ACE Activity Is Modulated by Kinin B₂ Receptor
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Abstract—Angiotensin-converting enzyme (ACE) is an ectoprotein able to modulate the activity of a plethora of compounds, among them angiotensin I and bradykinin. Despite several decades of research, new aspects of the mechanism of action of ACE have been elucidated, expanding our understanding of its role not only in cardiovascular regulation but also in different areas. Recent findings have ascribed an important role for ACE/kinin B₂ receptor heterodimerization in the pharmacological properties of the receptor. In this work, we tested the hypothesis that this interaction also affects ACE enzymatic activity. ACE catalytic activity was analyzed in Chinese hamster ovary cell monolayers coexpressing the somatic form of the enzyme and the receptor coding region using as substrate the fluorescence resonance energy transfer peptide Abz-FRK(Dnp)P-OH. Results show that the coexpression of the kinin B₂ receptor leads to an augmentation in ACE activity. In addition, this effect could be blocked by the B₂ receptor antagonist icatibant. The hypothesis was also tested in endothelial cells, a more physiological system, where both proteins are naturally expressed. Endothelial cells from genetically ablated kinin B₂ receptor mice showed a decreased ACE activity when compared with wild-type mice cells. In summary, this is the first report showing that the ACE/kinin B₂ receptor interaction modulates ACE activity. Taking into account the interplay among ACE, ACE inhibitors, and kinin receptors, we believe that these results will shed new light into the arena of the controversial search for the mechanism controlling these interactions. (Hypertension. 2008;51:1-7.)

Key Words: ACE ■ kinin B₂ receptor ■ dimerization ■ enzyme activity ■ ACE inhibitors ■ icatibant

Angiotensin-converting enzyme (ACE) is a transmembrane zinc metallopeptidase that cleaves carboxy-terminal dipeptides from several substrates and is abundant in vascular endothelial cells. An ACE soluble form found in plasma is derived from the membrane-bound form by shedding. ACE plays a major role in regulating cardiovascular functions, because it converts the decapeptide angiotensin (Ang) I into the vasoconstrictor and proliferative octapeptide angiotensin II and inactivates the vasodilatory nonapeptide bradykinin (BK). The enzyme also hydrolyzes other substrates like angiotensin 1-7, Ac-SDKP, substance P, cholecystokinin, hemopressin, and amyloid β-protein (reviewed by Reference 6). ACE inhibition is a valuable therapy for the management of hypertension and cardiac failure. Several data support the proposal that many effects of ACE inhibitors are because of the inhibition of Ang II generation. However, accumulating evidence indicates that several antihypertensive and cardioprotective effects of ACE inhibitors may be because of reduced BK degradation, with resultant increased endogenous BK levels. Numerous studies suggest that these compounds not only facilitate the accumulation of locally formed BK but also directly affect kinin B₂ receptor signaling, resulting in an enhanced response to BK. For instance, ACE inhibitors enhance BK-mediated effects in the presence of subthreshold kinin concentrations and amplify the BK-induced response in the presence of an ACE-resistant B₂ receptor agonist. In addition, ACE inhibitors are believed to stabilize the B₂ receptor in a high-affinity state, thereby preventing and/or reversing the BK-induced sequestration to caveolae before internalization. Minshull et al. have suggested that ACE inhibitors augment the BK effects on the receptor by inducing a cross-talk between ACE and the kinin B₂ receptor at the membrane; however, this is still controversial. In addition, it has been proposed that this indirect activation might depend on the steric relationship of the enzyme to the receptor, because ACE has to be in the immediate vicinity of the receptor for the activation to occur, possibly because of heterodimer formation.

Despite the large number of studies describing the potentiation of BK effects by ACE inhibitors, information about a possible modulation of ACE activity by kinin receptors was...
never in focus, according to our knowledge. Therefore, the present study was addressed to investigate a possible role of kinin receptor expression on ACE catalytic activity. We have explored this possibility in transfected Chinese hamster ovary (CHO) cells expressing ACE or ACE and kinin B₂ receptors using the fluorescence resonance energy transfer peptide Abz-FRK(Dnp)P-OH. In addition, native endothelial cells from wild-type (WT) and kinin B₂ receptor knockout mice were also analyzed to evaluate the influence of kinin receptors on ACE activity in a physiological system. Our findings show for the first time that kinin B₂ receptor expression positively modulated ACE activity, showing further evidence of an interaction among kinin B₂ receptor and ACE molecules.

Materials and Methods

Materials

If not otherwise indicated, all of the reagents were purchased from Sigma, and cell culture media and supplements were purchased from Gibco-BRL.

Animals

Experiments were carried out with kinin B₂ receptor knockout mice (B₂⁻/⁻). and C57Bl/6 animals (Taconic) were used as controls. Animals were obtained from the Centro de Desenvolvimento de Modelos Experimentais para a Medicina e Biologia at the Federal University of São Paulo. All of the experiments reported were conducted as stated in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by a local animal care and use committee. Animals were maintained on standard mouse chow at 22°C on a 12-hour light-dark cycle and allowed ad libitum access to food and tap water.

Expression of B₂ Receptor in Transfected CHO Cells

CHO cells were cultured according to Sabatini et al. Briefly, cells were previously transfected with a plasmid carrying the somatic form of the rat ACE coding region (using neomycin resistance gene for selection), and individual clones were screened for ACE activity. Clones were then stably transfected either with the empty pcDNA3 plasmid (mock-transfected cells) or with the plasmid containing the rat B₂ receptor coding region using LipofectAMINE 2000. After transfection, cells were selected in Ham’s F-12 medium containing 0.5 mg/mL of hygromycin B, and resistant clones were propagated. Clones with high expression levels of B₂ receptors mRNA were selected by real-time PCR. Total RNA (750 ng) was reverse-transcribed using Moloney murine leukemia virus (Invitrogen) to cDNA according to the manufacturer’s instructions. The reaction product was amplified by real-time PCR on the 7000 Sequence Detection System (ABI Prism, Applied Biosystems) using the TaqMan Universal PCR Master Mix (Applied Biosystems). The thermal cycling conditions and the data analysis were performed as described by the TaqMan real-time PCR. Standard and melting curves were performed in parallel to check reaction efficiency and specificity, respectively.

Expression of ACE mRNA in B₂⁻/⁻ Endothelial Cells

ACE and β-actin mRNA expression in WT and B₂⁻/⁻ endothelial cells were assayed by SYBR Green real-time PCR using 25 ng of total cDNA, SYBR Green Universal Master Mix (Applied Biosystems), and the following set of primers independently: ACE (5'-CTGTCGAGGTGATCCAC-3', 5'-CTCCATGTTCCAGAGGATAC-3'); GenBank accession No. NM_207624.2) and β-actin (5'-CTGGCTCTAGTCTCCACCT-3', 5'-CGGACCTACTCGTACTCCGTT-3'); GenBank accession No. NM_007393.1). The thermal cycling conditions and the data analysis were performed as described by DeBlasi et al. Functional Assays

The Cytosensor microphysiometer (Molecular Devices) was used to measure the acidification rate in the extracellular microenvironment because of cellular metabolic activity. CHO cells expressing the receptor and coexpressing ACE/B₂ were plated into capsule polycarbonate cups at a density of 10³ cells per cup in DMEM, pH 7.4. After a 48-hour culture, the cells were washed with PBS. The tissues of the lung surface were cut into small pieces separately. Pieces were placed into cell culture dishes (35 mm) and cultured in DMEM supplemented with 20% FBS containing 40 mg/L of gentamicin. After a 48-hour culture, the tissues were discarded. The medium was changed every 48 hours, and cells were subcultured between the days 6 and 8 by harvesting with trypsin-EDTA. Semicolon confluent (80-90%) cells were used until the fifth passage.

Measurement of ACE Activity in Transfected CHO and Endothelial Cells

ACE activity in CHO cells was performed according to Sabatini et al. The endothelial cells were plated in 6-well plates (Corning) at 1.5×10⁵ cells per well, washed with Hanks’ balanced salt solution buffer containing 10 μmol/L of ZnCl₂ (pH 7.4) and incubated in the same buffer with 10 μmol/L of Abz-FRK(Dnp)P-OH. Aliquots of culture supernatant were collected in different times (0 to 40 minutes), the reaction stopped at 0°C, and the fluorescence produced by the hydrolysis of the substrate by ACE determined as described above. The arbitrary fluorescence units were converted into micro-
moles of substrate hydrolyzed based on a calibration curve obtained using a standard solution of complete substrate.\textsuperscript{18, 23} ACE activity was inhibited by 2.5 μmol/L of the specific ACE inhibitor lisinopril used as a control in the assays. ACE shedding was not observed in the time period of our assays.\textsuperscript{14} The protein content of each well was determined by the method of Lowry et al.\textsuperscript{18} using BSA as standard. Measurements were performed in triplicate, and ACE activity values were reported as micromoles of substrate hydrolyzed per minute per milligram of protein (μmol·min⁻¹·mg⁻¹), represented as 1 unit.

ACE Protein Expression in Transfected CHO Cells

Protein expression was determined by Western blotting analysis. Subcellular fractionation was performed according to Bogan et al.\textsuperscript{25} Cell extracts were separated by SDS-PAGE (10%) and transferred to a polyvinylidene fluoride membrane. The membranes were blocked for 60 minutes in Tris-buffered saline Tween containing 5% nonfat milk. The immunoblot was performed with an anti-ACE-specific primary antibody (anti-ACE, clone 3C5, Chemicon, Millipore Corporation) 1:1000 dilution, overnight at 4°C, followed by the membrane incubation for 2 hours at room temperature with a secondary antibody (Stabilized goat anti-mouse horseradish peroxidase–conjugated, Pierce, Thermo Fisher Scientific Inc). The internal control was measured by membrane incubation with GAPDH antibody (anti-GAPDH rabbit monoclonal antibody, Cell Signaling) 1:2000 at 4°C overnight, followed by the incubation with the secondary antibody (stabilized goat anti-rabbit horseradish peroxidase–conjugated, Pierce, Thermo Fisher Scientific Inc) for 2 hours at room temperature. The immunoreactive bands were visualized with the chemiluminescence reagent Super Signal West Dura for the chemiluminescent detection of horseradish peroxidase on Western blotting (Pierce), according to the procedure described by the supplier.

Statistics

Differences of data among groups in individual experiments were analyzed for statistical significance by 1-way ANOVA, followed by the Bonferroni test. A value of $P<0.05$ was considered significant.

Results

ACE and Kinin B₂ Receptor Expression in Transfected CHO Cells

CHO cells were permanently transfected initially with the plasmid containing the coding region for somatic ACE. ACE expression was evaluated by RT-PCR and Western blot,\textsuperscript{18} and one clone expressing the enzyme in high amounts was selected and transfected either with an empty plasmid (mock-transfected cells) or with the same plasmid containing the kinin B₂ receptor coding region. The expression of the kinin B₂ receptor gene in these cells was evaluated by real-time PCR, by radioligand binding studies, and by a functional assay in the microphysiometer Cytosensor. The figure (Figure 1). In the binding study, cells expressing only the B₂ receptor or both ACE/B₂ receptor in the presence of lisinopril were able to bind specifically the B₂ agonist BK (Figure 1B), whereas cells expressing only ACE or ACE/B₂ receptor in the absence of lisinopril displayed no binding for the B₂ agonist (Figure 1B). Furthermore, BK was able to elicit a concentration-response effect on the extracellular acidification rate in CHO cells expressing the B₂ receptor or ACE/B₂ receptor in the presence of lisinopril (Figure 1C). This effect was blocked by the specific B₂ antagonist icatibant, showing the presence of a functional receptor at the membrane in both cases (data not shown). The coexpression of the receptor and the enzyme did not change the affinity of the B₂ agonist BK to the receptor (Table).
ACE Activity in CHO Cells Transfected With the Kinin B2 Receptor

By using a selective fluorescence substrate assay for ACE activity determination,23 we could evaluate ACE activity in CHO cell monolayers expressing both ACE and ACE with the B2 receptor (Figure 2). Unexpectedly, the results show that the coexpression of the kinin B2 receptor in these cells increased significantly ACE activity. Cells expressing the receptor presented ACE activity 41% higher (P<0.05) when compared with the cells expressing only ACE. The specificity of the assay was demonstrated by the complete inhibition of the catalytic activity in the presence of 2.5 μmol/L of lisinopril (data not shown). Furthermore, the antagonist icatibant could lower the increase in ACE activity induced by coexpression of the kinin B2 receptor (Figure 2). On the other hand, the B1 antagonist DesArg9-Leu8-BK used at a high concentration (10−6 mol/L) did not change ACE activity, and the B2 antagonist had no effect in CHO cells expressing only the enzyme (Figure 2). Because the level of ACE expression could vary in transfected cells, and this level would evidently interfere with the enzyme activity, we evaluated the levels of ACE by Western blot. Western blot analysis showed that the coexpression of the B2 receptor with ACE did not significantly alter the amount of ACE expressed in the cells, taking into account total protein (Figure 3A) or membrane fraction protein (Figure 3B), when compared with the mock-transfected cells.

Influence of B2 Receptor on ACE Activity in Endothelial Cells

To prove the hypothesis that the kinin B2 receptor could directly interact with ACE and modulate the enzyme activity in a more physiological environment, endothelial cells obtained from lungs of WT and kinin B2 receptor knockout mice were cultured and analyzed for ACE activity. Results show that cells from WT mice, expressing both ACE and the B2 receptor, possess higher ACE activity (Figure 4A) without ACE mRNA (Figure 4B) or ACE protein (Figure 4C) alterations, when compared with the activity obtained in cells from the B2 knockout mice. In addition, icatibant could partially inhibit ACE activity significantly in endothelial cells obtained from WT but not in the ones obtained from the B2 knockout animals (Figure 4A).

**Discussion**

ACE is a multisubstrate peptidyl-dipeptidase known to interfere with different systems involved in cardiovascular regulation, inflammation, pain, glucose homeostasis, and angiogenesis, among others. Ang I and BK, classic substrates for ACE, are converted to Ang II and hydrolyzed to inactive peptides, respectively, and have opposing effects in most systems. These agonists promote their effects by activation of specific G protein–coupled receptors (Ang II type 1 and Ang II type 2 for Ang II and B1 and B2 for BK). The interaction of ACE with Ang I and kinin B2 receptors has been extensively described in the literature.11,14–25 These studies also show that this interaction is able to alter the pharmacological profile of these receptors. In addition, it is known that ACE inhibitors can directly bind and activate kinin B2 receptors.31–32 Despite the long-held view that G protein–coupled receptors function as monomeric signaling units (ie, a single receptor couples to a G protein on ligand binding), a growing body of evidence suggests that these receptors form higher oligomeric structures through direct interactions among either identical or nonidentical family members.33,34 Recently, Chen et al35 have successfully shown heterodimer formation between the ACE and B2 receptor and colocalization of both proteins at the plasma membrane. However, the underlying mechanisms involved in receptor oligomerization and the exact role of this phenomenon in G protein–coupled receptor function remain largely unknown. In the present article we investigated the occurrence of ACE/B2 receptor interaction from a completely different aspect: we reasoned that this interaction between both proteins could lead to an alteration in ACE catalytic activity. Therefore, this hypothesis that takes into account the cross-talk between ACE and the kinin B2 receptor was analyzed basically by 2 different approaches: one using an artificial system, where CHO cells were transfected with both ACE and the B2 receptor and another one where a natural system expressing both proteins, like lung

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**Table. IC50 and Bmax Values for BK Binding to CHO Cells Transfected Only With the B2 Receptor, With the B2 Receptor+ACE, and With ACE**

<table>
<thead>
<tr>
<th>Cells</th>
<th>IC50, nM</th>
<th>n</th>
<th>Bmax, fmol per 10^6 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>B2</td>
<td>0.4±0.1</td>
<td>4</td>
<td>21±6</td>
</tr>
<tr>
<td>B2+ACE</td>
<td>NB</td>
<td>3</td>
<td>ND</td>
</tr>
<tr>
<td>B2+ACE (+ lisinopril)</td>
<td>0.4±0.1</td>
<td>3</td>
<td>1.3±0.4</td>
</tr>
<tr>
<td>ACE</td>
<td>NB</td>
<td>3</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are means±SEMs and the number of independent experiments in duplicate is indicated. NB indicates no binding; ND, not determined.

**References**

1. [125I-Tyr0-BK was used as radioligand. IC50 (the concentration of bradykinin required for 50% inhibition of [125I-Tyr0-BK binding)](http://hyper.ahajournals.org/)

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**Figure 2.** ACE activity in CHO cells expressing ACE or ACE/B2 receptors. ACE activity was evaluated in CHO-transfected cells using the fluorescence resonance energy transfer peptide Abz-FRK(Dnp)P-OH in those untreated, pretreated with the B1 receptor antagonist DesArg9-Leu8-BK (DLBK; 10−6 mol/L), and pretreated with the B2 receptor antagonist icatibant at different concentrations. The values represent means±SEMs of 6 experiments using 3 different clones of ACE-CHO (black) and ACE/B2 receptors (white). *P<0.05.
endothelial cells, was used. Our results using both systems show that the presence of the kinin B2 receptor can significantly increase ACE activity toward Abz-FRK(Dnp)P-OH and that the specific blockade of the receptor is sufficient to cause inhibition of the enzyme, demonstrating that antagonist occupancy of the B2 receptor negatively affects ACE catalytic activity toward this substrate. In accordance with this hypothesis, previous data from our laboratory show that deletion of the kinin B1 receptor in mice leads to an inhibition of ACE activity in stomach fundus. This finding was evidenced by the lack of the potentiating effect of captopril on the contractile responses to BK in this preparation, indicating a potential modulation of ACE activity by kinin B1 receptors. However, quantitative analysis of enzymatic activity in transfected cells is a difficult task. This analysis should take into account the level of protein expression, which can vary in transfected cells depending on transfection efficiency, and this level can evidently interfere with the enzyme activity. Therefore, to avoid transfection efficiency bias, we cotransfected the kinin B2 receptor on primarily ACE-transfected CHO cells. In addition, the levels of ACE in the cotransfected cells were also evaluated by Western blot and were shown not to differ significantly from the levels from those cells expressing only ACE. Therefore, we could rule out an alteration on ACE activity exclusively based on expression levels. Furthermore, we also evaluated the density of B2 receptors in the membrane of transfected CHO cells by binding assays. Although the initial amount of DNA used in the transfection assays was similar, we could observe a higher number of binding sites of B2 receptors in cells expressing only the kinin receptors (420 fmol/mg of protein) when compared with that coexpressing ACE and B2 receptors (26 fmol/mg of protein). Higher values of B2 receptor binding sites were also shown by Minshall et al. in CHO cells similarly transfected with the B2 receptor (900 to 1250 fmol/mg of protein) in comparison with cells transfected with both ACE and the B2 receptor (303 to 753 fmol/mg of protein). However, despite this variation in receptor density, the affinity of the agonist for the receptor was not altered, as shown by radioligand binding. Furthermore, the functionality of the B2 receptor in the transfected cells was evaluated by the Cytosensor microphysiometer, evidencing the correct expression and functionality of these receptors at the cellular membrane.

A complex formation between ACE and the B2 receptor is thought to be a bimolecular reaction, with the kinetics dependent on the reagents concentration. However, despite
the low concentration of the B₂ receptor in our cells, determined by the binding assay, the effect of icatibant on ACE activity shows that B₂ receptor concentration in our system is enough for the complex to be mounted and functional. Thus, our data bring new evidence to support a functional interaction between ACE and the B₂ receptor. One striking result shown in the present work was the inhibitory effect of the kinin B₂-specific antagonist icatibant on ACE activity. According to our findings, it can be inferred that binding of this molecule to the kinin B₂ receptor suggests the communication between ACE and B₂ receptor. This assumption is corroborated by the fact that the incubation of the cells only expressing ACE with icatibant did not result in alteration in the activity of the enzyme. In addition, incubation of icatibant with the soluble ACE present in plasma was not able to interfere with the enzyme activity. Furthermore, it is described that icatibant is highly stable against enzymatic degradation, not being a competitive substrate for ACE type kininase II. Although the molecular mechanism by which the B₂ receptor regulates the activity of ACE is not fully elucidated, some possibilities may be suggested. Because icatibant, a selective kinin B₂ receptor antagonist, is able to interfere in ACE activity, one possible mechanism by which B₂ receptor could regulate this activity is by dimerization between both proteins. This hypothesis is corroborated by the fact that both proteins colocalize and generate functional heterodimers.14,35 The physiological relevance of our findings is still not evident but could be related to the control of BK and Ang II levels, 2 potent and antagonizing peptides, where both ACE and the kinin B₂ receptor proteins are expressed. Thus, interaction between these proteins may contribute to the potentiation of angiotensin II effects in some tissues. Furthermore, this mechanism may act as a negative feedback to reduce BK formation after B₂ receptor stimulation. Despite the fact that the specificity of the assay has been confirmed by the complete inhibition of the hydrolysis of Abz-FRK(Dnp)P-OH by the specific ACE inhibitor lisinopril, the modulation in ACE activity by the B₂ receptor should be further studied with the physiological agonists Ang I and BK.

Perspectives

Thus, considering the physiological relevance of ACE and the role of its inhibitors in the clinic, we believe that these results will highlight the interplay between these molecules and kinin receptors and will help us to understand the mechanism controlling these interactions.

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Disclosures

None.

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