N-Domain–Specific Substrate and C-Domain Inhibitors of Angiotensin-Converting Enzyme

Angiotensin-(1–7) and Keto-ACE

Peter A. Deddish, Branislav Marcic, Herbert L. Jackman, Huan-Zhu Wang, Randal A. Skidgel, Ervin G. Erdős

Abstract—We used the isolated N- and C-domains of the angiotensin I–converting enzyme (N-ACE and C-ACE; ACE; kininase II) to investigate the hydrolysis of the active 1–7 derivative of angiotensin (Ang) II and inhibition by 5-S-5-benzamido-4-oxo-6-phenylhexanoyl-L-proline (keto-ACE). Ang-(1–7) is both a substrate and an inhibitor; it is cleaved by N-ACE at approximately one half the rate of bradykinin but negligibly by C-ACE. It inhibits C-ACE, however, at an order of magnitude lower concentration than N-ACE; the IC50 of C-ACE with 100 μmol/L Ang I substrate was 1.2 μmol/L and the Ki was 0.13. While searching for a specific inhibitor of a single active site of ACE, we found that keto-ACE inhibited bradykinin and Ang I hydrolysis by C-ACE in approximately a 38- to 47-times lower concentration than by N-ACE; IC50 values with C-ACE were 0.5 and 0.04 μmol/L. Furthermore, we investigated how Ang-(1–7) acts via bradykinin and the involvement of its B2 receptor. Ang-(1–7) was ineffective directly on the human bradykinin B2 receptor transfected and expressed in Chinese hamster ovary cells. However, Ang-(1–7) potentiated arachidonic acid release by an ACE-resistant bradykinin analogue (1 μmol/L), acting on the B2 receptor when the cells were cotransfected with cDNAs of both B2 receptor and ACE and the proteins were expressed on the plasma membrane of Chinese hamster ovary cells. Thus like other ACE inhibitors, Ang-(1–7) can potentiate the actions of a ligand of the B2 receptor indirectly by binding to the active site of ACE and independent of blocking ligand hydrolysis. This potentiation of kinins at the receptor level can explain some of the well-documented kininlike actions of Ang-(1–7). (Hypertension. 1998;31:912-917.)

Key Words: bradykinin receptor ■ signal transduction ■ arachidonic acid ■ enzymes

Ang-(1–7) is an active metabolite released by peptidases from Ang I or II.1 Neutral endopeptidase 24.11 (neprilysin) cleaves the decapetide Ang I to a tripeptide and to Ang-(1–7).2 Prolylendopeptidase and metalloendopeptidase 24.15 catalyze the same reaction.3,4 Ang II is converted by prolylcarboxypeptidase to a heptapeptide at its Pro7-Phe8 bond.5 The resulting heptapeptide is biologically active, but its actions differ from those of the parent peptide. It is not dipsogentic and does not stimulate aldosterone release. Instead of being a vasoconstrictor as Ang II is, it is a vasodilator on coronary arteries. In addition, the heptapeptide is antiproliferative and releases neurotransmitters and vasopressin.6–9

Although the existence of a receptor for Ang-(1–7) was reported,10–12 many of its effects involve BK, and they cannot be explained by the presence of a specific receptor for the heptapeptide. Thus the vasodilator effect of Ang-(1–7) on porcine, canine, or human arterial strips was attributed to NO release, very likely mediated by BK.9,13 This vasodilation and hypotension by Ang-(1–7) were also abolished by the BK B2 receptor antagonist HOE 140.14 In addition, Ang-(1–7) inhibits purified canine ACE.9 It is hypothesized that Ang-(1–7) is synergistic with BK because it either has a different Ang receptor subtype, is a ligand for the B2 receptor, or inhibits the enzymatic inactivation of BK.9

Ang I and BK are among the physiologically important substrates of Ang I–converting enzyme (kininase II; ACE); thus ACE inhibitors have dual actions in blocking hydrolysis of both peptides.15–18 The molecular cloning and sequencing of the complementary DNA for human and animal ACE revealed that ACE (somatic ACE) has two homologous domains,19–21 each containing an active center. According to their position in the N-terminal half or in the C-terminal half of the single chain protein, they are designated N- or C-domain, which will be referred to here as N-ACE and C-ACE. The synthesis of ACE is directed by a single gene in the body. The testicular form of the enzyme (germinal ACE) is shorter than the somatic ACE (732 versus 1306 residues)

Received October 2, 1997; first decision November 4, 1997; revision accepted December 1, 1997.
From the University of Illinois College of Medicine (Chicago).
Presented in part at the 51st Annual Fall Conference and Scientific Sessions of the Council for High Blood Pressure Research, American Heart Association, Washington, DC, September 16–19, 1997; and published in abstract form (Hypertension. 1997;30;494.).
Correspondence to Ervin G. Erdős, MD, University of Illinois College of Medicine at Chicago, Department of Pharmacology (M/C 868), 835 S Wolcott Ave, Chicago, IL 60612-7344.
E-mail EGErdos@uic.edu
© 1998 American Heart Association, Inc.
Selected Abbreviations and Acronyms

ACE = angiotensin-converting enzyme
Ang = angiotensin
BK = bradykinin
CHO = Chinese hamster ovary (cells)
HT-BK = more ACE-resistant BK derivative
keto-ACE = 5-S-5-benzamido-4-oxo-6-phenylhexanoyl-l-proline

and contains only the C-domain, which is attached to cell membranes,\textsuperscript{16,17,20–25} thus it lacks the N-domain active site.\textsuperscript{15,20–27}

Because keto-ACE inhibited the hydrolysis of a tripeptide representing the C-terminus of Ang I at an order lower concentration than it inhibited hydrolysis of a tripeptide similar to the C-terminus of BK,\textsuperscript{26} it was tested to see whether it would be a relatively specific inhibitor for one of the active centers. We also report here that Ang-(1–7) is cleaved to Ang-(1–5) and His-Pro, mainly by N-ACE, as it is only very slowly hydrolyzed by C-ACE. As such, it is a more potent inhibitor of the C-domain active site than N-ACE. Although Ang-(1–7) is an inhibitor, it potentiates the effects of BK independent of blocking its metabolism. In this regard, it acts similarly to classic ACE inhibitors that induce "crosstalk" between the enzyme and the receptor.\textsuperscript{29,30}

Methods

Materials

Hippuryl-glycyl-glycine (Hip-Gly-Gly), BK, angiotensin, and their metabolites, tissue culture medium, buffers, and reagents were purchased from Sigma Chemical Co. Phenyl-4-(n)-[\textsuperscript{3}H]-hippuryl-glycyl-glycine ([\textsuperscript{3}H]-Hip-Gly-Gly) and 5,6,8,9,11,12,14,15-[\textsuperscript{3}H]-arachidonic acid ([\textsuperscript{3}H]-AA) were purchased from Amersham and hippuryl histidyl-leucine (Hip-His-Leu) from Bachem. The keto-ACE was a gift from Dr R.G. Ahlquist at the Stanford Research Institute (Stanford, Calif). CHO cells were obtained from the American Type Culture Collection. The more ACE-resistant BK derivative HT-BK was from Novabiochem. Fetal bovine serum (FBS) was from Atlanta Biologicals. The cDNAs of ACE and B\textsubscript{2} receptor were kindly donated by Prof P. Corvol, College de France, and Dr K. Jarnigan, Syntex Co (Palo Alto, Calif). Enalaprilat was provided by Merck, Sharp & Dohme Research Division. Human recombinant ACE, expressed from a cDNA mutated to have only a functional C-domain active site (ACE K361, K365), was a gift of Dr Francois Alhenc-Gelas of INSERM U367, Paris.

Enzyme Purification

The N-ACE, C-ACE, and somatic ACE were purified as described.\textsuperscript{31} N-ACE was purified from ileal fluid collected after colostomy with the collaboration of N. Davidson of the University of Chicago, somatic ACE from human cadaver kidneys, and C-ACE from rabbit testicles obtained from Pel-Freeze Co. Approval for human tissue studies was granted by the Institutional Review Board at the University of Chicago and for animal tissue study by the Animal Care and Usage Committee at the University of Illinois at Chicago.

Protein Assay

The protein concentration of purified enzymes was determined as before.\textsuperscript{32}

Enzyme Assays

ACE activity was determined by several techniques. In a recording spectrophotometric assay, Hip-His-Leu was the substrate. In a fluorometric assay, the dipeptide cleaved from this substrate was coupled to o-phthalic dicarboxaldehyde.\textsuperscript{33} A radiometric assay used [\textsuperscript{3}H]Hip-Gly-Gly. The cleavage of longer peptide substrates was assessed by high-pressure liquid chromatography (HPLC).\textsuperscript{32}

Analysis of Hydrolysis Products

The hydrolysis of the various peptides by the ACE enzymes was assayed by HPLC. After incubation of enzymes with a peptide, ice-cold 5% trifluoroacetic acid (TFA) was added to 1.7% final concentration to stop the reaction. Peptides and their hydrolysis products were separated on a Waters µBondapak C\textsubscript{18} reverse-phase column with an increasing (5% to 40%) linear gradient of acetonitrile/0.05% TFA in H\textsubscript{2}O/0.05% TFA and detected with a Waters 484 detector at a wavelength of 214 nm.\textsuperscript{32} It was established that cleavage of the substrates followed zero-order kinetics to the time point of the assay.

Hydrolysis of Peptides

The rates of hydrolysis of the BK and angiotensin peptides were generally determined at 37°C in 50 mmol/L Tris maleate buffer, pH 7.4, containing 150 mmol/L NaCl, 100 µmol/L substrate, and 2 mmol/L enzyme. The effect of chloride concentration was assessed in the same buffer containing from 0 to 150 mmol/L NaCl. The kinetics of Ang-(1–7) hydrolysis were established by incubating a 2-nmol/L concentration of either N-ACE, somatic ACE, or a 300-nmol/L concentration of C-ACE with the peptide in five different concentrations varying from 10 to 200 µmol/L in 50 mmol/L Tris maleate, pH 7.4, and 150 mmol/L NaCl at 37°C. The amount of Ang-(1–5) formed was calculated from the peak area by comparison to a known standard. Kinetic parameters were calculated from Lineweaver-Burk plots.

Inhibition Studies

The effects of both keto-ACE and Ang-(1–7) on the hydrolysis of various substrates by N-ACE or C-ACE were determined by preincubating the enzyme for 30 minutes at 4°C with the potential inhibitor before addition of the substrate. A range of inhibitor concentrations was used and IC\textsubscript{50} was calculated from the inhibition curve. The K\textsubscript{i} was then calculated with kinetic parameters determined for uninhibited enzymes, with the following equation\textsuperscript{34}:

\[ v = \frac{V}{1 + \frac{K_i}{s}} \]

Transfection of CHO Cells and Expression of ACE and B\textsubscript{2} Receptor

Construction

An EcoRI fragment of B\textsubscript{2} receptor cDNA (177 to 1770 bp) containing the whole coding region was cloned into the EcoRI site of pcDNA1. The direction of the insert was determined by restriction enzyme digestion and sequencing. A 4-kb EcoRI fragment of human ACE cDNA was also cloned into the EcoRI site of pcDNA3, and its direction was determined by restriction enzyme digestion.

Transfection

Human ACE-pcDNA3, BK B\textsubscript{2} receptor–pcDNA1, and pH\textsuperscript{2}AP-3p-neo containing plasmids were used to transfect CHO cells with Lipofectin Reagent (Gibco BRL) as reported by us.\textsuperscript{35} Cell monolayers (approximately 50% confluent in 60- or 100-mm diameter dishes) were washed three times with serum-free Ham’s F-12. Lipofectin (30 to 80 µL) and DNA (5 to 15 µg/µL) were first individually diluted to 200 µL and then mixed together and incubated at room temperature for 15 minutes. An aliquot of the mixture (200 µL) was then diluted to 2 or 4 mL in serum-free Ham’s F-12 and applied to the cell culture monolayers. After 24 hours, the cells were washed twice and fed 5 or 10 mL of media containing 10% FBS. Two days later, the monolayers were subcultured after being treated with 0.5 mL trypsin/EDTA (0.025% trypsin/0.1 mmol/L EDTA) and plated at
TABLE 1. Hydrolysis of Ang-(1–7) and BK by N-ACE and C-ACE

<table>
<thead>
<tr>
<th>Enzyme*</th>
<th>Substrate Hydrolyzed, nmol†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ang-(1–7)</td>
</tr>
<tr>
<td>N-ACE</td>
<td>1.9±0.3</td>
</tr>
<tr>
<td>C-ACE</td>
<td>0</td>
</tr>
</tbody>
</table>

*Enzyme concentration, 2 nmol/L.
†Enzyme incubated with 100 μmol/L substrate for 1 hour at 37°C; all values expressed as mean±SEM (n=3).

low density in media containing 10% FBS and 600 μg/mL geneticin. Clonal colonies that grew in the presence of the selection medium were isolated with glass cloning rings, fed every 48 to 72 hours, and subcultured as needed. Cells that expressed B1 receptor only were designated as CHO-3B cells. Cotransfected cells expressing both active ACE and B2 receptor were the CHO-15AB cells.

**Effect of Ang-(1–7) on Arachidonic Acid Release by HT-BK**

Cells were grown on six-well plates in Ham’s F-12 medium containing 10% FBS. After reaching 60% to 80% confluence, the culture medium was replaced with Ham’s F-12 medium containing 0.5% FBS and 1 μCi/mL of [3H] arachidonic acid (loading medium). Cells were incubated in this medium for 24 hours. Monolayers were then washed three times with the release buffer (RB) (25 mmol/L HEPES, 150 mmol/L NaCl, 5 mmol/L KCl, 5.5 mmol/L dextrose, 0.8 mmol/L MgSO4, 1 mmol/L CaCl2, and 0.1% fatty acid-free BSA, pH 7.4). Triplicate wells were then incubated at 37°C for 30 minutes with one of the following treatments: (1) 1 mL of RB alone to measure basal [3H] arachidonic acid release (this value was later subtracted from each triplicate); (2) 1 μmol/L HT-BK in 1 mL RB; (3) 1 μmol/L HT-BK plus 1 μmol/L of Ang-(1–7) in 1 mL of RB; (4) 1 μmol/L HT-BK plus 10 μmol/L of Ang-(1–7) in 1 mL of RB; (5) 1 μmol/L HT-BK plus 10 μmol/L of Ang II in 1 mL RB; or (6) 10 μmol/L of Ang-(1–7) in 1 mL of RB without added HT-BK. After this time period, RB from each well was simply transferred to scintillation vials (released [3H] arachidonic acid is bound to fatty acid–free BSA and counted).

Results

**Hydrolysis of Ang-(1–7) and BK by N-ACE and C-ACE**

The decrease in the amount of unhydrolyzed substrate and the increase in hydrolysis products [Ang-(1–5), BK-(1–7), and Hip-His-Leu] were determined by comparison of the peak areas with peak areas of known standards in HPLC.

The results of the hydrolysis of Ang-(1–7) and BK by N-ACE and C-ACE are given in Table 1. Note that whereas BK was hydrolyzed approximately equally by both N-ACE and C-ACE under the conditions used (2 nmol/L enzyme, 100 μmol/L substrate, Cl− 150 mmol/L), the amount of Ang-(1–7) cleaved by the two enzymes differed greatly. N-ACE hydrolyzed Ang-(1–7) at about one half of the rate at which it hydrolyzed BK, whereas C-ACE in the concentration used did not cleave Ang-(1–7). These results are taken to show that Ang-(1–7) is primarily a substrate of the N-domain active site of ACE.

**Effect of Cl− Concentration on Ang-(1–7) Hydrolysis**

To determine this, the substrate was exposed to the enzymes at Cl− concentrations ranging from 0 to 300 mmol/L. The hydrolysis of Ang-(1–7) by N-ACE was stimulated less than twofold when the Cl− concentration was raised to 300 mmol/L from 0. (Because the N-ACE was not dialyzed, the activity at 0 Cl− may in fact be due to a trace of Cl− present.) There was still no detectable hydrolysis of Ang-(1–7) by C-ACE at any chloride concentration used.

**Kinetics of Ang-(1–7) Hydrolysis**

Because Ang-(1–7) was not hydrolyzed when the peptide was incubated with 2 nmol/L C-ACE for up to 3 hours, a much higher concentration of the enzyme was tried. Measurable activity was obtained when 100 μmol/L Ang-(1–7) was incubated with 300 nmol/L C-ACE for 3 hours. The product was Ang-(1–5), and the reaction was completely blocked by 1 μmol/L enalaprilat, indicating that the hydrolysis was due to ACE (data not shown). Therefore, the kinetics of hydrolysis of Ang-(1–7) were determined with 300 nmol/L C-ACE, 2 nmol/L N-ACE, and 2 nmol/L somatic ACE with various substrate concentrations (Table 2). The IC50 values of Ang-(1–7) with the three representative forms of ACE were 4.3, 6.8, and 6.6 μmol/L for N-ACE, C-ACE, and somatic ACE, respectively. The kcat value for N-ACE (27 min−1) was 75 times higher than the kcat for C-ACE (0.36 min−1) and quite similar to the kcat for somatic ACE (23.7 min−1). These values yield a specificity constant (kcat/Km) for N-ACE (6.1) that is 100 times larger than that for C-ACE (0.06) and 1.6 times greater than that for somatic ACE (3.7).

Ang-(1–7) was incubated with another source of C-ACE, somatic ACE, where His in positions 361 and 365 was mutated to Lys, thereby rendering the N-ACE domain inactive. Ang-(1–7) in these experiments was not hydrolyzed by the C-ACE (10 nmol/L), although the enzyme cleaved Hip-His-Leu (data not shown). Higher concentration of enzyme was not tried because of the limited amount of available material.

**Inhibition of C-ACE and N-ACE by Ang-(1–7)**

The low Km with both active sites (lower than that of Ang I)21 and the very low kcat of Ang-(1–7) with C-ACE indicated that Ang-(1–7) may be an inhibitor of the enzyme. This idea was tested by determining the IC50 values and Ki of Ang-(1–7) with Hip-His-Leu, Ang I, and BK as substrates of C-ACE and N-ACE. Table 3 shows that Ang-(1–7) inhibited C-ACE in μmol/L concentrations. The IC50 values of Ang-(1–7) with Ang I, BK, and Hip-His-Leu hydrolysis were 1.2, 3.9, and 8.2 μmol/L, respectively. With N-ACE, the IC50 values were 9 to 23 times higher (28, 46, and 71 μmol/L for inhibition of Ang I, BK, and Hip-His-Leu hydrolysis, respectively). The Ki values are also indicated in Table 3.
The reported pattern of keto-ACE inhibition of hydrolysis of various substrates by somatic ACE 28 indicated that it may be a relatively specific inhibitor for one of the ACE active sites. We therefore studied the inhibition of the hydrolysis of BK and Ang I by both N-ACE and C-ACE. Table 4 shows that keto-ACE is indeed relatively actively site-specific. With equal concentrations of N-ACE and C-ACE, the IC50 of keto-ACE was 47 times lower for C-ACE (0.51 μmol/L) than for N-ACE (24 μmol/L) with BK as the substrate. When Ang I was the substrate, the IC50 was 38 times lower for C-ACE (0.04 μmol/L) than for N-ACE (1.5 μmol/L). Thus keto-ACE is a more potent inhibitor of the C-domain active site of ACE.

Effect of Ang-(1–7) on BK-Induced Arachidonic Acid Release From Transfected CHO Cells

We reported that ACE inhibitors potentiate the effect of BK and analogues on B2 receptors, and this potentiation in the systems used is not due to inhibition of BK hydrolysis by ACE. 29,30 Therefore we tested the effect of Ang-(1–7) on the response of CHO cells grown in culture and transfected to express both ACE and B2 receptor (CHO-15AB cells) or B2 receptor only (CHO-3B). As ligand, the BK analogue HT-BK, 29,30 which is more resistant to hydrolysis by ACE than BK, was used (Figure). Arachidonic acid released in response to HT-BK in the absence or presence of Ang-(1–7) was measured. Values were normalized to the amount of arachidonic acid released by HT-BK stimulation alone. The addition of either 1 μmol/L or 10 μmol/L of Ang-(1–7) to CHO-15AB cells increased the arachidonic acid release by HT-BK approximately twofold or threefold but only from cells that contained both ACE and B2 receptor (CHO-15AB). As ligand, the BK analogue HT-BK, 29,30 which is more resistant to hydrolysis by ACE than BK, was used (Figure). Arachidonic acid released in response to HT-BK in the absence or presence of Ang-(1–7) was measured. Values were normalized to the amount of arachidonic acid released by HT-BK stimulation alone. The addition of either 1 μmol/L or 10 μmol/L of Ang-(1–7) to CHO-15AB cells increased the arachidonic acid release by HT-BK approximately twofold or threefold but only from cells that contained both ACE and B2 receptor (CHO-15AB).

Ang-(1–7) potentiates the release of arachidonic acid (AA) by HT-bradykinin (HT-BK) from CHO cells transfected both with ACE and B2 receptor (CHO-15AB). Bottom, Ang-(1–7) is inactive if only B2 receptor is transfected and expressed (CHO-3B). Cells were stimulated by 1 μmol/L HT-BK alone (open bar), HT-BK + 1 μmol/L Ang-(1–7) (diagonally lined bar), HT-BK + 10 μmol/L Ang-(1–7) (cross-hatched bar), HT-BK + 10 μmol/L Ang II (vertically lined bar); and 10 μmol/L Ang-(1–7) (filled bar). Ang II did not potentiate the effect of HT-BK, and Ang-(1–7) without added B2 agonist was inactive. Values are mean ± SEM, *P < .05. Ordinate equals the relative release of labeled AA, with release by HT-BK alone taken as 1.

TABLE 3. Inhibition of C-ACE and N-ACE by Ang-(1-7)

<table>
<thead>
<tr>
<th>Substrate, Concentration</th>
<th>IC50 and K (μmol/L)</th>
<th>IC50 and K (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hip-His-Leu, 1 mmol/L</td>
<td>8.2 ± 1.2</td>
<td>71 ± 6</td>
</tr>
<tr>
<td>Ang I, 100 μmol/L</td>
<td>1.2 ± 0.4</td>
<td>28</td>
</tr>
<tr>
<td>BK, 10 μmol/L</td>
<td>3.9 ± 0.6</td>
<td>46</td>
</tr>
</tbody>
</table>

*Expressed as mean ± SEM; n = 3 for each substrate.
†Values are the average (and range) of two experiments.

TABLE 4. Inhibition of Hydrolysis of BK and Ang I by Keto-ACE

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Keto-ACE IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>BK</td>
<td>24 ± 5</td>
</tr>
<tr>
<td>Ang I</td>
<td>1.5 ± 0.3</td>
</tr>
</tbody>
</table>

*Substrate concentration, 100 μmol/L.
†Enzyme concentration, 2 nmol/L. All values expressed as mean ± SEM (n = 3).

Inhibition of ACE by Keto-ACE

Effect of Ang-(1–7) on BK-Induced Arachidonic Acid Release From Transfected CHO Cells

We reported that ACE inhibitors potentiate the effect of BK and analogues on B2 receptors, and this potentiation in the systems used is not due to inhibition of BK hydrolysis by ACE. 29,30 Therefore we tested the effect of Ang-(1–7) on the response of CHO cells grown in culture and transfected to express both ACE and B2 receptor (CHO-15AB cells) or B2 receptor only (CHO-3B). As ligand, the BK analogue HT-BK, 29,30 which is more resistant to hydrolysis by ACE than BK, was used (Figure). Arachidonic acid released in response to HT-BK in the absence or presence of Ang-(1–7) was measured. Values were normalized to the amount of arachidonic acid released by HT-BK stimulation alone. The addition of either 1 μmol/L or 10 μmol/L of Ang-(1–7) to CHO-15AB cells increased the arachidonic acid release by HT-BK approximately twofold or threefold but only from cells that contained both ACE and B2 receptor (CHO-15AB)
Acid release by the ligand of the B₂ receptor in CHO-15AB cells when both human ACE and B₂ receptor were coexpressed, and this effect was not due to inhibition of BK inactivation. The parent peptide Ang II was inactive, since it is neither a substrate or inhibitor of ACE, possibly because its C-terminus is Pro²-Phe³ and not Pro² as in Ang-(1–7). C-terminal proline is an important component of ACE inhibitors for interaction with the enzyme.¹⁵,¹⁷

In isolated heart tissue, guinea pig ileum (R.D. Minshall, S.F. Rabito, R. Iigic, E.G. Erdös, unpublished observations, 1997), or cultured cells cotransfected with B₂ receptor and ACE,²⁹ the activation of B₂ receptor by ACE inhibitors was not primarily due to blocking kinin breakdown because ACE-resistant B₂ ligands were also potentiated. Addition of an active ACE inhibitor immediately enhanced BK activity as much as severalfold on all preparations tested.²⁹,³⁰ This immediate response cannot be correlated with the much slower hydrolysis of kinins in the same preparations. Ang-(1–7) acted similarly to other ACE inhibitors, demonstrated in more extensive studies.³⁹ In these investigations,²⁹ ACE inhibitors enhanced the number of BK binding sites, protected high-affinity sites, abolished the desensitization of the receptor, and delayed endocytosis. That Ang-(1–7) inhibits ACE was also shown by others.⁹ However, the inhibition, because of the differences in activity of the two domains, is more complex. Ang-(1–7) is a substrate of N-ACE but it also inhibits N-ACE, although at higher concentration than C-ACE. C-ACE cleaves Ang-(1–7) approximately 100 times slower than N-ACE, but Ang-(1–7) inhibits it at an order of magnitude lower concentration.

In ACE, the active site on the N-domain may more readily be exposed to bloodstream substrates and inhibitors; the enzyme is inserted into the plasma membrane by a transmembrane anchor peptide of the C-domain.¹⁵,²²–²⁵,³⁵,³⁶ ACE inhibitors bind to both active sites, but depending on their structure, they may differ in their affinities, primarily because of differences in dissociation rates from the two active sites.³²,³⁷,³⁸ We discovered in human ileal fluid collected after surgery a naturally occurring, short form of ACE having only the C-domain functional (not shown) because two His residues were mutated to Lys.²³,²⁴ The results were similar to those obtained with rabbit testicular ACE, and this human C-ACE also did not cleave Ang-(1–7).

It appears that vasodilation and probably NO release by Ang-(1–7)⁹,¹³,¹⁴ can be due to indirect potentiation of BK as an agonist of the B₂ receptor. This explains why Ang II receptor blockers were inactive, but a B₂ receptor blocker abolished Ang-(1–7) effects.⁵,¹⁴ It also follows that ACE inhibitors may block some of the activity of Ang-(1–7) by similar effects and competing for ACE.⁹,²⁹,³⁰

Because of the differences in the cleavage of biological substrates by the two domains, it would be important to develop a second generation of ACE inhibitors that react mainly with one of the active sites of ACE.⁹,⁴⁰ Ang-(1–7) appears to be a relatively specific substrate of N-ACE but an inhibitor of C-ACE. The other inhibitor we used, keto-ACE, also inhibits C-ACE at much lower concentration than N-ACE. Keto-ACE is a ketomethylene derivative of a blocked tripeptide substrate, Bz-Phe-Gly-Pro.⁴¹ The IC₅₀ for N-ACE was 38 to 47 times higher than for C-ACE with Ang I and BK substrates. The reported inhibition of short substrates²⁸,³² by keto-ACE can be interpreted now as caused by the different affinities of substrates and inhibitors for the two active sites.

In conclusion, we have shown that Ang-(1–7) is both a substrate and an inhibitor of ACE. Some of its pharmacological effects may be attributed to an indirect potentiation of BK action on its B₂ receptor by binding to the active site of ACE. This potentiation is not due primarily to inhibition of BK inactivation by ACE but to an induction of “crosstalk” between B₂ receptor and ACE on plasma membranes, which is triggered by ACE inhibitors.²⁹

Acknowledgments

These studies were supported in part by MERIT grant HL-36473 from the National Heart, Lung, and Blood Institute, National Institutes of Health. We thank Richard D. Minshall, PhD, and William Campbell, PhD, for helpful discussions; Dr Bernhard A. Schölkens for a gift of HOE-140; and Sara Thorburn, MA, for editorial assistance.

References


N-Domain–Specific Substrate and C-Domain Inhibitors of Angiotensin-Converting Enzyme: Angiotensin-(1–7) and Keto-ACE

Peter A. Deddish, Branislav Marcic, Herbert L. Jackman, Huan-Zhu Wang, Randal A. Skidgel and Ervin G. Erdös

_Hypertension_. 1998;31:912-917
doi: 10.1161/01.HYP.31.4.912

_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1998 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/31/4/912

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

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

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