Angiotensin 1-9 and 1-7 Release in Human Heart
Role of Cathepsin A

Herbert L. Jackman, Malek G. Massad, Marin Sekosan, Fulong Tan, Viktor Brovkovych, Branislav M. Marcic, Ervin G. Erdös

Abstract—Human heart tissue enzymes cleave angiotensin (Ang) I to release Ang 1-9, Ang II, or Ang 1-7. In atrial homogenate preparations, cathepsin A (deamidase) is responsible for 65% of the liberated Ang 1-9. Ang 1-7 was released (88% to 100%) by a metallopeptidase, as established with peptidase inhibitors. Ang II was liberated to about equal degrees by ACE and chymase-type enzymes. Cathepsin A’s presence in heart tissue was also proven because it deamidated enkephalinamide substrate by immunoprecipitation of cathepsin A with antiserum to human recombinant enzyme and by immunohistochemistry. In immunohistochemistry, cathepsin A was detected in myocytes of atrial tissue. The products of Ang I cleavage, Ang 1-9 and Ang 1-7, potentiated the effect of an ACE-resistant bradykinin analog and enhanced kinin effect on the B_2 receptor in Chinese hamster ovary cells transfected to express human ACE and B_2 (CHO/AB), and in human pulmonary arterial endothelial cells. Ang 1-9 and 1-7 augmented arachidonic acid and nitric oxide (NO) release by kinin. Direct assay of NO liberation by bradykinin from endothelial cells was potentiated at 10 nmol/L concentration, 2.4-fold (Ang 1-9) and 2.1-fold (Ang 1-7); in higher concentrations, Ang 1-9 was significantly more active than Ang 1-7. Both peptides had traces of activity in the absence of bradykinin. Ang 1-9 and Ang 1-7 potentiated bradykinin action on the B_2 receptor by raising arachidonic acid and NO release at much lower concentrations than their 50% inhibition concentrations (IC_{50}s) with ACE. They probably induce conformational changes in the ACE/B_2 receptor complex via interaction with ACE. (Hypertension. 2002;39:976-981.)

Key Words: peptides • angiotensin-converting enzyme • receptors, bradykinin • nitric oxide

Millions of patients are treated with angiotensin I–converting enzyme (ACE) or kininase II inhibitors against hypertension, congestive heart failure, diabetic nephropathy and other conditions. The dual effects of these inhibitors in blocking angiotensin (Ang) II release and the catabolism of bradykinin (BK) cannot account for all their actions. ACE inhibitors enhance the activity of BK, kallidin (Lys_1-BK), and the ACE-resistant BK peptide analog on their B_2 receptor in cultured cells. They induce protein-protein interaction, an enzyme-to-receptor crosstalk, which initiates a different signal transduction pathway than triggered by BK alone on the B_2 receptor. The activation of this receptor leads to an enhanced release of mediators into circulation, such as nitric oxide (NO) or prostaglandins.

In addition, these inhibitors may increase potentially the role of other enzymes that hydrolyze Ang I and BK by elevating the peptide substrate concentrations. Carboxypeptidases M and N liberate des-Arg^9-BK, a ligand of B_1 receptor. Human neutral endopeptidase 24.11 (neprilysin) releases Ang 1-7, which opposes Ang II activity and potentiates BK. Ang 1-9, liberated by carboxypeptidase-type enzymes, cannot be converted to Ang II by ACE.

Carboxypeptidase A of mast cells, an ACE variant (ACE II) that cleaves single C-terminal amino acid of Ang I, and a serine peptidase from platelets named deamidase, can all release Ang 1-9. Deamidase is very likely identical with cathepsin A (CATA), also called lysosomal protective protein or lysosomal carboxypeptidase A. CATA cleaves peptide bonds optimally at acid pH, but esters and amide bonds of C-terminal amino acids at a neutral pH. It was reported that in micromolar concentration, Ang 1-9 inhibits ACE and potentiates BK action on its B_2 receptor. Here, we report that CATA is abundantly present in human heart tissues. Its product of Ang I hydrolysis, Ang 1-9, and, at a somewhat higher concentration, Ang 1-7, enhances the effect of a kinin agonist on the B_2 receptor. They increase the release of arachidonic acid (AA) and NO at concentrations well below their 50% inhibition concentrations (IC_{50}) value for ACE.

Materials and Methods

Peptides and reagents were purchased as described previously. Ang 1-9 and Dansyl-Phe-Leu-Arg were synthesized. Cell cultures were performed with Chinese hamster ovary cells trans-
fected to express human ACE and B<sub>2</sub> receptor (CHO/AB) or with human pulmonary arterial endothelial (HPAE) cells.

**Preparation of Heart Tissues**

Human right atrial or left ventricular tissues obtained during surgery with permission of the University of Illinois-Chicago (UIC) Institutional Review Board, were homogenized in 200 mmol/L sodium acetate, pH 5.5, or 200 mmol/L sodium phosphate, pH 7.0, buffer. The reactions were stopped with trifluoroacetic acid, centrifuged at 16 000g for 10 minutes, and assayed in high-performance liquid chromatography (HPLC).<sup>19</sup>

**Enzyme Assays**

Routinely, 100 μmol/L Ang I was the substrate in 200 mmol/L sodium acetate, pH 5.5, or 200 mmol/L sodium phosphate, pH 7.0, buffer. The reactions were stopped with trifluoroacetic acid, centrifuged at 16 000g for 10 minutes, and assayed in high-performance liquid chromatography (HPLC).<sup>19</sup>

**Deamidation by Human Heart Tissue**

The substrate was 300 μmol/L D-Ala<sup>2</sup>-Leu<sup>5</sup>-enkephalinamide, and the enzyme was 25 μL of human atrial Prep 1, assayed at 25°C and pH 7.<sup>19</sup>

**Immunoprecipitation**

Aliquots of 16 000g human atrial or ventricular preparations (Prep 1) were immunoprecipitated with rabbit antiserum (1:100 v/v) elicited to 10<sup>9</sup>/H11002 human pulmonary arterial endothelial (HPAE) cells.

**Potentiation of Arachidonic Acid Release**

Cultured cells<sup>7,26</sup> in 6-well plates were loaded with [H]<sup>11002</sup>AA. The BK, ACE resistant BK analog (BKan),<sup>7,28</sup> and other agents were then added to cells.<sup>29</sup>

**Recombinant CATA**

A cDNA coding for the human CATA was isolated from a Lambda gt 10 human kidney cDNA library, using a 700 bp PCR fragment. The DNA of the expression vector DA-pVL1392 was cotransfected into High Five cells (Invitrogen) with linearized BaculoGold baculovirus DNA. Eighty percent of rCATA proenzyme was secreted, then activated with trypsin, measured with Dansyl-Phe-Leu-Arg,<sup>22,24</sup> then measured with Dansyl-Phe-Leu-Arg,<sup>23</sup> and purified on S-Sepharose column.

**Truncated CATA**

Human CATA is composed of 32 kDa and 20 kDa peptide chains.<sup>23</sup> The truncated rCATA contains 19 amino acids of the heavy chain, coupled to the N-terminal 29 residues in the light chain, forming a metallopeptidase and enhanced the reaction. Phenanthroline possibly protected the substrate against break-down by a metallopeptidase and enhanced the reaction.

**Peptidase Activity**

The peptidase activities of the Prep 1 extract of the right atrium and left ventricle were compared. At pH 5.5, they released approximately the same low amount of Ang II (2 nmol/h per milligram of protein) from Ang I. However, the atrial preparations (n=9) converted substantially more Ang I to Ang 1-9 and Ang 1-7 (77.1±8 SEM, 44.7±9 SEM) than the ventricular extracts (n=4) (19.8±6, 8.6±1). For Ang 1-9 release, the difference was significant at the level P<0.001; for Ang 1-7, P<0.02 at pH 5.5. As expected from the peptidase activity of CATA<sup>19,20,23</sup> in the atrial preparation, it released more Ang 1-9 at pH 5.5 than

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**Deamidation**

The presence of CATA in the heart was shown with a variety of techniques. First, deamidation (Table 1) was assayed by right atrial Prep 1 with D-Ala<sup>2</sup>-Leu<sup>5</sup>-enkephalinamide substrate.<sup>19</sup> CATA converts this peptide to enkephalin by cleaving the CONH<sub>2</sub> bond of Leu<sup>5</sup>, and the D-Ala<sup>2</sup> residue protects it against aminopeptidases. The atrial extract converted the peptide substrate to enkephalin at a rate of 19.7±4 nmol per h/mg protein at neutral pH. The activity was inhibited by serine peptidase inhibitors, diisopropylfluorophosphate (DFP) 63% and ebelactone B 54%;<sup>20,27,31</sup> The metallopeptidase inhibitor, 1,10-phenanthroline, instead of inhibiting, enhanced the reaction by 13% and even more at pH 5.5 (45%). At that pH, the rate of conversion of enkephalinamide increased to 29.3 (Table 1), but inhibitors of CATA were much less effective (Table 1).

The lower rate of inhibition by DFP and ebelactone B at lower pH is in agreement with the reported neutral pH optimum of CATA for cleaving C-terminal amide bonds.<sup>19</sup> The results suggest the presence of additional enzyme(s) in heart tissue that can convert enkephalinamide to enkephalin at an acid pH. Phenanthroline possibly protected the substrate against break-down by a metallopeptidase and enhanced the reaction.

**Ang 1-9 Release by rCATA**

Figure 1 shows the HPLC tracings of Ang 1-9 release from Ang I and its subsequent conversion to Ang II by human rCATA.

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<table>
<thead>
<tr>
<th>TABLE 1. Conversion of D-Ala&lt;sup&gt;2&lt;/sup&gt;-Leu&lt;sup&gt;5&lt;/sup&gt;-Enkephalinamide to Enkephalin by Human Right Atrial Prep 1*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibitors</td>
</tr>
<tr>
<td>pH</td>
</tr>
<tr>
<td>7.0</td>
</tr>
<tr>
<td>5.5</td>
</tr>
</tbody>
</table>

DFP indicates diisopropylfluorophosphate; Prep 1, preparation 1.

* n=3; values are the mean percent decrease (−) or increase (+) in activity. †nmol per h/mg protein±SEM.

**Statistics**

Means and SE were calculated and statistical significance of differences between means were tested by one-way analysis of variance with post hoc test.

An expanded Methods section can be found in an online data supplement available at http://www.hypertensionaha.org.

**Results**

**Presence of CATA in Human Heart**

**Deamidation**

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**Ang 1-9 Release by rCATA**

Figure 1 shows the HPLC tracings of Ang 1-9 release from Ang I and its subsequent conversion to Ang II by human rCATA.
TABLE 2. Percent Inhibition of Ang I Hydrolysis by Human Atrial Preparations at pH 7

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Products</th>
<th>Ang II*</th>
<th>Ang 1–9</th>
<th>Ang 1–7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I</td>
<td>II</td>
<td>I</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>(2.7±1)</td>
<td>(41.1±17)</td>
<td>(19.4±3)</td>
</tr>
<tr>
<td>Ebelactone B, 10 μmol/L</td>
<td></td>
<td>13±8</td>
<td>0</td>
<td>65±4</td>
</tr>
<tr>
<td>1,10 Phenanthroline, 1 mmol/L</td>
<td></td>
<td>35±12</td>
<td>16±6</td>
<td>22±7</td>
</tr>
<tr>
<td>Soybean trypsin inhibitor, 0.5 mg/ml</td>
<td></td>
<td>57±14</td>
<td>49±25</td>
<td>0</td>
</tr>
<tr>
<td>Enalaprilat, 1 μmol/L</td>
<td></td>
<td>71±11</td>
<td>44±21</td>
<td>0</td>
</tr>
<tr>
<td>Phosphoramidon, 10 μmol/L</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

I. 16 000g supernatant without Chaps detergent, Prep 1; II. 1000g supernatant with 1% Chaps, Prep 2.

*Assay at 25°C at pH 7.0, n=5, percent change±SEM; †Numbers in parentheses represent uninhibited activity in nmol/h per milligram±SEM; ‡Increase in activity (%).
In addition to the release of AA, Ang 1-7 and Ang 1-9 potentiated the release of NO by bradykinin kininase resistant analog (BKan) in human pulmonary arterial endothelial cells. Solid bar: BKan 10 nmol/L. Open bar: BKan+Ang 1-7 10 nmol/L or BKan+Ang 1-9 10 nmol/L. Cross-lined bar: BKan+Ang 1-7 or BK+Ang 1-9, 100 nmol/L. Cross-hatched bar: BKan+Ang 1-7 or Ang 1-9, 100 nmol/L. Ordinate: NO nmol per 3 minutes. Ang 1-7 and Ang 1-9 released little NO in the absence of BK.

In HPAE cells of the fourth through seventh passages, Ang 1-9 (30 nmol/L and 100 nmol/L) enhanced the activity of 100 nmol/L BKan 2.6-fold and 3.3-fold (n=4), whereas 100 nmol/L Ang 1-7 doubled the effect of BKan. These concentrations are over an order of magnitude below the concentration values of the dose that produces 50% inhibition (IC50) of these two peptides.15,21,25 Consequently, both Ang 1-9 and Ang 1-7 increased the activity of the ACE-resistant B2 ligand at a concentration that would not inhibit ACE significantly in solution. Furthermore, Ang 1-9 is active per se without being converted to Ang 1-7.

**NO Release**

In addition to the release of AA, Ang 1-7 and Ang 1-9 potentiated [Ca2+] elevation by BK (Herbert L. Jackman, unpublished observation, 2001). Consequently, we investigated NO synthesis in HPAE cells; BKan (10 nmol/L) was the ligand (Figure 2). Both Ang 1-7 and Ang 1-9 significantly enhanced NO level in a concentration-dependent manner, from 10 to 100 and to 1000 nmol/L. The corresponding numbers for Ang 1-7 are as follows: a 2-fold enhancement followed by a 2.6- and finally a 4-fold increase in the effect of BKan over that of the peptide added to cells only with medium. Ang 1-9 enhanced BK effects in 10, 100, and 1000 nmol/L concentrations 2.4-, 3.8-, and 5-fold, respectively. These experiments were done in triplicate (n=3) and the results were significantly different from control at P<0.01. Ang 1-9, at 100 and 1000 nmol/L concentrations, was significantly (P<0.05) more active than Ang 1-7 in enhancing BK effect. As in the other studies, in the absence of BK, Ang 1-7 and Ang 1-9 released only trace amounts (10% to 15%) of NO.

**Immunostaining of CATA**

Immunostaining of human paraffin-embedded atrial tissue with IgG fraction of antiserum to rCATA showed strong focal positive staining for CATA in the cytoplasm of myocytes (Figures 3A and 3B), but not in endothelium, mesothelial lining, or smooth muscle in vessel walls. The majority of myocytes stained positive, usually in a perinuclear location.

As a positive control, we used embedded kidney tissues. The kidney stained strongly positive for CATA predominantly in the cytoplasm of cells from the proximal convoluted tubules and also in the lumen of the proximal tubules (not shown). Weaker staining was seen in the cells of the loop of Henle and distal convoluted tubules. The glomeruli, collecting ducts, and blood vessels did not stain.

**Discussion**

Human heart has enzymes, which, by cleaving Ang I, release products that may counteract the actions of Ang II. Ang 1-7 opposes Ang II and enhances the effect of BK, as shown in animal experiments and in tissues.13,14 In cultured cells, Ang 1-7 and Ang 1-9 potentiated BK on its B2 receptor in micromolar concentrations15 if ACE was also expressed. Ang 1-7 was cleaved by the N-domain of ACE, but it only inhibited the C-domain,16 whereas Ang 1-9 inhibited both domains in micromolar concentrations. The enhancement of BK activity by the 2 peptides goes beyond protecting BK against enzymatic breakdown.5-7 as also shown by using BKan, a B2 receptor peptide agonist that is resistant to ACE.7,28 The peptides potentiated the effects of BKan in concentrations lower than their IC50 values (Figure 2), when both ACE and B2 receptor were expressed and their calculated median effective concentrations (EC50) were about 2 orders of magnitude lower than the reported IC50s. These derivatives of Ang I did not act directly on the B2 receptor and, in the absence of BK, had only about 10% to 15% activity, and HOE 140 abolished 80% to 90% of the activity (not shown).

The term used for CATA, lysosomal carboxypeptidase A,24 is not quite correct, because it also cleaves C-terminal basic amino acids,19 just as carboxypeptidase B does. CATA is a serine peptidase with 2 different pH optima. It cleaves peptide bonds best at an acid pH, whereas the esterase and deamidase activities are optimal at neutral pH.19 The enzyme is present in high concentrations, for example, in kidney, urine, placenta, fibroblasts, platelets, brain, and endothelial cells15,34-37 but it generally is unstable in solution above neutrality. As the lysosomal protective protein, it is complexed with neuraminidase and β-galactosidase intracellularly23,24,28 and protects them against breakdown. A lack of this function causes the genetically determined disease, galactosialidosis.38

We purified the enzyme first from human platelets and found it highly concentrated in macrophages and endothelial cells.19,27,36 Besides Ang I and BK, CATA cleaves endothelin fastest below neutrality.36 Because it breaks a C-terminal amide bond at neutral pH, we called it deamidase. Many bio-active peptides have a protected C-terminus amino acid containing CONH2 instead of a –COOH group, but less is known about enzymes that cleave CONH2 bond than about carboxypeptidases that release C-terminal amino acids. Among the other substrates of the CATA-deamidase are oxytocin, substance P, peptides administered against malignant tumors39 and others.40

Lysosomal and cytosolic enzymes either metabolize peptides after their release or are active on cell membranes as cathepsin G is.51 CATA can be stabilized at neutrality by KCl and sucrose42 and was released by epinephrine from platelet granules.19
The activity of Ang 1-9 is not due to conversion to Ang 1-7; the inhibition of Ang I hydrolysis (Table 2) suggests that different enzymes are involved. Besides, Ang 1-9 potentiated BK at a lower concentration than Ang 1-7 in cultured cells. Thus, both derivatives of Ang I augment BK activity on its B2 receptor, probably by combining with the active site of ACE, inducing a conformational change in a heterodimer complex formed by ACE and B2 receptor. Because they augment the release of 3H-AA and NO by BK, both G\textsubscript{i} and G\textsubscript{q} proteins are involved in the process.

Human heart tissues may counteract the effects of renin by cleaving Ang I to derivatives not converted by ACE or chymase to Ang II or by breaking down the generated Ang II further. Ang 1-7 has its own receptor in some tissues. Inhibitors of ACE or both ACE and neprilysin (eg, omapatrilat) may enhance the release of Ang 1-9 by raising Ang I concentration. Heart tissue is rich in CATA but also contains other enzymes that cleave Ang I at His9-Leu, including a carboxypeptidase A-type enzyme, mast cell carboxypeptidase, and ACE II. The pH optimum for CATA peptidase is at pH 5.5 with short synthetic substrates, but with longer active peptides, the pH curve is not sharp. Substantial activity is present at neutral pH as with other enzymes.

In conclusion, the two derivatives of Ang I, Ang 1-9 and Ang 1-7, liberated by enzymes in heart tissues, can enhance the local effects of kinins by augmenting NO and arachidonic acid release. In that respect, Ang 1-9 was more potent than Ang 1-7 in the tests employed. Human heart tissues have a repertoire of enzymes that, besides converting Ang I to Ang II, can release potential antagonists of Ang II or liberate Ang II from Ang I stepwise, and they could be of special importance with the use of ACE and combined ACE-neprilysin inhibitors.

Acknowledgments
These studies were partially supported by the National Heart, Lung, and Blood Institute grants HL36473 and HL58118, and by Program Project HL60678. We are grateful for the advice of Dr. Peter Deddish and for the editorial assistance of Ms. Sara Bahnmaier.

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Hypertension. 2002;39:976-981
doi: 10.1161/01.HYP.0000017283.67962.02

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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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