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ACE- Versus Chymase-Dependent Angiotensin II Generation in Human Coronary Arteries

A Matter of Efficiency?

Beril Tom, Ingrid M. Garrelds, Elizabeth Scalbert, Alexander P.A. Stegmann, Frans Boomsma, Pramod R. Saxena, A.H. Jan Danser

Objective—The objective of this study was to investigate ACE- and chymase-dependent angiotensin I-to-II conversion in human coronary arteries (HCAs).

Methods and Results—HCA rings were mounted in organ baths, and concentration-response curves to angiotensin II, angiotensin I, and the chymase-specific substrate Pro^{11}-D-Ala^{12}–angiotensin I (PA–angiotensin I) were constructed. All angiotensins displayed similar efficacy. For a given vasoconstriction, bath (but not interstitial) angiotensin II during angiotensin I and PA–angiotensin I was lower than during angiotensin II, indicating that interstitial (and not bath) angiotensin II determines vasoconstriction. PA–angiotensin I increased interstitial angiotensin II less efficiently than angiotensin I. Separate inhibition of ACE (with captopril) and chymase (with C41 or chymostatin) shifted the angiotensin I concentration-response curve ≈5-fold to the right, whereas a 10-fold shift occurred during combined ACE and chymase inhibition. Chymostatin, but not captopril and/or C41, reduced bath angiotensin II and abolished PA–Ang I–induced vasoconstriction. Perfused HCA segments, exposed luminally or adventitially to angiotensin I, released angiotensin II into the luminal and adventitial fluid, respectively, and this release was blocked by chymostatin.

Conclusions—Both ACE and chymase contribute to the generation of functionally active angiotensin II in HCAs. However, because angiotensin II loss in the organ bath is chymase-dependent, ACE-mediated conversion occurs more efficiently (ie, closer to AT_1 receptors) than chymase-mediated conversion. **(*Arterioscler Thromb Vasc Biol*. 2003;23: 251-256.)**

Key Words: angiotensin ■ angiotensin-converting enzyme ■ chymase ■ human coronary artery ■ Pro^{11}-D-Ala^{12}–angiotensin I

ACE inhibitors are widely used for the treatment of hypertension and heart failure. Their beneficial effects are believed to be attributable to blockade of the generation of angiotensin (Ang) II from Ang I. In contrast with this concept, during chronic ACE inhibitor therapy, plasma and tissue Ang II levels are unchanged or elevated compared with the pretreatment situation.[1,2] This is not a methodological artifact.[1,2] Several explanations may therefore be put forward. First, the ACE inhibitor dose may have been too low to obtain sufficient ACE inhibition. Indeed, high-dose ACE inhibition seems to be more effective than low-dose ACE inhibition.[3] Second, the rise in renin and Ang I that occurs when Ang II no longer suppresses renin release may overcome ACE inhibition, at least in part.[4,5] Third, ACE upregulation is known to occur both as a consequence of chronic ACE inhibitor therapy and during the progression of cardiovascular diseases.[2] Fourth, in vitro studies have shown that there are alternative enzymes capable of converting Ang I into Ang II.[6–8] The most important of these is the serine protease chymase. Although one in vivo study recently reported that the chymase-specific substrate Pro^{11}-D-Ala^{12}–Ang I (PA–Ang I) induced AT_1 receptor–mediated vasoconstriction in human dorsal hand veins,[9] in vivo evidence for chymase-dependent Ang II generation from native Ang I could not be obtained in humans.[10]

In this respect, it is important to realize that the location of ACE and chymase differs greatly. ACE is a membrane-associated enzyme that, because of proteolytic cleavage of its membrane anchor, also occurs in a soluble form in the extracellular fluid. ACE-expressing cells include endothelial cells, vascular smooth muscle cells, and cardiomyocytes.[11,12] In contrast, chymase is located intracellularly, mainly in the cytosol of mast cells that are present in the adventitia.[13] It has also been demonstrated in endothelial[13] and vascular smooth muscle cells[14] and in the extracellular matrix,[13] although not all studies agree on this matter.[15] Based on this distribution

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pattern, it is generally believed that chymase is predominantly active in the interstitial space.[16] However, studies investigating interstitial Ang II generation could not[17] or only partly[16] confirm this concept, either because interstitial fluid contains endogenous inhibitors of chymase (eg, α_1-antitrypsin)[18] or because of species differences.[19]

It was the aim of the present study to provide an explanation for the discrepancy between in vivo and in vitro studies with regard to the contribution of chymase to Ang I-to-II conversion in humans. Previous in vitro studies measured either Ang II generation in tissue homogenates[6,19] or evaluated Ang II–mediated constriction after the application of Ang I or PA–Ang I to intact human arteries.[8] In this study, using human coronary arteries (HCAs), we measured Ang II release, Ang II–mediated contractile responses, and interstitial Ang II levels after the application of PA–Ang I and native Ang I with or without inhibitors of ACE and/or chymase. The underlying assumption of our studies was that because of the different location of ACE and chymase, chymase-dependent generation of functionally active Ang II occurs less efficiently, ie, further away from AT_1 receptors, and thus (because of rapid angiotensin metabolism in the interstitial space) requires more Ang I to lead to vasoconstriction. HCAs are particularly useful for this purpose, because they contain ACE, chymase, and AT_1 receptors.[7]

Methods

Drugs

Ang I, Ang II, captopril, chymostatin, PD123319, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), and substance P were purchased from Sigma. C41 (3-[3,4-dimethoxyphenylsulfonyl]-1-3,4-dimethylphenylimidazoline-2,4-dione) was synthesized by Dr M.C. Viaud, Tours, France. Irbesartan was a gift from Bristol-Myers Squibb, and PA–Ang I (purity >95%) was a gift from the Institut de Recherches Internationales Servier. Chymostatin and C41 were dissolved in dimethylsulfoxide, and irbesartan was dissolved in ethanol. All other compounds were dissolved in distilled water.

Tissue Collection and Preparation

HCAs were obtained from 17 heart-beating organ donors (6 men, 11 women; ages 18 to 58 years, mean±SEM 46±3 years) who died of noncardiac causes (14 cerebrovascular accident, 3 head trauma) <24 hours before the heart was taken to the laboratory. Hearts were provided by the Rotterdam Heart Valve Bank after removal of the aortic and pulmonary valves for transplantation purposes. The study was approved by the Ethics Committee of the Erasmus MC. Immediately after circulatory arrest, the hearts were stored in an ice-cooled sterile organ-protecting solution.[7] After arrival in the laboratory, the HCA (diameter ≈3 to 4 mm) was removed and stored overnight in a cold, oxygenated Krebs bicarbonate solution of the following composition (in mmol/L): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, and glucose 8.3; pH 7.4. Vessels containing macroscopically visible atherosclerotic lesions were excluded.

Organ Bath Studies

Vessels were cut into segments of ≈4 mm length, suspended on stainless steel hooks in 15-mL organ baths containing Krebs bicarbonate solution, aerated with 95% O_2/5% CO_2, and maintained at 37°C. Segments were allowed to equilibrate for at least 30 minutes, and the organ bath fluid was refreshed every 15 minutes during this period. Changes in tissue contractile force were recorded with a Harvard isometric transducer (South Natick). The segments, stretched to a stable force of ≈15 mN, were exposed to 30 mmol/L KCl twice. Subsequently, segments were exposed to 100 mmol/L KCl to determine the maximal contractile response to KCl. The segments were then allowed to equilibrate in fresh organ bath fluid for 30 minutes. Next, segments were preincubated for 30 minutes with or without the AT_1 receptor antagonist irbesartan (1 μmol/L), the AT_2 receptor antagonist PD123319 (1 μmol/L), the chymase inhibitor chymostatin (100 μmol/L), the ACE inhibitor captopril (10 μmol/L), and/or the chymase inhibitor C41 (10 μmol/L). Thereafter, concentration response curves (CRCs) to Ang I, Ang II, and PA–Ang I were constructed. Endothelial integrity was verified at the end of each experiment by observing relaxation to 1 nmol/L substance P after precontraction with 1 μmol/L $PGF_{2\alpha}$.

To measure Ang II in organ bath fluid, a 50 μL-sample was obtained from the organ bath at the time the vasoconstrictor response had reached a plateau. Samples were rapidly mixed with 10 μL angiotensinase inhibitor solution[7] and stored at −80°C. Tissue Ang II was measured in a limited number of vessel segments only. Based on previous measurements in vessel segments showing that vascular Ang II remains stable for at least 1 hour after the addition of Ang I,[20] the segments were removed 1 hour after the addition of 1 μmol/L Ang I or PA–Ang I, washed in fresh Krebs solution, dried on tissue paper, frozen in liquid nitrogen, and stored at −80°C.

Perfusion Studies

Vessels were cut into segments of 1 to 2 cm length. Side branches, if present, were tied off with silk sutures. Each segment was mounted horizontally in a double-jacketed 4-mL organ bath and perfused from a 6-mL reservoir using a roller pump (Ismatec IPS; flow 1 mL/min). Both organ bath and reservoir contained Krebs bicarbonate solution, aerated with 95% O_2/5% CO_2, and were maintained at 37°C. The perfusate was collected in the reservoir and reperfused through the vessel, thus creating a closed perfusion circuit,[21] allowing both adventitial (ie, into the bath) and luminal (ie, into the reservoir) drug application. Ang I (1 μmol/L) was added to the bath or the reservoir, under control conditions, in the presence of 10 μmol/L captopril and in the presence of 10 μmol/L captopril plus 100 μmol/L chymostatin, respectively. Luminal and adventitial fluid samples (50 μL) were obtained as described above immediately before and 30 and 60 minutes after the addition of Ang I. Before each subsequent Ang I application, bath and reservoir fluid were refreshed. All experiments were performed in the presence of 1 μmol/L irbesartan to prevent AT_1 receptor–mediated vasoconstriction.

Biochemical Measurements

Ang II in organ bath and reservoir fluid was measured with sensitive radioimmunoassays.[7] Ang II in vessel segments was measured by radioimmunoassay after SepPak extraction and HPLC separation.[5]

Data Analysis

Data are given as mean±SEM. Contractile responses are expressed as a percentage of the maximal contraction to 100 mmol/L KCl (63.9±4.9 mN, n=11). CRCs were analyzed as described earlier[7] to obtain pEC_{50} ($-^{10}\log EC_{50}$) values. Statistical analysis was performed by one-way ANOVA, followed by post hoc evaluation (according to Tukey or Dunnett where appropriate). $P<0.05$ was considered significant.

Results

Organ Bath Studies

Ang I, Ang II, and PA–Ang I displayed similar maximal effects (Figure 1, Table). Irbesartan, but not PD123319, prevented the PA–Ang I–induced vasoconstriction (Figure 1), indicating that its contractile effects, like those of Ang I in this model,[7] are mediated via AT_1 receptors. PA–Ang I was less potent ($P<0.05$) than Ang I and Ang II. At the moment of constriction, Ang II levels in the organ bath fluid during both Ang I and PA–Ang I amounted to <5% of the Ang I and PA–Ang I levels (Figure 2), and the pEC_{50} values calculated

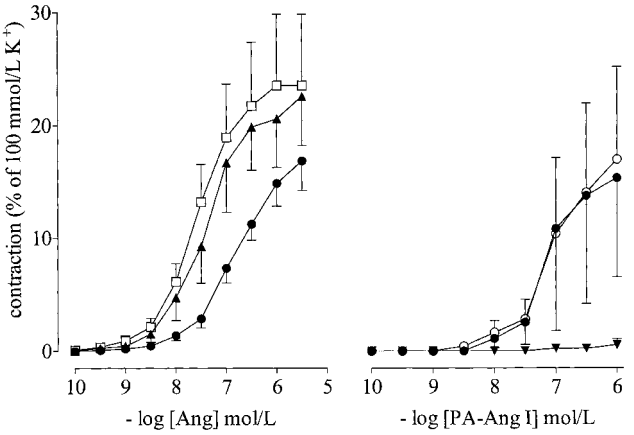

Figure 1. Left, contractions of HCAs to Ang I (▲), Ang II (□), and PA–Ang I (●). Contractions (mean±SEM, n=8) are expressed as a percentage of the response to 100 mmol/L KCl. Right, contractions of HCAs to PA–Ang I in the absence (●) or presence of irbesartan (▼) or PD123319 (○). Contractions (mean±SEM, n=3) are expressed as a percentage of the response to 100 mmol/L KCl.

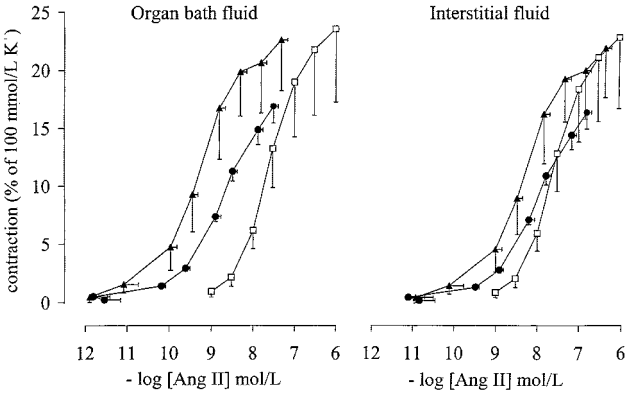

Figure 2. Contractions of HCAs versus organ bath fluid (left) and interstitial (right) Ang II levels at the time of constriction after the addition of Ang I (▲), Ang II (□), and PA–Ang I (●). Contractions (mean±SEM, n=8) are expressed as a percentage of the response to 100 mmol/L KCl.

from these Ang II levels (9.20±0.25 and 8.90±0.15 for Ang I and PA–Ang I, respectively; P=NS) were 20 to 40 times higher ($P<0.01$) than the pEC_{50} for Ang II. Thus, for a given constriction, the organ bath fluid Ang II level was lower during Ang I and PA–Ang I application than during Ang II application.

Captopril (P=NS), C41 (P=NS), and chymostatin ($P<0.05$) modestly shifted the Ang I CRC to the right, whereas a ≈5- to 10-fold rightward shift ($P<0.01$) occurred during C41 plus captopril (Figure 3, Table). Chymostatin, but not captopril and/or C41, abolished the effects of PA–Ang I. C41 did not affect the Ang II CRC (Table).

Chymostatin reduced the Ang II release into the organ bath during Ang I and PA–Ang I application by >75% ($P<0.05$, Figure 4). Captopril, C41, or their combination did not significantly affect the Ang II levels in the organ bath during Ang I and PA–Ang I application.

Interstitial Ang II and Vasoconstriction

One hour after the application of 1 μmol/L Ang I to the organ bath, the level of Ang II in the arterial segment (wet weight 24±2 mg; n=24) was 18.7±4.3 pmol/g wet weight (n=4). Vascular Ang II was not affected by irbesartan (18.9±6.0 pmol/g; n=4) or PD123319 (29.4±12.1 pmol/g; n=4). Comparable observations were made after the application of 1 μmol/L PA–Ang I (vascular Ang II, respectively, 11.4±1.4, 9.9±1.4 and 7.6±2.4 pmol/g; n=4 for each condition), although the vascular Ang II levels during PA–Ang I were 2 to 4 times ($P<0.01$) lower than during Ang I. The organ bath fluid levels of Ang II in these experiments were not affected by irbesartan or PD123319 and resembled those in Figure 4 (data not shown).

The lack of effect of irbesartan and PD123319 on vascular Ang II indicates that most tissue Ang II is not bound to either cell surface or internalized AT_1 or AT_2 receptors and thus is located extracellularly (ie, in interstitial fluid). Because interstitial fluid accounts for ≈15% of tissue weight,[20] it can be calculated that the interstitial Ang II levels during Ang I and PA–Ang I application are 148.7±35.6 and 64.3±5.1 nmol/L (mean of all measurements, including those with AT receptor blockers), respectively. This is 9.4±4.2 and 5.1±1.5 ($P<0.05$ for difference) times higher than the organ bath fluid Ang II levels at the time of vasoconstriction. The interstitial Ang II levels during Ang II application are as high as the Ang II levels in the organ bath.[20] The right panel of Figure 2 illustrates the consequence of this concept. It is clear that for a given vasoconstriction, the interstitial Ang II levels during Ang I, PA–Ang I, and Ang II are comparable. However, it

pEC_{50} and E_{max} of Ang I, Ang II, and PA-Ang I in Human Coronary Arteries With or Without Inhibitors

	Ang I		PA–Ang I		Ang II	
Inhibitor	pEC_{50}	E_{max}, % of 100 mmol/L K^+	pEC_{50}	E_{max}, % of 100 mmol/L K^+	pEC_{50}	E_{max}, % of 100 mmol/L K^+
None	7.35±0.13	23±4.4	6.87±0.14*	17±2.7	7.63±0.05	24±6.3
10 μmol/L captopril	7.25±0.13	20±3.9	7.00±0.12	17±2.9		
10 μmol/L C41	7.14±0.12	18±3.7	6.75±0.11	14±2.7	7.55±0.06	17±3.8
10 μmol/L captopril+10 μmol/L C41	6.58±0.05†	21±6.2	6.67±0.07	17±3.7		
100 μmol/L chymostatin	6.90±0.13‡	18±4.4	<6‡	6.7±1.7‡§		

Data are mean±SEM of 8 experiments.

*$P<0.05$ vs Ang I and Ang II. †$P<0.05$ and ‡$P<0.01$ vs none. §Contractile response at the highest concentration of Ang I that was tested.

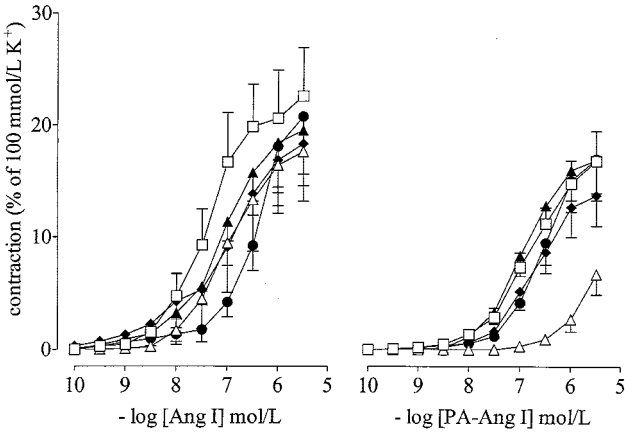

Figure 3. Contractions of HCAs to Ang I (left) and PA–Ang I (right) in the absence (□) or presence of chymostatin (△), captopril (▲), C41 (◆), or captopril+C41 (●). Contractions (mean±SEM, n=8) are expressed as a percentage of the response to 100 mmol/L KCl.

requires more PA–Ang I than Ang I to reach a certain interstitial Ang II level.

Perfusion Studies

Luminal Ang I application resulted in the appearance of Ang II in luminal but not adventitial fluid, whereas adventitial Ang I application resulted in the appearance of Ang II in adventitial but not luminal fluid (Figure 5). Ang II levels increased over time and were ≈4 times higher in adventitial fluid after adventitial Ang I application than in luminal fluid after luminal Ang I application. Chymostatin plus captopril, but not captopril alone, fully prevented luminal and adventitial Ang II release.

Discussion

The present study compares, in HCAs mounted in organ baths, the vasoconstrictor efficiency of Ang II generated by ACE and by chymase, using both native Ang I and the chymase-specific substrate PA–Ang I. The results show that

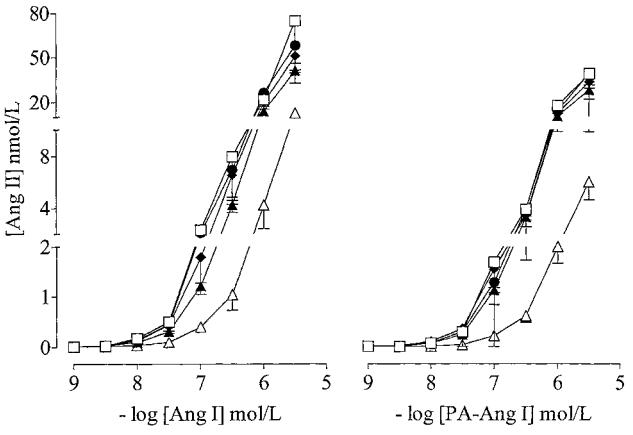

Figure 4. Ang II levels in organ bath fluid measured at the moment of contraction of HCAs during the construction of CRCs to Ang I (left) and PA–Ang I (right) in the absence (control; □) or presence of chymostatin (△), captopril (▲), C41 (◆), or captopril+C41 (●). Data are mean±SEM of 8 experiments.

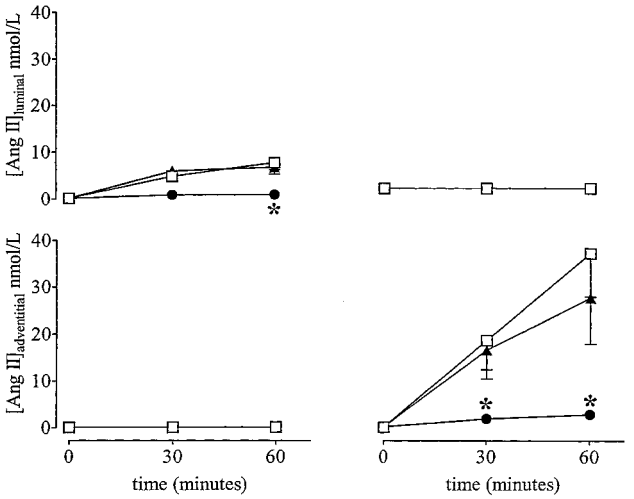

Figure 5. Luminal and adventitial Ang II levels after the luminal (left panels) or adventitial (right panels) application of Ang I (1 μmol/L) under control conditions (□), in the presence of captopril (▲), or in the presence of captopril+chymostatin (●). In the panels providing the control data only, Ang II was undetectable under all conditions. SEM, if not shown, is smaller than the symbol. Data are mean±SEM of 5 experiments (*$P<0.05$ vs control).

Ang I is 5 to 10 times more potent than PA–Ang I and that Ang II release into the organ bath is almost exclusively chymase-dependent. Furthermore, chymase inhibition with chymostatin is sufficient to block the vasoconstrictor effects of PA–Ang I, whereas in the case of Ang I, combined inhibition of ACE and chymase results in a better blockade of vasoconstriction than separate inhibition of ACE and chymase. Thus, both ACE and chymase contribute to the generation of functionally active Ang II from Ang I in isolated HCAs, but the generation of Ang II by chymase is far less efficient, resulting in loss of Ang II into the organ bath fluid, thereby requiring more Ang I to obtain a certain degree of constriction. These data may offer an explanation for the discrepancy between in vitro and in vivo findings with regard to the enzymes contributing to Ang I-to-II conversion.[7,8,10]

In agreement with previous in vitro[7] and in vivo[10] studies, Ang I was as potent as Ang II in the present experimental setup. This is remarkable in view of the low Ang II concentrations that were detected in the organ bath fluid during the construction of the Ang I CRC. In fact, for a given constriction, the organ bath fluid concentrations during Ang I were ≈40 times lower than those during Ang II. The explanation for this apparent discrepancy, confirmed in the present study, is that the vascular interstitial Ang II concentrations at equimolar Ang I and Ang II levels are comparable[20] and thus that vasoconstriction is determined by interstitial rather than organ bath fluid Ang II. The findings on PA–Ang I parallel those on Ang I. Taken together, therefore, the levels of Ang II in the organ bath fluid are not representative of the Ang II levels seen by the receptor and rather represent nonfunctional Ang II that has been lost in the organ bath. Similarly, coronary constriction in the isolated perfused rat heart during exposure to either renin or Ang II did not relate to the Ang II levels in the effluent, suggesting that also in this preparation

(representative for resistance vessels, as opposed to the conduit-type artery used in the present study), constriction is determined by tissue (interstitial) Ang II.[22]

Chymostatin, but none of the other inhibitors used in this study (including the chymase-selective inhibitor C41), prevented the release of Ang II into the organ bath fluid during the application of both Ang I and PA–Ang I. The lack of effect of 10 μmol/L C41, despite its potent inhibitory effects toward chymase in human heart homogenates (IC_{50} 22 nmol/L),[8] suggests either that C41 does not have sufficient access to chymase in intact tissue or that chymostatin, being a nonselective chymase inhibitor, inhibits alternative converting enzymes (eg, cathepsin G). The latter conclusion would also imply that PA–Ang I is not a chymase-specific substrate, as has already been suggested based on studies in rat arteries.[23] However, in humans using aprotinin (an inhibitor of cathepsin G but not chymase), evidence for cathepsin G–mediated Ang I-to-II conversion could not be obtained.[6] Thus, the most likely explanation for the limited effect of C41 compared with chymostatin, in agreement with a recent study in human mammary arteries,[8] is that, at the concentration we used, its inhibitory capacities are more modest than those of chymostatin. It does have access to chymase, as evidenced by our observation that the Ang I CRC is shifted to the right in the presence of captopril and C41, but not when these inhibitors are given separately. The limited availability of C41 prevented us from constructing Ang I and PA–Ang I CRCs at higher C41 concentrations.

Captopril, at a dose that is sufficient to obtain complete ACE inhibition,[21,24] did not reduce Ang II release in the organ bath. In contrast, ACE inhibition resulted in complete suppression of Ang II release during incubation of porcine arteries with Ang I and during perfusion of rat hearts with renin or Ang I.[20–22] Thus, although ACE-dependent Ang II release does occur in vitro, in human arteries it is apparently modest compared with the chymase-dependent Ang II release. In view of the fact that chymase is predominantly located adventitially,[13] whereas ACE is largely present on endothelial cells,[12] we attempted to distinguish endothelial from adventitial Ang II release by quantifying Ang II in luminal and adventitial fluid obtained from perfused HCAs after the application of Ang I to either the luminal or the adventitial compartment. The data confirm the concept that chymase is located predominantly adventitially, because the chymase-dependent Ang II release after adventitial Ang I application was 4 times higher than that after luminal Ang II release. Angiotensin diffusion from the adventitial compartment to the luminal compartment or vice versa did not occur, in agreement with earlier work suggesting that such exchange probably takes place at the level of the capillaries.[21,25] Remarkably, however, ACE inhibition did not affect luminal Ang II release. This contrasts with in vivo studies measuring venous Ang II levels during arterial Ang I infusion,[10] as well as with studies in Ang I–perfused porcine arteries.[21] Because chymase has been demonstrated in endothelial cells,[13] release of this chymase under in vitro conditions may have resulted in a greater contribution of chymase to endothelial Ang II release ex vivo, thus making it impossible to detect ACE-dependent Ang II release. Along the same lines of reasoning, the contribution of adventitial chymase is probably also larger ex vivo. Explanations for the greater role of chymase in vitro than in vivo are, first, that tissue storage and handling may have resulted in chymase release from intracellular storage sites and, second, the absence of endogenous chymase inhibitors (eg, α_1-antitrypsin and secretory leukocyte protease inhibitor)[18,26] in isolated vessel preparations.

Irrespective of the cause of the major contribution of chymase to Ang II generation in isolated HCAs, it is clear that ACE is located more strategically than chymase, ie, closer to AT_1 receptors, thereby allowing it to contribute to Ang II generation in a highly efficient manner, with little or no loss of Ang II to the organ bath. The higher interstitial Ang II levels during Ang I application compared with PA–Ang I application confirm this concept. Similarly, efficient ACE-dependent Ang II generation has been demonstrated in porcine femoral arteries and in cultured rat cardiomyocytes.[20,27]

The question arises whether chymase does play a role at all in vivo. In view of its location (adventitial, largely intracellular), it may not face the same Ang I levels as ACE. Moreover, endogenous inhibitors in interstitial fluid suppress its activity, thereby counteracting its more efficient cleavage of Ang I compared with ACE (k_{cat}/K_m 198 versus 125 μmol/L^{-1} min^{-1}).[6,18,26] Thus, it will probably only generate Ang II when Ang I levels are high, eg, during ACE inhibition. In support of this concept, all in vivo studies using the chymase-specific substrate PA–Ang I[9,23,28,29] show that the concentrations of this substrate required to induce vasoconstriction are 10 to 100 times higher than the concentrations of Ang I required to reach the same degree of vasoconstriction, despite the fact that the affinities of Ang I and PA–Ang I for chymase are similar.[8] Furthermore, even when Ang II is generated by adventitial chymase at high Ang I levels, its functional activity may be limited, because it is will be exposed to significant metabolism on its way to AT_1 receptors.[17,30]

In conclusion, ACE-dependent Ang II generation results in more efficient AT_1 receptor stimulation than chymase-dependent Ang II generation, and the importance of the latter pathway is overestimated under in vitro conditions.

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