming activities increased after treatment with aprotinin contain aprotinin-sensitive Ang I–or Ang II–degrading enzymes, since after aprotinin treatment, peaks other than those of Ang I or Ang II in the HPLC analyses did not differ from those for no treatment, indicating no further degradation of Ang I or Ang II by aprotinin-sensitive enzymes. In addition, the increased Ang II–forming activities with aprotinin in those tissues were considerably inhibited by pretreatment with chymostatin (H.U., M.A., unpublished observations, 1997). These results indicate that most of the chymostatin-inhibitable Ang II–forming activity was due to chymase. Chymase is inhibited by chymostatin but not by aprotinin, whereas cathepsin G is inhibited by both inhibitors. For this reason we measured aprotinin-sensitive Ang II–forming activity to confirm the specificity of our assay.

In conclusion, each organ in each species has a unique profile regarding the pathways of Ang II formation. In humans, chymase-dependent Ang II formation was predominant in the lung, aorta, and heart, which supports the clinical significance of ACE-independent Ang II formation. When studying the pathophysiological roles of ACE-independent Ang II formation in animal models, one should choose species and/or organs in which the Ang II–forming system has a profile similar to that in humans.

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References


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