Selective Angiotensin-Converting Enzyme C-Domain Inhibition Is Sufficient to Prevent Angiotensin I–Induced Vasoconstriction

Joep H.M. van Esch, Beril Tom, Vincent Dive, Wendy W. Batenburg, Dimitris Georgiadis, Athanasios Yiotakis, Jeanette M.G. van Gool, René J.A. de Bruijn, René de Vries, A.H. Jan Danser

Abstract—Somatic angiotensin-converting enzyme (ACE) contains 2 domains (C-domain and N-domain) capable of hydrolyzing angiotensin I (Ang I) and bradykinin. Here we investigated the effect of the selective C-domain and N-domain inhibitors RXPA380 and RXP407 on Ang I–induced vasoconstriction of porcine femoral arteries (PFAs) and bradykinin-induced vasodilation of preconstricted porcine coronary microarteries (PCMAs). Ang I concentration-dependently constricted PFAs. RXPA380, at concentrations \( > 1 \mu M \), shifted the Ang I concentration-response curve (CRC) 10-fold to the right. This was comparable to the maximal shift observed with the ACE inhibitors (ACEi) quinaprilat and captopril. RXP407 did not affect Ang I at concentrations \( \leq 0.1 \mu M \). Bradykinin concentration-dependently relaxed PCMAs. RXPA380 (10 \( \mu M \)) and RXP407 (0.1 \( \mu M \)) potentiated bradykinin, both inducing a leftward shift of the bradykinin CRC that equaled \( 50\% \) of the maximal shift observed with quinaprilat. Ang I added to blood plasma disappeared with a half-life \( t_{1/2} \) of 42 ± 3 minutes. Quinaprilat increased the \( t_{1/2} \) 4-fold, indicating that 71 ± 6% of Ang I metabolism was attributable to ACE. RXPA380 (10 \( \mu M \)) and RXP407 (0.1 \( \mu M \)) increased the \( t_{1/2} \) 2-fold, thereby suggesting that both domains contribute to conversion in plasma. In conclusion, tissue Ang I–II conversion depends exclusively on the ACE C-domain, whereas both domains contribute to conversion by soluble ACE and to bradykinin degradation at tissue sites. Because tissue ACE (and not plasma ACE) determines the hypertensive effects of Ang I, these data not only explain why N-domain inhibition does not affect Ang I–induced vasoconstriction in vivo but also why ACEi exert blood pressure–independent effects at low (C-domain–blocking) doses. (Hypertension. 2005;45:120-125.)

Key Words: angiotensin ▪ bradykinin ▪ angiotensin-converting enzyme

Angiotensin-converting enzyme (ACE) is a dipeptidyl carboxypeptidase that generates angiotensin II (Ang II) and inactivates bradykinin, 2 peptides that play a key role in regulation of blood pressure. Somatic ACE has 2 homologous domains, each containing an active center. According to their positions (N-terminal and C-terminal), these domains are designated as the N-domain and C-domain, respectively. Bradykinin potentiation by ACE inhibitors correlates directly with ACE C-domain and N-domain blockade1,2 (ie, half maximal potentiation is observed during blockade of 1 domain, and full potentiation occurs during blockade of both domains). In contrast, selective inhibition of the N-domain with the phosphinic peptide RXP407 did not affect blood pressure responses to Ang I bolus injections in mice,3 nor did targeted inactivation of the N-domain in mice affect blood pressure or plasma Ang II levels.4 Although this suggests that the C-domain is the dominant Ang I–converting site, a recent study2 demonstrated that selective inhibition of either the N-domain or the C-domain fully prevents conversion of systemically administered Ang I in mice. Moreover, in the latter study, it was simultaneously demonstrated that when using soluble ACE, full inhibition of Ang I cleavage is obtained only during blockade of both ACE active sites. Thus, Ang I–II conversion by membrane-bound and soluble ACE appears to obey to different mechanisms, and C-domain–selective ACE inhibitors such as quinaprilat,5 Ang-(1–7),6 and the new phosphinic peptide RXPA3802 may fully suppress tissue Ang I–II conversion at concentrations that neither fully block Ang I–II conversion in plasma nor fully potentiate bradykinin.

To study this possibility, we compared the inhibitory effects of the C-domain–selective inhibitors RXPA380,2...
quinaiprilat, and Ang-(1–7) (selectivity for C-domain versus N-domain, respectively, ≈1000- to 200-fold) and the N-domain–selective inhibitors captopril and RXP407 (selectivity for N-domain versus C-domain, respectively, ≈20- and 300-fold) toward membrane-bound and soluble ACE. Ang I–II conversion by membrane-bound ACE was quantified by investigating Ang I–induced contractions of porcine femoral arteries (PFAs). These contractions do not involve enzymes other than ACE. Ang I–II conversion by soluble ACE was studied by quantifying Ang II generation after the addition of Ang I to human blood plasma. Bradykinin potentiation was studied in porcine coronary arteries (PCAs) and porcine coronary microarteries (PCMAs). We excluded the possibility that the Ang-(1–7)–induced potentiation of bradykinin is mediated via the recently cloned Ang-(1–7) receptors. Finally, we studied the consequences of C-domain inhibition toward bradykinin in human coronary microarteries (HCMAs). The consequences of such inhibition toward Ang I–II conversion could not be studied in HCMAs because in isolated human coronary vessels, Ang I conversion depends on chymase rather than ACE.

Methods

Tissue and Blood Collection

HCMAW were obtained from 6 heart-beating organ donors (3 men and 3 women; ages 13 to 61 years) who died of noncardiac causes (3 subarachnoidal bleeding, 2 head trauma, 1 postanoxic encephalopathy) <24 hours before the heart was taken to the laboratory. Hearts were provided by the Rotterdam Heart Valve Bank after removal of the valves for transplantation purposes. The study was approved by the ethics committee of the Erasmus MC. Immediately after circulatory arrest, hearts were stored in an ice-cooled sterile organ-protecting solution. At arrival in the laboratory, tertiary branches of the left anterior descending coronary artery (diameter 280 to 300 μm; mean 420 μm) were removed and stored overnight in a cold (4°C) oxygenated Krebs bicarbonate solution of the following composition (mmol/L): 118 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, and 8.3 glucose 3.8, pH 7.4.

PCAs, PCMAs, and PFAs were obtained from 32 2- to 3-month-old pigs (Yorkshire×Landrace; weight 10 to 15 kg) that had been used in vivo in experiments studying the effects of calcitonin-gene related peptide receptor (ant)agonists under pentobarbital (600 mg IV) anesthesia and from 14 slaughterhouse pigs. The ethics committee of the Erasmus MC approved the protocol for this investigation. Arteries were either removed at the end of the experiment or after the heart had been brought to the laboratory in cold Krebs bicarbonate solution. Vessels were stored overnight in cold oxygenated Krebs bicarbonate solution. Blood (50 mL) was collected from 6 healthy volunteers (4 men and 2 women; ages 25 to 41 years) as described previously. Plasma was stored at −70°C.

Functional Studies

After overnight storage, PCAs and PFAs were cut into segments of ~4-mm length and mounted in 15-mL organ baths. HCMAs and PCMAs were cut into segments of ~2-mm length and mounted in Mulvany myographs (J.P. Trading) with separated 6-mL organ baths. HCMAs rather than large human coronary arteries were used because only the former relax to bradykinin. PCMAs were used in the bradykinin studies involving RXP380 and RXP407 because of the limited availability of these drugs. All baths contained Krebs bicarbonate solution at 37°C and were aerated with 95% O₂ and 5% CO₂. Endothelial integrity was verified by observing relaxation to 10 nmol/L substance P after preconstriction with the thromboxane A₂ analogue U46619. Subsequently, to determine the maximum contractile response, the tissue was exposed to 100 mmol/L KCl. Segments were then allowed to equilibrate in fresh organ bath fluid for 30 minutes in the presence or absence of RXP380, RXP407, quinaiprilat, captopril, or Ang-(1–7). The mixture was incubated at 37°C and 200-μL samples were taken at 0, 5, 10, and 30 minutes (1:2 diluted plasma) or at 0, 60, 120, and 240 minutes (1:20 diluted plasma). Samples were immediately mixed with inhibitor solution and stored at −80°C until analysis. Ang I and II were measured with sensitive radioimmunoassays.

Metabolism Studies

To study Ang I–II conversion in plasma, 2.5 pmol Ang I was added to blood plasma diluted 1:2 or 1:20 in phosphate buffer (pH 7.4) in the presence or absence of increasing concentrations of RXP380, RXP407, quinaiprilat, captopril, or Ang-(1–7). The mixture was incubated at 37°C and 200-μL samples were taken at 0, 5, 10, and 30 minutes (1:2 diluted plasma) or at 0, 60, 120, and 240 minutes (1:20 diluted plasma). Samples were immediately mixed with inhibitor solution and stored at −80°C until analysis. Ang I and II were measured with sensitive radioimmunoassays.

Data Analysis

Data are given as mean±SEM. CRCs were analyzed as described previously to obtain pEC50 (−log[EC50]) values. Statistical analysis was by ANOVA, followed by post hoc evaluation according to Dunnett. P<0.05 was considered significant.

Results

Bradykinin-Induced Relaxations

Bradykinin relaxed U46619-preconstricted HCMAs (n=6), PCAs (n=4), and PCMAs (n=14; pEC50 8.3±0.2, 7.4±0.3, and 8.5±0.2, respectively; Figures 1 and 2). Quinaiprilat (10 μmol/L) shifted the bradykinin CRC in all 3 vessel types (HCMAs, PCAs, and PCMAs) ≈10-fold to the left (pEC50 9.5±0.2, 8.5±0.1, and 9.7±0.2, respectively; P<0.01 versus control for all). Ang-(1–7) also shifted the bradykinin CRC in HCMAs to the left, and at a concentration of 10 μmol/L, its effect (pEC50 9.1±0.3; P<0.05 versus control) was comparable to that of 10 μmol/L quinaiprilat (Figure 1). The leftward shift of 10 μmol/L Ang-(1–7) in PCAs (pEC50 7.9±0.5; P<0.05 versus control) was not affected by the Ang-(1–7) receptor antagonist d-Ala–Ang-(1–7) (10 μmol/L;
shifted the Ang I CRC to the right in a concentration-dependent manner, and a maximum (≈10-fold) shift occurred at concentrations of 1 μmol/L, 10 μmol/L, and 100 μmol/L, respectively (Figures 3 and 4). Ang-(1–7), up to a concentration of 1 μmol/L, did not shift the Ang I CRC to the right (n=6; Figure 4), although it did reduce the maximum constrictor effect of Ang I by >60% at the latter concentration. At a 10-fold higher concentration, Ang-(1–7) virtually abolished all Ang I–induced effects (Figure 3). This high–Ang-(1–7) concentration also reduced the maximum effect of Ang II (from 96±23% to 14±4% of the response to 100 mmol/L K⁺; n=3), thereby indicating that its blocking effects toward Ang I are attributable to Ang II type 1 (AT₁) receptor antagonism rather than ACE inhibition. RXP407, up to a concentration of 100 μmol/L, did not significantly affect the Ang I CRC (Figures 3 and 4).

Ang I Metabolism in Human Blood Plasma

The half life of Ang I added to human plasma (diluted 1:2 in phosphate buffer) was 7±0.2 minutes (n=4). Quinaprilat, captopril, and Ang-(1–7) increased the Ang I half life in 1:2 diluted plasma in a concentration-dependent manner to maximally 35±9, 29±4, and 32±6 minutes, respectively (n=4 for each; Figure 5). From these data, it can be calculated that in the absence of inhibitors, 75±3% of the Ang I metabolism in human plasma is attributable to Ang I–II conversion by ACE (Figure 4). The highest quinaprilat and captopril concentrations were tested in this study fully prevented the appearance of Ang II in the incubation mixture (data not shown). Ang-(1–7), at concentrations ≥1 μmol/L, did not affect the generation of Ang II or the half life of Ang I. Higher concentrations of Ang-(1–7) interfered with the Ang II (but not the Ang I) assay, thus not allowing us to demonstrate that these concentrations also suppressed generation of Ang II. However, the latter is highly likely in view of the similar increase in Ang I half life in the presence of the highest Ang-(1–7) concentration as in the presence of the highest quinaprilat and captopril concentrations.


Figure 2. Top panels, Relaxations of U46619-preconstricted PCMAs to bradykinin in the absence (control) or presence of the inhibitors RXPA380 (left) and RXP407 (right). For comparison, the effect of 10 μmol/L quinaprilat is also shown. Data (mean±SEM of 4 to 7 experiments) are expressed as a percentage of the contraction induced by U44169. Bottom panels, Change in \(-\log[EC_{50}]\) of the bradykinin CRC in the presence of increasing concentrations of RXPA380 or RXP407. An increase in \(-\log[EC_{50}]\) represents a leftward shift of the bradykinin CRC. con indicates control. Significant differences (P<0.05) vs control were obtained at the highest 2 concentrations only.

Figure 3. Constrictions of PFAs to Ang I in the absence (control; ○) or presence (●) of 100 μmol/L RXPA380, 10 μmol/L quinaprilat, 10 μmol/L Ang-(1–7), 100 μmol/L captopril, or 100 μmol/L RXP407. Although a wide range of inhibitor concentrations were tested (also see Figure 4), for the sake of clarity, only the CRC obtained in the presence of the highest inhibitor concentration is shown in each panel. Data (mean±SEM of 5 to 8 experiments) are expressed as a percentage of the contraction to 100 mmol/L K⁺.
Importantly, the quinaprilat concentration that maximally shifted the Ang I CRC to the right (10 nmol/L; Figure 4) reduced Ang I conversion by plasma ACE by only 50%, and a 100-fold higher ($P<0.01$) quinaprilat concentration (1 mmol/L) was needed to fully suppress conversion (Figure 4). Conversely, the captopril concentration required to block plasma ACE by 50% ($IC_{50}$) was $\approx 5$-fold lower ($P=NS$) than the captopril concentration required to cause a half-maximal shift of the Ang I CRC ($-\log[IC_{50}] 6.5\pm 0.2$ versus $6.0\pm 0.3$). No such comparisons could be made for Ang-(1–7) because of its AT1 receptor-blocking capacities in the organ bath experiments.

RXPA380 and RXP407, up to concentrations of 100 μmol/L, did not affect Ang I metabolism in 1:2 diluted plasma (n=4; data not shown). Subsequent measurement of these inhibitors in plasma revealed strong plasma protein binding, which reduced their free concentrations by 100-fold (V. Dive, unpublished observations, 2004). Therefore, to minimize the problems arising from protein binding, we studied the effects of these inhibitors in 1:20 diluted plasma. Under these conditions, the half life of Ang I was 42 minutes (n=4; Figure 5). Quinaprilat (10 μmol/L) increased the Ang I half life to 166 minutes ($P<0.01$), thereby demonstrating that 71% of the Ang I metabolism in these samples is attributable to ACE ($P=NS$ versus 1:2 diluted samples). RXPA380 and RXP407 increased the Ang I half life in 1:20 diluted plasma in a concentration-dependent manner to maximally 156 minutes ($P=NS$ versus quinaprilat).
and 80±9 (P<0.01 versus quinaprilat) minutes, respectively (Figure 5). Only the highest RXPA380 concentration (but not the highest RXP407 concentration) fully prevented the appearance of Ang II in the incubation mixture (data not shown). Importantly, the RXPA380 concentration required to fully block Ang I–II conversion in plasma (100 µmol/L) was 100× higher (P<0.01) than the concentration required to fully shift the Ang I CRC to the right (Figure 4).

Discussion
The present study shows that low (C-domain–selective) concentrations of RXPA380 and quinaprilat are sufficient to fully prevent Ang I–induced contractions of PFAs (ie, to cause a maximum rightward shift of the Ang I CRC), whereas high concentrations (capable of blocking the C-domain and N-domain) are required to fully block Ang I–II conversion in human blood plasma. Selective N-domain inhibition with RXP407 did not affect Ang I–induced contractions and reduced Ang I–II conversion in plasma by ≈50%. Together, these data suggest that only the C-domain contributes to Ang I–II conversion by membrane-bound ACE, and that both domains contribute to Ang II generation by soluble ACE. In contrast, bradykinin degradation by membrane-bound ACE depends on both domains because, in agreement with the biphasic quinaprilat-induced leftward shift observed previously in PCAs,1 RXPA380 as well as RXP407 shifted the bradykinin CRC to the left, and a maximum leftward shift was observed only when both domains were blocked. Using the same selective inhibitors, it has already been shown that both domains contribute to bradykinin degradation by soluble ACE.

Our data on captopril, a modestly selective N-domain inhibitor,7 are in full agreement with the above concept of C-domain–dependent Ang I–II conversion by membrane-bound ACE. When using this inhibitor, the concentrations required to shift the Ang I CRC to the right, if anything, were higher than the concentrations required to block Ang I–II conversion by circulating ACE. This directly opposes our findings with quinaprilat and RXPA380. The lack of a significant difference in the present study most likely relates to the modest (∼20-fold) selectivity of captopril toward the N-domain.

We also evaluated the effects of Ang-(1–7), an angiotensin metabolite that selectively blocks the ACE C-domain.6 Studies investigating the metabolism of Ang I in isolated human and porcine vessels have already shown that under the present experimental conditions, Ang-(1–7) will not be generated in sufficient amounts to exert effects.8,9,12,13 First, we investigated the potentiating capacity of Ang-(1–7) toward membrane-bound ACE in HCMAs. To this end, we constructed bradykinin CRCs rather than Ang I CRCs because as a result of the presence of chymase in human coronary arteries, ACE inhibition will not result in a significant rightward shift of the Ang I CRC in human vessels.12,13 As expected, Ang-(1–7) shifted the bradykinin to the left in a concentration-dependent manner, reaching the same maximal leftward shift as quinaprilat at a concentration of 10 µmol/L. The inhibitory capacities of Ang-(1–7) toward human ACE were further supported by the fact that this inhibitor increased the Ang I half life in human blood plasma to exactly the same degree as the ACE inhibitors quinaprilat and captopril. Second, we excluded the possibility that the Ang-(1–7)–induced leftward shift of the bradykinin CRC depends on the activation of Ang-(1–7) receptors rather than ACE inhibition, using the selective Ang-(1–7) receptor antagonist d-Ala–Ang-(1–7).11 As shown in Figure 1, this antagonist indeed did not affect the Ang-(1–7)–induced leftward shift of the bradykinin CRC in PCAs, although it does block the direct vasodilator effects of Ang-(1–7) in the isolated rabbit afferent arteriole.18 Finally, we studied the effects of Ang-(1–7) toward Ang I–induced contractions in PFAs. PCAs are not suitable for such experiments because of their limited reactivity to Ang II.13 In agreement with our findings on quinaprilat, Ang-(1–7) markedly shifted the Ang I CRC to the right, at concentrations that in PCAs selectively blocked the ACE C-domain.1 However, it simultaneously reduced the maximum effect of Ang I, and identical observations were made toward Ang II.19,20 This suggests that the Ang-(1–7) concentrations that selectively block the C-domain are also capable of blocking AT1 receptors, thereby not allowing us to demonstrate the functional consequence of selective C-domain inhibition by Ang-(1–7) toward Ang I.

Our observation that N-domain inhibition does not block Ang I–II conversion by membrane-bound ACE is in full agreement with a previous study demonstrating no effect of the N-domain selective inhibitor RXP407 on the blood pressure responses to Ang I in mice.1 In apparent contrast with our current data, as well as with the data on Ang I pressor responses in mice, Georgiadis et al2 observed that RXP407, at N-domain selective doses, did block conversion of systemically administered Ang I in mice. This discrepancy may be explained in several ways. First, it could relate to in vitro Ang II generation at the time of blood sampling21 in the absence (but not in the presence) of RXPA380 and RXP407. To avoid such in vitro generation, blood samples need to be collected with a syringe containing an angiotensinase inhibitor cocktail. Georgiadis et al2 circumvented this problem by collecting arterial blood samples directly in 20% trifluoroacetic acid. In addition, RXPA380 and RXP407 displayed equivalent in vivo ACE-inhibitory potencies in the study by Georgiadis et al2, and the strong protein binding of these inhibitors would have reduced their capacity to fully block soluble ACE ex vivo. Thus, it is unlikely that in vitro Ang II generation fully explains this discrepancy. A second explanation, based on a recent study showing enhanced ACE-mediated outside-in signaling in the presence of ACE inhibitors,22 is that ACE-induced signaling is determined by the N-domain. If true, RXP407 but not RXPA380 would exert effects through ACE that need to be taken into consideration when investigating Ang I–induced vasoconstriction. Finally, the differences may relate to the fact that the Ang I–II conversion in the study by Georgiadis et al2 reflects conversion in the pulmonary vascular bed. In this vascular bed, in contrast with other vascular beds, de novo Ang II production could be fully attributed to conversion of circulating Ang I.23

Perspectives
This study is the first to show that low concentrations of (C-domain–selective) ACE inhibitors are sufficient to fully
inhibit Ang II generation by membrane-bound ACE. Such selective inhibition of Ang II generation at tissue sites (eg, in the heart) could underlie previous studies showing that low doses of ACE inhibitors exert beneficial effects in the absence of blood pressure reduction. Selective inhibition of tissue Ang II generation (in addition to AT1 receptor blockade) might also explain why the beneficial effects of Ang-(1–7) generation at tissue sites (eg, in heart) could underlie previous studies showing that low doses of ACE inhibitors exert beneficial effects in the absence of blood pressure reduction. Selective inhibition of Ang II generation at tissue sites (eg, in heart) could underlie previous studies showing that low doses of ACE inhibitors exert beneficial effects in the absence of blood pressure reduction.

References


5. Perich RB, Jackson B, Johnston CI. Structural constraints of inhibitors for binding to two active sites on somatic angiotensin converting enzyme. Eur J Pharmacol. 1994;266:201–211.


Selective Angiotensin-Converting Enzyme C-Domain Inhibition Is Sufficient to Prevent Angiotensin I–Induced Vasoconstriction

Joep H.M. van Esch, Beril Tom, Vincent Dive, Wendy W. Batenburg, Dimitris Georgiadis, Athanasios Yiotakis, Jeanette M.G. van Gool, René J.A. de Bruijn, René de Vries and A.H. Jan Danser

Hypertension. 2005;45:120-125; originally published online December 6, 2004; doi: 10.1161/01.HYP.0000151323.93372.f5

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/45/1/120

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org/subscriptions/