Hydrolysis of Angiotensin Peptides by Human Angiotensin I–Converting Enzyme and the Resensitization of B2 Kinin Receptors

Zhenlong Chen, Fulong Tan, Ervin G. Erdös, Peter A. Deddish

Abstract—We measured the cleavage of angiotensin I (Ang I) metabolites by angiotensin I–converting enzyme (ACE) in cultured cells and examined how they augment actions of bradykinin B2 receptor agonists. Monolayers of Chinese hamster ovary cells transfected to stably express human ACE and bradykinin B2 receptors coupled to green fluorescent protein (B2GFP) or to express only coupled B2GFP receptors. We used 2 ACE-resistant bradykinin analogues to activate the B2 receptors. We used high-performance liquid chromatography to analyze the peptides cleaved by ACE on cell monolayers and found that Ang I-9 was hydrolyzed 18/11003 slower than Ang I and 30% slower than Ang I-7. Ang I-7 was cleaved to Ang I-5. Although μmol/L concentrations of slowly cleaved substrates Ang I-7 and Ang I-9 inhibit ACE, they resensitize the desensitized B2GFP receptors in nmol/L concentration, independent of ACE inhibition. This is reflected by release of arachidonic acid through a mechanism involving cross-talk between ACE and B2 receptors. When ACE was not expressed, the Ang I-9, Ang I-7 peptides were inactive. Inhibitors of protein kinase C–, phosphatases and Tyr-kinase blocked this resensitization activity, but not basal B2 activation by bradykinin. Ang I-9 and Ang I-7 enhance bradykinin activity, probably by acting as endogenous allosteric modifiers of the ACE and B2 receptor complex. Consequently, when ACE inhibitors block conversion of Ang I, other enzymes can still release Ang I metabolites to enhance the efficacy of ACE inhibitors. (Hypertension. 2005;46:1-6.)

Key Words: angiotensin ■ angiotensin-converting enzyme ■ bradykinin ■ enalapril ■ protein kinases

Angiotensin I–converting enzyme (ACE) inhibitors are used by tens of millions of patients to block conversion of angiotensin I (Ang I) to Ang II and inactivation of bradykinin; the latter contributes significantly to the beneficial effects.1–4 After ACE inhibitors increase Ang I concentration, it can be converted to derivatives such as Ang I-9 and Ang I-7.5,6 Ang I-7 actively antagonizes the vasoconstrictor and proliferative effects of Ang II.7,8 Ang I-9, believed to be inactive until converted by ACE to the active Ang I-7, which is subsequently inactivated by release of the C-terminal dipeptide to form Ang I-5. The heptapeptide has its own receptor, its specific activities differ from those of Ang II,9,10 and it also enhances the activity of bradykinin on its B2 receptors.11,12

The major enzymes that cleave Ang I to derivatives other than Ang II are ACE213–15 and deamidase or cathepsin A.12 ACE2 is a carboxypeptidase instead of a peptidyl dipeptidase like ACE.16 ACE2 hydrolyzes Ang II orders of magnitude faster than Ang I.15 ACE2 generates Ang I-7 by releasing the C-terminal Phe8 of Ang II much more efficiently than cleaving the C-terminal Leu10 of Ang I (Figure 1). ACE2 commanded much attention because a genetically determined lack of the enzyme can block normal heart development13,14 and because of its identity with the corona virus receptor.13 Ang I is rapidly converted to Ang I-9 by deamidase or cathepsin A, found in the heart and throughout the body.12 A carboxypeptidase A-type enzyme17 also releases Ang I-9, which inhibits ACE at relatively high concentrations.11

To determine whether Ang I-9 is active per se or it becomes active only after conversion to Ang I-7 under our conditions, we examined the metabolism of Ang I, Ang I-9, and Ang I-7 in stably transfected Chinese hamster ovary (CHO) cells that express human ACE and human bradykinin B2 receptors coupled to green fluorescent protein (B2GFP). We then used the membranes of living cells to establish that Ang I cleavage products, in addition to potentiating bradykinin activity,12,18,19 also resensitized the B2GFP receptors desensitized by agonist. We found that they were active at much lower concentrations than suggested by their IC50 values for ACE. Because ACE, or kininase II, is a major bradykinin inactivator,3 we used an ACE-resistant bradykinin analogue (BKan)20 and synthesized another, didansyl-lysyl-bradykinin (DidnsKBK), as B2GFP receptor agonists.

Materials and Methods

The Materials section is available online at http://www.hypertensionaha.org.
digested with *Xho*I and *Hind*III. The open reading frame representing the coding sequence of B2GFP was fully sequenced before its expression and analysis.

**Construction of CHO/ACE–B2GFP Cells**

The cDNA of B2GFP was stably transfected into CHO-K1 cells, and transfectants were selected by fluorescence-activated cell sorting. Selected clones were tested for B2GFP function by measuring [Ca\(^{2+}\)] and response to bradykinin stimulation. Cells with high expression of B2GFP (CHO/BG) were selected and transfected with cDNA of human ACE and the transfectants selected with Geneticin. Then a clone of cells with high ACE activity, CHO/ACE-BG (CHO/ABG), was chosen.

**Synthesis of DidnsKBK**

The molecular mass of samples was determined with electron spray mass spectrometry, corresponding closely to the calculated values for monodansyl-KBK (1421.8) and DidnsKBK (1655.1). The αNH₂ group in the monodansyl compound and both α-amino and ε-amino groups of Lys in DidnsKBK were coupled (please see online supplement, available at http://www.hypertensionaha.org).

**Hydrolysis of Peptides by Cell Monolayers**

CHO/ABG cells were grown in DMEM containing 10% FBS in 12-well plates. Cell monolayers were washed with serum-free DMEM, and 400 μL serum-free DMEM containing 50 to 800 μmol/L angiotensin peptide was added to each well. Plates were incubated at 37°C with shaking for appropriate times. 140-μL aliquots were drawn and added to 70 μL of 5% ice-cold TFA. Fifty-microliter aliquots were analyzed by reversed-phase HPLC. Less than 1% ACE activity was released spontaneously into the medium.

**Lisinopril Titration of ACE**

ACE activity on cell monolayers was titrated with the tight binding inhibitor lisinopril. Cells were grown to confluence in 12-well plates (≈10\(^{4}\) cells per well). After washing, 0 to 375 nmol/L lisinopril was incubated at 37°C with shaking for 30 minutes. Then 50 μmol/L Ang I was added and incubation was continued for 5 minutes. Hydrolysis products were analyzed.

**Hydrolysis of Peptides by Cell Monolayers**

For kinetics, Ang I and Ang 1-9 (50 to 800 μmol/L) were incubated with 2 or 10 nmol/L purified human ACE in 50 mmol/L Tris–maleate buffer with 150 mmol/L NaCl, pH 7.8, at 37°C for 30 minutes. Ice-cold 5% trifluoroacetic acid (TFA) precipitated the enzyme. Percent hydrolysis was determined by changes in substrate peak area in high-performance liquid chromatography (HPLC).

**Analysis of Hydrolysis Products**

Peptides and their hydrolysis products were separated on a Bond–clone 10μ C18 reversed-phase column (Phenomenex) with a 10% to 28% linear gradient of acetonitrile/0.05% TFA in H₂O/0.05% TFA over 30 minutes. Products were detected at 214-nm wavelength. The cleavage of peptides followed zero-order kinetics during the assay, and amounts of peptide fragments were determined with standard peptides.

**Resensitization of Bradykinin Receptors in CHO/ABG Cells**

After desensitization of B2GFP receptors by initial exposure of cells to bradykinin or BKan to induce tachyphylaxis, we assessed the
extent of resensitization, or the return of sensitivity to kinin in the medium, by measuring [3H]AA release from cells. Cells were stimulated and desensitized with 1 μmol/L bradykinin, BKan, or DidnsKBK (10 μmol/L) for 30 minutes, then exposed again to agonist or Ang 1-7, Ang 1-9, or enalaprilat for an additional 5 minutes. [3H]AA release was determined by sampling AA release during the first 30 minutes and corrected for the spontaneous release. B2 receptor antagonist HOE 140 (1 μmol/L) was added as a control 10 minutes before Ang 1-7 or Ang 1-9.

**Effect of Inhibitors on Resensitization**

CHO/ABG cells were incubated with 1 of the following inhibitors: 1.0 μmol/L staurosporine, 0.5 μmol/L calphostin C, 1.0 μmol/L okadaic acid, 0.1 μmol/L calyculin A, 0.1 μmol/L Gö 6976, or 20 μmol/L genistein for 30 minutes at 37°C. The cell monolayers were then washed with fresh medium before testing for resensitization.

**Statistics**

Data are expressed as mean ± SEM (n=3). Statistical evaluation was performed by 1-way ANOVA for matched values.

**Results**

**Expression of B2GFP Receptor and ACE**

CHO cells transfected with B2GFP cDNA were selected for high expression; then we transfected them with ACE cDNA and isolated CHO/ABG clones with high ACE activity. We determined the number of B2 receptors expressed per cell by saturation binding of [3H]bradykinin and the number of ACE molecules per cell by active site titration of ACE in cell monolayers with lisinopril.15 CHO/BG and CHO/ABG cells had ∼1×10⁶ B2 receptors per cell, and the CHO/ABG cells had ∼5×10⁶ ACE molecules per cell.

**Hydrolysis of N-Terminal Protected BKan Analogue**

To study resensitization, we synthesized a derivative of bradykinin with a bulky N terminus that would protect it from inactivation by ACE but would allow it to act as an agonist of the B2 receptor. KBK was dansylated at its NH2 and at the αNH2 and εNH2 groups of lysine (Table 1). Whereas human ACE cleaved monodansyl-KBK at about one third the rate of KBK, DidnsKBK was only 0.4% hydrolyzed in 30 minutes.

**Kinetics of Hydrolysis of Peptides by Cell Monolayers**

Table 2 shows that Ang I had the highest Michaelis constant (Km; 119 μmol/L) when CHO/ABG cell monolayers hydrolyzed Ang peptides, followed by Ang 1-9 (61 μmol/L) and Ang 1-7 (35 μmol/L). The catalytic constant (kcat) of Ang I was also the highest (2890/min⁻¹) and was 18× higher than that of Ang 1-9 and 12× higher than Ang 1-7. Ang 1-7 was 4× less efficiently hydrolyzed (kcat/Km=6.5 [μmol/L]⁻¹ min⁻¹)

**Comparison of Kinetics of Hydrolysis by Purified Human ACE and Cell Monolayers**

Table 2 also compares the hydrolysis of Ang I and Ang 1-9 by purified human renal soluble ACE with the plasma membrane–bound ACE of CHO/ABG cells. The apparent Km for the cell surface enzyme was approximately twice that of the purified enzyme (119 versus 67 μmol/L for Ang I; 61 versus 28 μmol/L for Ang 1-9). The kcat of ACE on the plasma membrane of intact cells was much higher than with purified enzyme, which suggests some loss of activity of during purification or a lack of negative cooperativity between the 2 active sites of ACE on the cell membrane. Also, the specificity constant (kcat/Km) for each peptide was lower for the purified enzyme than for the cell surface enzyme (1.3 versus 2.6 μmol/L/min) for Ang 1-9. The lower Km with the purified enzyme did not offset the lower kcat.

**Resensitization of B2GFP Receptors by Ang 1-7 and Ang 1-9**

The bradykinin B2 receptor was desensitized by pretreating CHO/ABG cells with 1 μmol/L BKan, and the agonist was tested again to establish that desensitization had occurred. Ang 1-7 or Ang 1-9 had the lowest specificity constant (2.6 [μmol/L]⁻¹ min⁻¹), ~10× less than that of Ang I.

**Table 1.** Hydrolysis of Angiotensin Peptides by Human ACE on Intact CHO Cell Surface* and Purified Human ACE

<table>
<thead>
<tr>
<th>Peptide</th>
<th>ACE (μmol/L)</th>
<th>kcat (min⁻¹)†</th>
<th>kcat/Km (μmol/L⁻¹ min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ang 1 Cells</td>
<td>119</td>
<td>2890</td>
<td>24.3</td>
</tr>
<tr>
<td>Purified</td>
<td>67</td>
<td>520</td>
<td>7.8</td>
</tr>
<tr>
<td>Ang 1-9 Cells</td>
<td>61</td>
<td>156</td>
<td>2.6</td>
</tr>
<tr>
<td>Purified</td>
<td>28</td>
<td>35</td>
<td>1.3</td>
</tr>
<tr>
<td>Ang 1-7 Cells</td>
<td>35</td>
<td>228</td>
<td>6.5</td>
</tr>
<tr>
<td>Purified</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

*Peptide concentration = 100 μmol/L. Human ACE = 2 nmol/L. †Values = mean ± SEM of 3 experiments done in duplicate.
ACE using hippuryl-his-leucine as substrate (data not shown).

Effect of Protein Kinase C and Protein Phosphatase Inhibitors on Resensitization

Figure 3 shows the effects of the protein kinase C (PKC), phosphatase, and PKCa inhibitors on the resensitization of B2 receptors in CHO/ABG cells. Several diverse signal transduction inhibitors abolished the resensitization of the B2 receptor by Ang 1-9: staurosporine, calphostin C; okadaic acid, calyculin A, or 0.1 μmol/L G6976, washed, desensitized with BKan (1 μmol/L), then exposed to medium alone or 1 μmol/L BKan or 10 nmol/L Ang 1-9. Values are mean±SEM (n=3). †P<0.01 vs medium control. Kinase or phosphatase inhibitors blocked resensitization also by Ang 1-7 and enalaprilat (data not shown).

Effect of Tyrosine Kinase Inhibitor on Resensitization

The tyrosine kinase inhibitor genistein also completely blocked resensitization of CHO/ABG cells by Ang 1-7, Ang 1-9, or enalaprilat (EPT) (Figure 4). As with PKC inhibitors, preincubation of the cells with genistein did not block the initial release of [3H]AA to 1 μmol/L BKan before resensitization.

Discussion

Although the role of angiotensin in hypertension was well established during the past century,23 this peptide has become increasingly relevant to various pathological conditions other than hypertension. Ang II, released by ACE, acting on its angiotensin type 1 (AT1) receptor is usually noxious, whereas activation of the Angiotensin type 2 (AT2) receptor can counteract some of these effects, possibly through the bradykinin B2 receptor.24 ACE is also kininase II;3,25 its inhibitors block the inactivation of kinins. Because of the proven and probable pathological functions of Ang II and the widely reported counter-regulatory actions of Ang 1-7,7–9 we wished to establish how Ang II metabolites influence bradykinin activity. We included Ang 1-9 because it has not been well studied and is thought to be active only after conversion to Ang 1-7 (Figure 1). Both peptides inhibited ACE11,19 but only in micromolar concentrations. We reported that Ang 1-7 was cleaved only by one of the active sites of ACE, by the so-called N domain and not by native testicular C domain.19 Rice et al described that Ang 1-9 was converted more readily...
to Ang 1-7 by the separate recombinant C domain of ACE than by the N domain, but as shown above, they also found that Ang 1-9 was hydrolyzed much slower than Ang I.\textsuperscript{15} Cleavage of peptides by ACE can be influenced by its high carbohydrate content; for example, the degrees of glycosylation of ACE from human lung and kidney differ.\textsuperscript{20} Carbohydrate structures of enzymes can depend on their tissue and cellular origin.

To learn more about Ang I derivatives, we transfected CHO cells to express human ACE and bradykinin B\textsubscript{2} receptors. Lisinopril titration of active ACE sites on the cell surface indicated \( \approx 5 \times 10^{6} \) ACE molecules on the apical surface. Using saturation binding with labeled bradykinin, we found \( \approx 1 \times 10^{6} \) B\textsubscript{2} receptors per cell. This estimate assumes 2 active sites per molecule of ACE rather than an apparent single active site attributable to negative cooperativity between the 2 sites.\textsuperscript{27,28}

We compared the rates of hydrolysis of Ang I and its derivatives by ACE in CHO cells. Ang 1-9 was converted to Ang 1-7 at a rate \(< 6\%\) of Ang II release with a 90\% lower specificity constant. Ang 1-7 was inactivated to Ang 1-5 by ACE at a relatively low turnover rate and specificity constant (Table 1). Thus, Ang 1-7 and Ang 1-9 are more resistant to hydrolysis by ACE than Ang I. Although Ang 1-7 and Ang 1-9 have a slow turnover rate, their \( K_{m} \) was lower than that of Ang I but still much higher than the \( K_{m} \) for bradykinin: 1 to 0.1 \( \mu\text{mol/L}. \textsuperscript{25,26} \) Consequently, nephrilysin (neutral endopeptidase 24.11) is the major enzyme that liberates Ang 1-7 from Ang I\textsuperscript{28} and ACE2,\textsuperscript{15} and prolylcarboxypeptidase releases it from Ang I.\textsuperscript{29} Human chymase liberates only Ang II from Ang I\textsuperscript{30} (Table 1).

Ang I is hydrolyzed only very slowly by ACE 2,\textsuperscript{15} whereas deamidase (cathepsin A) converts it effectively to Ang 1-9.\textsuperscript{12,23} Ang 1-9 is more than a poor precursor of Ang 1-7 attributable to low conversion rate by ACE; it is also an active derivative of Ang I because enhances bradykinin actions in cultured cells and reactivates its receptor. Ang 1-9 may antagonize Ang II; just as Ang 1-7 does in laboratory animals.\textsuperscript{7,8} Ang 1-7 and Ang 1-9 inhibited ACE only at relatively high \( \mu\text{mol/L} \) concentrations, but in human pulmonary endothelial cells, they increased NO release by bradykinin at a much lower concentration than the \( IC_{50} \) values.\textsuperscript{12}

ACE inhibitors acted beyond ACE inhibition when they potentiated kinins in cultured cells or surviving organs.\textsuperscript{32} ACE and B\textsubscript{2} can colocalize and form heterodimers.\textsuperscript{20} These findings suggest that ACE inhibitors can function as indirect allosteric enhancers\textsuperscript{33} of B\textsubscript{2} bradykinin receptor agonists by inducing conformational changes via ACE.

Indeed, because Ang 1-7 and Ang 1-9 resensitize the receptors to bradykinin, they may be endogenous allosteric enhancers for B\textsubscript{2} receptor agonists if ACE and B\textsubscript{2} are expressed in the same cells. The peptides resensitized receptors at concentrations nearer to physiological levels than their \( IC_{50} \) values.

To show that resensitization of the receptors cannot be explained by blocking bradykinin inactivation,\textsuperscript{11,20,32} we used ACE-resistant BK\textsubscript{ans}. One ACE-resistant kinin analogue has a modified Phe\textsuperscript{8}–Arg\textsuperscript{9} bond;\textsuperscript{20} in the other, we dansylated both amino groups of lysine in Lys\textsuperscript{1}-bradykinin. The size of the peptide substrate restricts ACE activity, which drops beyond undecapeptide.\textsuperscript{34} Because B\textsubscript{2} receptors are more permissive and accept N-terminally enlarged agonists, such as dextran-coupled bradykinin with intact C terminus,\textsuperscript{35} we synthesized the DidnsKBK analogue. This modified kinin was hydrolyzed by ACE at \(< 3\%\) of the rate for Lys\textsuperscript{1}-bradykinin; it was not a competitive inhibitor and was blocked by HOE 140 on the B\textsubscript{2} receptor. As with BK\textsubscript{an}, CHO/ABG cells desensitized to DidnsKBK were resensitized by Ang 1-9 or enalaprilat. We believe that resensitization depends on the induction of conformational changes in ACE–B\textsubscript{2} complex and certainly not on blocking a potential inactivation of the ACE-resistant BK\textsubscript{an}.

Inhibition of protein kinases abolished potentiation\textsuperscript{12} and resensitization without affecting basal bradykinin activities. This process unlikely involves phosphorylation of the cytosolic portion of ACE\textsuperscript{36} because truncating the enzyme there did not affect resensitization by enalaprilat.\textsuperscript{20} Resensitization was blocked by inhibitors of PKC, PKC\textsubscript{\alpha}, tyrosine kinase, and serine phosphatases, and the findings indicate that resensitization depends on rapid phosphorylation and dephosphorylation of the receptor with PKC\textsubscript{\alpha} as a key component.\textsuperscript{37}

B\textsubscript{2} receptor activation liberates prostaglandins and other arachidonic acid metabolites\textsuperscript{38,39} and involves first \( G_{\alpha} \) protein leading to downstream activation of phospholipase A\textsubscript{2}. Because inhibition of PKC\textsubscript{\alpha} abolished resensitization, that suggests a complex cascading signal transduction pathway, which could result in the downstream opening of a transient receptor potential channel-1 Ca\textsuperscript{2+} channel.\textsuperscript{40}

Perspectives

ACE inhibitors block Ang I conversion, enhancing its concentration as substrate for enzymes such as nephrilysin or deamidase. ACE2 and prolylcarboxypeptidase convert Ang II to Ang 1-7. The well-documented effects of Ang 1-7 underscore the potential importance of Ang 1-9 as an active metabolite of Ang I. Ang 1-9 and Ang 1-7 are quite resistant to ACE on the plasma membrane of cultured cells, indicating that these peptides are relatively stable derivatives. Ang 1-7 and 1-9 potentiate bradykinin B\textsubscript{2} receptor agonists and resensitize the desensitized receptors to ACE-resistant kinins independent of ACE inhibition, provided human ACE and B\textsubscript{2} receptors are expressed. Peptides resensitized B\textsubscript{2} receptor similarly to enalaprilat, although Ang 1-7 and Ang 1-9 are poor ACE inhibitors. Resensitization of receptors restored their ability to react to the agonist in the medium and initiate signal transduction. The fact that serine/threonine kinase and even tyrosine kinase inhibitors can block the process without affecting basal bradykinin activity suggests different pathways of transduction leading to phospholipase A\textsubscript{2} activation and arachidonic acid release. ACE appears to be an “acceptor” of Ang 1-7 and Ang 1-9, which, in low concentrations, may modify the conformation of an enzyme–receptor complex. By countering the actions of Ang II, the angiotension-derived peptides can enhance the therapeutic usefulness of ACE inhibitors.

Acknowledgments

These studies were supported by National Institutes of Health, National Heart, Lung, and Blood Institute grants HL36473 and HL68580.
References


34. Skidgel RA, Erdös EG. Angiotensin converting enzyme (ACE) and neprilysin hydrolyze neuropetides: a brief history, the beginning and follow-ups to early studies. Peptides. 2003;24:521–525.


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Hypertension, published online October 24, 2005:

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