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

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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 $1 \mu$mol/L, 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 $0.1 \mu$mol/L. Bradykinin concentration-dependently relaxed PCMAs. RXPA380 (10 $\mu$mol/L) and RXP407 (0.1 mmol/L) potentiated bradykinin, both inducing a leftward shift of the bradykinin CRC that equaled $\approx 50\%$ 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 $\mu$mol/L) and RXP407 (0.1 mmol/L) 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

An 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 RXPA3807 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

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quinaripril, and Ang-(1–7) (selectivity for C-domain versus N-domain, respectively, 1000-, 200-, and 10-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.7,9 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 bradykinin1,10 is mediated via the recently cloned Ang-(1–7) receptors.11 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 they were obtained from 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.12 At arrival in the laboratory, tertiary branches of the left anterior descending coronary artery (diameter 280 to 600 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, 2.5 NaHCO3, and 8.3 glucose 8.3, pH 7.4.

**Methods**

**Tissue and Blood Collection**

HCMAs 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.12 At arrival in the laboratory, tertiary branches of the left anterior descending coronary artery (diameter 280 to 600 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, 2.5 NaHCO3, and 8.3 glucose 8.3, pH 7.4.

**Data Analysis**

Data are given as mean ± SEM. CRCs were analyzed as described previously12 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). Quinaprilat (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 quinaprilat (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;
Figure 1), nor did this drug exert additional effects on top of quinaprilat (n=110; data not shown).

RXPA380 and RXP407, at concentrations of 10 μmol/L and higher, induced a leftward shift of the bradykinin CRC in PCMAs that equaled ≥50% of the maximal shift observed with quinaprilat (Figure 2).

**Ang I–Induced Constrictions**

Ang I constricted PFAs (pEC50 =8.1 ±0.1; n=32; Figure 3) to maximally 40% to 60% of the contraction to 100 mmol/L K⁺. RXPA380 (n=6), quinaprilat (n=8), and captopril (n=5) 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 mmol/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 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[EC50] of the bradykinin CRC in the presence of increasing concentrations of RXPA380 or RXP407. An increase in −log[EC50] 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 (10 μmol/L) was needed to fully suppress conversion (Figure 4). Conversely, the captopril concentration required to block plasma ACE by 50% (IC50) was 5-fold lower (P=NS) than the captopril concentration required to cause a half-maximal shift of the Ang I CRC (–log[IC50] 6.5±0.2 versus 6.0±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±3 minutes (n=4; Figure 5). Quinaprilat (10 μmol/L) increased the Ang I half life to 166±22 minutes (P<0.01), thereby demonstrating that 71±6% 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±19 (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, 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, 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. 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. 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. 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.

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), when infused at doses that do not block the ACE N-domain, differ from those of ACE inhibitors in heart failure.

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