Roles of the Two Active Sites of Somatic Angiotensin-Converting Enzyme in the Cleavage of Angiotensin I and Bradykinin
Insights From Selective Inhibitors

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Abstract—Somatic angiotensin-converting enzyme (ACE) contains two homologous domains, each bearing a functional active site. The in vivo contribution of each active site to the release of angiotensin II (Ang II) and the inactivation of bradykinin (BK) is still unknown. To gain insights into the functional roles of these two active sites, the in vitro and in vivo effects of compounds able to selectively inhibit only one active site of ACE were determined, using radiolabeled Ang I or BK, as physiological substrates of ACE. In vitro studies indicated that a full inhibition of the Ang I and BK cleavage requires a blockade of the two ACE active sites. In contrast, in vivo experiments in mice demonstrated that the selective inhibition of either the N-domain or the C-domain of ACE by these inhibitors prevents the conversion of Ang I to Ang II, while BK protection requires the inhibition of the two ACE active sites. Thus, in vivo, the cleavage of Ang I and BK by ACE appears to obey to different mechanisms. Remarkably, in vivo the conversion of Ang I seems to involve the two active sites of ACE, free of inhibitor. Based on these findings, it might be suggested that the gene duplication of ACE in vertebrates may represent a means for regulating the cleavage of Ang I differently from that of BK. (Circ Res. 2003;93:148-154.)

Key Words: angiotensin-converting enzyme ■ angiotensin ■ bradykinin ■ phosphinic peptide inhibitors

Somatic angiotensin-converting enzyme in vertebrates (ACE, EC 3.4.15.1) is a zinc metallopeptidase involved in the release of angiotensin II (Ang II) and the inactivation of bradykinin (BK), two peptide hormones that play a key role in the regulation of blood pressure, renal and cardiovascular functions. These peptides are end products of two highly regulated systems, respectively, the renin-angiotensin system (RAS) and the kallikrein-kinin system (KKS). It has been suggested that the plasma KKS could be the physiological counterbalance to the RAS. As ACE is strategically poised between the RAS and KKS systems, the question may arise as to whether the ACE activity is controlled to regulate the balance between the RAS and KKS systems and, if so, by which mechanism.

Somatic ACE is a two-domain protein resulting from a tandem gene duplication, with each domain possessing a functional active site. Since the discovery that ACE has two active sites, there has been much speculation about the functional significance of the presence of two active sites in the same enzyme. Although the two domains of ACE display in vitro a relatively broad substrate specificity, such as the ability to cleave Ang I and BK, there are some biochemical features that differentiate between the two active sites. For example, the hematoregulatory peptide, Ac-S-D-K-P, was proved in vitro to be specifically cleaved by the N-domain of ACE. The N-domain and C-domain have different in vitro patterns of chloride activation and can be differentiated by the relative potencies of some inhibitors. Among these, RXP407, a phosphinic peptide, was reported recently as the first example of a potent N-domain–specific inhibitor of ACE. Experiments performed with RXP4077 confirmed previous studies suggesting that Ac-S-D-K-P is specifically cleaved in vivo by the N-domain of ACE. Taken together, these data suggest that some differences occur between the two active sites of ACE.

However, the in vivo functional roles of these two active sites of ACE, if any, particularly toward Ang I and BK cleavage, are still unknown. To gain some additional insights on this question, we have chosen to develop inhibitors of the C-domain active site of ACE that are more specific than those currently available. Specific in vivo inhibition of either the N- or C-domain of ACE should help to determine the contribution of these active sites toward the cleavage of Ang I and BK. In the present study, we discuss the in vitro properties of a new phosphinic peptide inhibitor, called RXPA380, which turns out to be a potent and highly specific...
Materials and Methods

Enzymes

Preparation of human wild-type somatic ACE and two ACE mutants have been described. The term C-domain active site describes an ACE form in which the N-domain active site was inactivated. Purification of mouse ACE from plasma was performed as previously described. Human recombinant neutral endopeptidase 24.11 (NEP) was from R&D Systems (Europe).

In Vitro Enzyme Assays and Inhibition Studies

ACE assays were performed at 25°C in 50 mmol/L HEPES (pH 6.8), 200 mmol/L NaCl, and 10 μmol/L of ZnCl₂. All enzymatic studies were performed in order to remain below 10% of substrate hydrolysis (initial rate conditions). Continuous assays with the synthetic fluorogenic substrate Mca-Ala-Ser-Asp-Lys-DpaOH were performed as previously described. The extent of Ang I (5 μmol/L, 90 nCi) and BK (1 μmol/L, 75 nCi) hydrolysis by ACE was monitored by HPLC analysis. Conditions for the separation of the parent substrate and products, as well as for the detection of radiolabeled peptides, are described below. ACE concentration was chosen in order to observe a full range of inhibition percentages. Theoretical inhibition profiles of human recombinant ACE by RXPA380 and RXP407 and Ki values determination were obtained as previously described. In the case of mouse ACE, because Kᵢ values for the degradation of substrate utilized, by each active site of mouse ACE, could not be determined, Kᵢ(app) values were reported. NEP assays were performed as described.

Animals

Eighty-four male C57BL/6J mice (Ifa Credo), weighing 23 to 25 g, were included in this study. All experiments were conducted in accordance with the Décret sur l’Expérimentation Animal (French Law on rules for animal experimentation, Decree 87-848, October 19, 1987). Animals were anesthetized with 80 mg/kg sodium pentobarbital (SunoL), administered by intraperitoneal injection. The right carotid artery was isolated and cannulated with a catheter (PE10, 0.28 mm, 0.61, A-M Systems, Inc) for arterial blood collection. The right jugular vein was cannulated with a catheter (FEP, 0.12 × 0.67, Carnegie Medecin) that was connected to a liquid switch (CMA/110, Carnegie Medecin) for intravenous injections. Inhibitors or saline solution was perfused (compactS, B-Braun perfusor) for 30 minutes through the jugular vein. During drug administration and blood collection, animal body temperature was maintained at 38°C by placing the animal on an operating table, equipped with a servo-controlled heating plate.

In Vivo Effects of RXP407 and RXPA380

Inhibitor and Substrate Administration

RXPA380 (0.9, 3, 10, and 30 mg/kg) and RXP407 (10 mg/kg) in solution (isotonic solution adjusted to pH 7, 50 μL) were intravenously infused for 30 minutes to anesthetized mice. Control groups were infused with saline or perindopril (10 mg/kg) solution (Servier, France). At the end of the perfusion, intravenous bolus injection of substrate in isotonic solution (50 μL) was performed. Injected quantities of the different substrates were as follows: Ang I, mixture of unlabeled Ang I (2 μg) and 21 μCi of 11-125I-Ang I; BK, mixture of unlabeled BK (2 μg) and 11 μCi of 11-125I-BK; and Ac-SDKP, mixture of unlabeled Ac-SDKP (2 μg) and 17 μCi of 11-125I-Ac-SDKP.

Blood Collection

After substrate injection, arterial blood (50 μL) was collected in previously weighed propylene tubes containing 40 μL of water, 10 μL of 80% TFA, and 1 μL of heparin. The exact quantity of blood collected was determined by weighing the tubes. After adding 195 μL of water, tubes were kept for 10 minutes in an ice bath and
samples were centrifuged at 4°C to obtain plasma. Samples (2×100 μL) were immediately analyzed by HPLC. For Ang I or BK experiments, blood was collected 30 seconds after the start of substrate injection. For Ac-S-D-K-P, blood was collected 10 minutes after the start of substrate injection.

HPLC Analysis
The analysis of plasma aliquots was performed by liquid chromatography using a Perkin Elmer 200 HPLC system linked to a radioflow detector (Z 500-4 cell, Berthold). Sample injections (50 μL on-column) were made by auto-sampler. The chromatographic separations were performed on a Kromasil C18 150×4.6-mm column (AIT, France) with elution (1 mL/min) using a gradient method. The mobile phase consisted of 10%CH3CN/90%H2O/0.1%TFA (solvent A) and 90%CH3CN/10%H2O/0.1%TFA (solvent B). Gradients were as follows for the separation of Ang I samples: 0 to 30 minutes, 0% to 30% B, 30 to 35 minutes, 30% to 100% B; BK samples: 0 to 30 minutes, 0% to 25% B, 30 to 35 minutes, 30% to 100% B; and Ac-SDKP samples: 0 to 30 minutes, 0% to 30% B, 30 to 35 minutes, 30% to 100% B. The eluted peaks were identified by comparison of their retention times to those of unlabeled standards, corresponding to native substrate (Ang I, BK, and Ac-SDKP) and the expected cleavage products, respectively, Ang II, BK(1-7), and BK(1-5). These assignments were confirmed by electrospray mass analysis of the collected peaks. Quantitative measurements of Ang I, Ang II, BK, and Ac-SDKP were performed by integration of the area under the corresponding peaks on the chromatogram. These values were normalized according to the weight of blood collected for each animal. Six animals were used for each condition.

Data and Statistical Analysis
Data reported for Ang I and BK correspond to the analysis of blood samples collected 30 seconds after the substrate injection. For Ac-SDKP, the data reported correspond to analysis of blood collected 10 minutes after the substrate injection. Data are presented as mean±SD. Statistical comparisons were performed using the non-parametric Mann-Whitney U test (StatView 5 software).

Results
Potency and Selectivity of RXPA380 Toward Human Recombinant ACE
Previous studies have suggested that constrained inhibitors of ACE present some selectivity for the C-domain. More recently, we reported that particular BK potentiating peptides displayed binding selectivity for the C-domain of ACE. These peptides, which are characterized by the presence of several prolines in their sequences, can be viewed also as constrained structures. These observations led us to synthesize phosphinic peptides containing a proline in their P1' position (authors’ unpublished data, 2003). Evaluation of this series of compounds led us to identify RXPA380 as the most selective inhibitor for the C-domain of ACE (structure displayed in the Table). The inhibition profiles of the N-domain and C-domain mutants of human recombinant ACE by RXPA380 are reported in Figure 1A. As expected for a C-selective inhibitor, the concentrations of RXPA380 required to fully inhibit the activity of the C-domain mutant (IC50 2.5 mmol/L) are much lower than those needed to inhibit the N-domain mutant (IC50 10 μmol/L). The profile observed for the inhibition of wild-type human recombinant ACE by RXPA380 (Figure 1B), using the fluorogenic Mca-Ala peptide as substrate, has two inflection points corresponding to the titration of, respectively, the C-domain of somatic ACE at lower inhibitor concentrations followed by the titration of the N-domain of ACE at higher RXPA380 concentrations. Kvalues of 3 nmol/L and 10 μmol/L for the inhibition of the C- and N-domain of human recombinant ACE by RXPA380 were determined from this curve (Table). The inhibition of ACE by a mixture of RXPA380 and RXP407, in equivalent amounts, resulted in a monophasic titration profile (Figure 1B), a profile different from the one obtained with RXPA380 alone. Such monophasic titration profile is typical of an inhibitor blocking the two ACE active sites with the same potency (mixed inhibitor). The IC50 values of RXP407 and RXPA380 are 7 nmol/L and 3 mmol/L, respectively, for the N-domain and the C-domain of ACE (Table). Thus, a mixture of these two inhibitors, with similar IC50 values for these two sites of ACE, should behave like a mixed inhibitor of this enzyme, a statement in agreement with the experimental data. RXPA380 and RXP407 behaved as very weak inhibitors of human recombinant NEP (Table).

Inhibition of Mouse Somatic ACE by RXPA380 and RXP407
Inhibition of purified mouse somatic ACE by RXPA380 or RXP407, using Ang I as substrate, gave inhibition profiles characterized also by the presence of two inflection points.
Functional Roles of the Two ACE Active Sites

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In Vivo Stability of RXPA380

After intravenous infusion of tritiated RXPA380 in mouse (30 minutes, 10 mg/kg), the 0- to 24-hour recovery of radioactivity averaged 13% and 65% of the dose in urine and feces, respectively, indicating a slow and substantial biliary excretion of RXPA380. HPLC analysis of feces extracts indicated that the whole radioactivity corresponded to almost intact RXPA380 (see online data supplement, available at http://www.circresaha.org). After infusion of RXPA380 (t=30 minutes), plasma concentration of intact RXPA380 is 20-fold higher than the \( K_i \) value of RXPA380 for the inhibition of the C-domain of mouse ACE. In plasma, 85% of the radioactivity corresponds to intact RXPA380. Previous studies have shown that RXP407 is metabolically stable in mice.\(^{16,17}\)

In Vivo Effects of RXP407 or RXPA380 on Ac-SDKP Cleavage

In a previous study, RXP407 was shown to increase the basal levels of plasma endogenous Ac-SDKP in OF1 mice, with a maximal effect observed at 10 mg/kg.\(^{17}\) This dose was thus selected to determine the effects of RXPA380 and RXP407 infusion on the cleavage of Ac-SDKP injected in C57BL mice (mixture of unlabeled Ac-SDKP \( [2 \mu g] \) and 17 \( \mu Ci \) of \(^3\)H-Ac-SDKP). RXP407 at 10 mg/kg, compared with controls, increased the levels of exogenous Ac-SDKP in plasma by 16-fold. In contrast, the same dose of RXPA380 has no significant effects on the levels of Ac-SDKP, compared with controls under the same conditions.

In Vivo Effects of RXP407 or RXPA380 on Ang I Conversion

Levels of labeled Ang I and Ang II at 30 seconds in plasma, after intravenous injection of Ang I (mixture of unlabeled Ang I \( [2 \mu g] \) and 21 \( \mu Ci \) of \(^3\)H-Ang I), were determined in mice after infusion of the inhibitor and compared with controls. RXPA380 induced a dose-dependent decrease of the Ang II/Ang I ratio. The Ang II/Ang I ratio, which is a measure of the degree of ACE inhibition, was reduced by 50% (0.9 mg/kg) to 89% (30 mg/kg) with the selected doses of RXPA380. RXP407 treatment at 10 mg/kg was slightly more efficient for reducing the Ang II/Ang I ratio, compared with the effects observed with 10 and 30 mg/kg RXP380. The same reduction of the Ang II/Ang I ratio was observed when the animals were treated by a combination of RXP407 and RXPA380, at 10 mg/kg for each compound.

In Vivo Effects of RXP407 or RXPA380 on BK Cleavage

Levels of BK in plasma 30 seconds after the injection of labeled BK (mixture of unlabeled BK \( [2 \mu g] \) and 11 \( \mu Ci \) of \(^3\)H-BK) in control mice were below the detection limit. It was therefore not possible to calculate a percentage of BK protection due to inhibitor treatment compared with controls. This led us to treat mice with perindopril, a highly potent and mixed inhibitor of ACE in vivo.\(^{23}\) Levels of BK observed 30 seconds after the injection of labeled BK, under perindopril (10 mg/kg, 30-minute infusion), were arbitrarily taken as 100% of BK protection. Under the same experimental con-
conditions, at 10 mg/kg, RXPA380 infusion led to detectable plasma levels of labeled BK. Compared with perindopril, 10 mg/kg RXPA380 afforded 9.2% of BK protection. Comparable levels of BK protection were observed with RXP407 treatment, at the same dose. However, when the animals were infused with a combination of RXP407 and RXPA380, at the 10 mg/kg for each compound, the BK levels were increased by 6-fold, compared with the BK levels observed when the animals were treated with the inhibitor alone. BK protection of 65% was observed when the inhibitors were used in combination, compared with perindopril.

Discussion

RXPA380 is a novel phosphinic peptide inhibitor of ACE able to differentiate the two ACE active sites with a dissociation constant more than three orders of magnitude lower for the C-domain of the human or mouse ACE. Furthermore, RXPA380 was shown to be active in vivo, able to inhibit the conversion of exogenously administered Ang I into Ang II in a mouse model and to block the cleavage of exogenously administered BK when used in combination with RXP407. This compound, and RXP407, are weak inhibitors of NEP, a peptidase that in humans and rats also participates in the metabolism of Ang I and BK.23,24 Accordingly, the in vivo effects of RXPA380 and RXP407 on the cleavage of Ang I and BK, reported in this study, are not likely to reflect the inhibition of NEP, but more probably the selective inhibition of either the N- or C-domain of ACE.

The inhibition profiles of mouse ACE by RXPA380 and RXP407, using Ang I as substrate, are characterized by the presence of two titration domains (Figure 2A), corresponding to the sequential inhibition of the two ACE active sites by these highly selective inhibitors. This interpretation is supported by the monophasic shape of the inhibition profile observed when a mixture of RXPA380 and RXP407 is used to inhibit ACE. From the inhibition profiles obtained with RXPA380 or RXP407, one may conclude that in vitro, the two ACE active sites participate in the cleavage of Ang I. Also, these profiles imply that the inhibition of one ACE active site by these selective inhibitors does not prevent the hydrolysis of Ang I by the other active site, free of inhibitor. Two equivalents of RXPA380 or RXP407 can bind to one ACE equivalent. We previously demonstrated that the position of the inflection points in these profiles provides a good approximation of the inhibitor affinity for the N- and C-domain of ACE.16,20 Furthermore, we showed that the

\[ *P<0.05 \text{ compared with controls; } n=6 \text{ mice per group. a, } P=0.0104 \text{ compared with RXPA380 0.9 mg/kg. b, } P<0.01 \text{ compared with RXPA380 3 mg/kg. c, } P=0.14 \text{ compared with RXPA380 10 mg/kg. d, } P=0.065 \text{ compared with RXPA380 30 mg/kg. e, } P=0.055 \text{ compared with RXPA380 30 mg/kg. } \]

Effects of RXPA380 and RXP407 infusion on the levels of exogenously injected Ac-S-D-K-P (mixture of unlabeled Ac-SDKP (2 μg) and 17 μCi of 3H-Ac-SDKP) in mice. Levels of exogenous Ac-S-D-K-P reported are those determined 10 minutes after the injection of Ac-S-D-K-P. Experimental data are expressed as mean±SD. **P<0.01 and *P<0.05 compared with controls; n=6 mice per group. a, P=0.0104 compared with RXPA380 0.9 mg/kg. b, P<0.01 compared with RXPA380 3 mg/kg. c, P=0.14 compared with RXPA380 10 mg/kg. d, P=0.065 compared with RXPA380 30 mg/kg. e, P=0.055 compared with RXPA380 30 mg/kg. C, Effects of RXPA380 and RXP407 infusion on the levels of exogenously injected BK. Percentages of BK protection were calculated using the perindopril group (10 mg/kg) as controls. Levels of exogenous BK protection observed with perindopril were taken as 100%. Levels of BK correspond to those determined 30 seconds after the injection of BK (mixture of unlabeled BK [2 μg] and 11 μCi of 3H-BK). Experimental data are expressed as mean±SD. **P<0.01 and *P<0.05 compared with perindopril group; n=6 mice per group.

Figure 3. A, Effects of RXPA380 and RXP407 infusion on the levels of exogenously injected Ac-S-D-K-P (mixture of unlabeled Ac-SDKP (2 μg) and 17 μCi of 3H-Ac-SDKP) in mice. Levels of exogenous Ac-S-D-K-P reported are those determined 10 minutes after the injection of Ac-S-D-K-P. Experimental data are expressed as mean±SD. **P<0.01 compared with controls; n=6 mice per group. B, Effects of RXPA380 and RXP407 infusion on the cleavage of exogenously injected Ang I, expressed as the Ang II/Ang I ratio. Levels of exogenous Ang I and Ang II correspond to those determined 30 seconds after the injection of Ang I (mixture of unlabeled Ang I [2 μg] and 21 μCi of 3H-Ang I). Experimental data are expressed as mean±SD. **P<0.01 and *P<0.05 compared with controls; n=6 mice per group. C, Effects of RXPA380 and RXP407 infusion on the levels of exogenously injected BK. Percentages of BK protection were calculated using the perindopril group (10 mg/kg) as controls. Levels of exogenous BK protection observed with perindopril were taken as 100%. Levels of BK correspond to those determined 30 seconds after the injection of BK (mixture of unlabeled BK [2 μg] and 11 μCi of 3H-BK). Experimental data are expressed as mean±SD. **P<0.01 and *P<0.05 compared with perindopril group; n=6 mice per group.
percentage of inhibition observed after the titration of each active site reflects the specificity of each active site in cleaving the substrate used for the inhibition experiment.\textsuperscript{16,20} Thus, the 70\% inhibition observed at 200 nmol/L RXPA380 (Figure 2A) indicates that the C-domain of mouse ACE is slightly more efficient in cleaving Ang I than the N-domain. In agreement with this conclusion was the observation that 200 nmol/L of RXP407, a concentration that fully blocks the N-domain, only promotes 30\% inhibition of the ACE activity, using Ang I as substrate (Figure 2A). The inhibition profiles reported in Figure 2B, using BK as substrate, are very similar to those reported with Ang I (Figure 2A). Thus, the conclusions drawn for the Ang I also apply for the BK cleavage. Previous studies, using N- and C-domain mutants of human recombinant ACE, led to the conclusion that both sites equally participate in the cleavage of these substrates.\textsuperscript{10} Our results suggest that the C-domain of mouse ACE is slightly more efficient in cleaving Ang I and BK than the N-domain, at least in vitro.

In vivo, infusion of one inhibitor, either RXP407 or RXPA380, at 10 mg/kg afforded a modest protection against BK cleavage, less than 10\% protection, compared with perindopril treatment (Figure 3C). The same dose of RXP407 increased the levels of Ac-SDKP, an N-selective substrate of ACE, by 16-fold compared with controls (Figure 3A). Effective inhibition of the ACE N-domain is thus achieved by 10 mg/kg RXP407 treatment. The degree of BK protection was much higher when the two inhibitors were combined, 65\% of protection compared with 100\% protection observed with perindopril. From the above results, it can be concluded that the inhibition of one ACE active site is not sufficient to prevent the cleavage of BK. Probably, while one active site of ACE is blocked by either RXP407 or RXPA380, the other active site, free of inhibitor, is still able to hydrolyze BK and counterbalances the inhibitor effect. These results are in agreement with those derived from in vitro experiments.

In sharp contrast with the results reported for BK, administration of RXPA380 or RXP407 (10 mg/kg) reduced the Ang II/Ang I ratio by 90\% compared with controls. At this dose, RXPA380 did not inhibit the cleavage of Ac-SDKP, showing that under these conditions the N-domain active site of ACE is free from RXPA380. Thus, in vivo, the inhibition of either the N- or C-domain of ACE prevents the cleavage of Ang I, while in vitro inhibition of the Ang I cleavage requires the blockade of the two ACE active sites. That the inhibition of a single ACE active site is sufficient in vivo to prevent Ang I cleavage is supported by the experiments showing that animal treatment with the inhibitor alone or in combination had similar effects on the Ang II/Ang I ratio. It is worth noting that under these conditions, infusion of either RXPA380 or RXP407 did not prevent BK cleavage, thus ruling out the scenario that in vivo the binding of the inhibitor to one site produces the inactivation of the other active site, free of inhibitor.\textsuperscript{25} The hypothesis that in vivo Ang I is selectively cleaved by one active site can be discarded, as RXP407 and RXPA380 treatment results in the same effects on the Ang II/Ang I ratio. Thus, actually, the reason that the conversion of Ang I in vivo seems to require the two active sites of ACE, free of inhibitor, remains an elusive issue. However, it should be kept in mind that our in vitro experiments are based on a soluble form of mouse ACE, while the in vivo experiments are dependent on ACE in its membrane-bound form. In vivo effects of the inhibitors on Ang II/Ang I ratio were determined, as this ratio is considered a very good index of the tissue ACE inhibition.\textsuperscript{22,26,27} In our experimental setting, the intravenously infused Ang I traversed the pulmonary vascular bed before reaching the carotid artery, where blood was collected to determine the Ang II/Ang I ratio. Thus, the observed conversion of Ang I to Ang II likely occurred during the pulmonary circulation, performed by the membrane-bound form of ACE present in this tissue. Several studies supported the notion that in vivo the conversion of Ang I to Ang II is mostly performed by tissue-bound ACE activity and not by the soluble plasma ACE activity.\textsuperscript{28,29} Interestingly, genetically altered mice that lack the membrane-bound form of ACE have abnormally low blood pressure.\textsuperscript{30,31} Furthermore, recent studies performed by the Erdös group suggest that direct intermolecular interactions between membrane-bound ACE and BK B\(_2\) receptor might be an essential mechanism for the potentiation of BK.\textsuperscript{32} Taken together, these studies indicate that the membrane-bound form of ACE may control some physiological functions of ACE. Thus, in vitro experiments based on soluble forms of ACE may not properly reflect the function of ACE in its membrane environment.

Whatever the detailed molecular mechanisms involved in the conversion of Ang I, our observations may reflect an in vivo mechanism that regulates Ang II release from BK inactivation. Such mechanism will rely on the presence of two active sites in ACE. Thus, the presence of two active sites in ACE, resulting from a tandem gene duplication along the evolution, may have a functional significance in vertebrates, as far as the metabolism of Ang I and BK is concerned. We recently reported that natural peptides, like BK potentiating peptides found in snake venom, were able to differentiate the two ACE active sites and act as selective inhibitors of ACE.\textsuperscript{20} Whether such peptides may occur in mammalian species is still unknown, but according to the results reported here, such putative endogenous selective inhibitors of ACE would allow the inactivation of BK while blocking Ang II release.

Classical ACE inhibitors act both on the release of Ang II and the inactivation of BK. These effects have precluded a clear demonstration of whether the antihypertensive and cardioprotective benefits of ACE inhibitors are solely related to the inhibition of Ang II release or also to the inhibition of BK inactivation.\textsuperscript{4,33} Selective inhibitors of ACE, as those reported in the present study, may help to clarify this issue, as these inhibitors were observed to affect mainly Ang I conversion.

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**References**


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Supplementary information:

In vivo metabolism of RXPA380

Labelling of RXPA380: Tritiated trifluoroacetic acid (150µl) was first produced with tritium gaz $^3\text{H}_2$ (20Ci) in presence of palladium oxyde (26 mg). Then, RXPA380 (5mg) was labeled by hydrogen-tritium exchange (tryptophane side chain) performed with tritiated trifluoroacetic acid (150 µl, 10Ci). After 2 hours at room temperature, the tritiated solvant was removed under vacuum, and tritiated RXPA380 washed 4 times with methanol and then purified by HPLC on C18 Kromasyl column (250x10). 26 mCi of pure tritiated RXPA380 was obtained, with a specific radioactivity of 18,5 Ci/mmol.

Sample extraction and quantification: RXPA380 was infused for 30 min at a 10mg/kg dose. Aliquot of pooled homogenates faeces (0-24h) were extracted with methanol. This mixture was stirred for 24h to insure extraction of RXPA380 in the liquid phase. After centrifugation, the liquid phase was evaporated and dissolved with HPLC solvents. Total radioactivity in the sample was determined by liquid scintillation and analyzed by HPLC. Plasma was diluted 10 fold with methanol, after agitation, tubes were centrifuged, supernatant was diluted in HPLC solvents. Total
radioactivity in the sample was determined by liquid scintillation and analyzed by HPLC system linked to a radioflow detector.

The three radio-HPLC profiles correspond respectively to:

A) a solution of pure tritiated RXPA380 in water after 24 h at room temperature

B) faeces extract obtained 24h after the injection of RXPA380 to the animals.

C) plasma extract obtained 30 min after the infusion of RXPA380 (10mg/kg) to mice. Equivalent amount of radioactivity was injected in each case.