Release of Angiotensin-(1-7) From the Rat Hindlimb
Influence of Angiotensin-Converting Enzyme Inhibition

Mark C. Chappell, Martina N. Gomez, Nancy T. Pirro, Carlos M. Ferrario

Abstract—The results of recent studies have demonstrated that angiotensin (Ang)-(1-7) contributes to the antihypertensive actions of either combined ACE/Ang II type 1 receptor blockade or ACE inhibition alone. The vasculature is a key site of action for either drug regimen, and evidence favors a local Ang system within these tissues. Because ACE may degrade Ang-(1-7), we determined whether ACE inhibition alters Ang-(1-7) release from the rat hindlimb perfused with Krebs-Ringer buffer containing Ficoll. Ang-(1-7) release averaged 36±13 fmol (period 1, 15-minute collection) and 44±11 fmol (period 2) in the control buffer. The addition of the ACE inhibitor lisinopril to the perfusion buffer augmented levels of Ang-(1-7) in periods 3 (144±39 fmol) and 4 (163±35 fmol; P<0.05 versus 1 or 2, n=8). HPLC and radioimmunoassay of effluent from control or lisinopril treatment demonstrated a single immunoreactive peak with a retention time identical to that of Ang-(1-7). The addition of the neprilysin inhibitor SCH 39370 reduced Ang-(1-7) release in the lisinopril buffer from 177±32 (period 1) and 173±39 (period 2) fmol to 112±24 (period 3) and 87±23 fmol (period 4; P<0.05 versus 1 or 2, n=6). Ang I metabolism in the collected perfusate revealed the formation of Ang-(1-7) that was sensitive only to thimet oligopeptidase inhibition; Ang II generation was not detected. The present study demonstrates the recovery of endogenous Ang-(1-7) from the perfused hindlimb. The release of Ang-(1-7) is significantly influenced by inhibition of ACE, which may reflect both increased substrate (Ang I) levels and reduced metabolism of the peptide. Neprilysin inhibition reduced but did not abolish Ang-(1-7) release, which suggests that other endopeptidases may contribute to the release of the peptide. (Hypertension. 2000;35[part 2]:348-352.)

Key Words: angiotensin-converting enzyme ■ receptors, angiotensin II ■ angiotensin-(1-7)

Our studies demonstrate that angiotensin (Ang)-(1-7) contributes to the antihypertensive actions of either combined ACE/Ang II type 1 receptor blockade or ACE inhibition alone.1–3 The alternative formation of Ang-(1-7) from Ang I catalyzed primarily by the endopeptidase nepri-lysin (EC 3.4.24.11) may be 1 mechanism to offset the increased levels of Ang-(1-7), in periods 3 (144±39 fmol) and 4 (163±35 fmol; P<0.05 versus 1 or 2, n=8). HPLC and radioimmunoassay of effluent from control or lisinopril treatment demonstrated a single immunoreactive peak with a retention time identical to that of Ang-(1-7). The addition of the neprilysin inhibitor SCH 39370 reduced Ang-(1-7) release in the lisinopril buffer from 177±32 (period 1) and 173±39 (period 2) fmol to 112±24 (period 3) and 87±23 fmol (period 4; P<0.05 versus 1 or 2, n=6). Ang I metabolism in the collected perfusate revealed the formation of Ang-(1-7) that was sensitive only to thimet oligopeptidase inhibition; Ang II generation was not detected. The present study demonstrates the recovery of endogenous Ang-(1-7) from the perfused hindlimb. The release of Ang-(1-7) is significantly influenced by inhibition of ACE, which may reflect both increased substrate (Ang I) levels and reduced metabolism of the peptide. Neprilysin inhibition reduced but did not abolish Ang-(1-7) release, which suggests that other endopeptidases may contribute to the release of the peptide. (Hypertension. 2000;35[part 2]:348-352.)

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Studies to date, however, have not investigated the extent to which vascular formation of Ang-(1-7) occurs. In this regard, we determined the dynamics of Ang-(1-7) release and the influence of acute ACE and neprilysin inhibition on the recovery of the peptide from the isolated rat hindlimb perfused with an artificial buffer.

Methods

Animals

Experiments were conducted in male Sprague-Dawley rats (12 to 14 weeks old) obtained from Harlan. Rats were housed in an ALACC-approved facility and maintained on a 12-hour light/dark cycle; the animals had free access to rat chow and water.

Hindquarter Perfusion

The rat hindlimb protocol was based on previous methods used to measure Ang II21,22 and bradykinin23 release. Rats were anesthetized with pentobarbital (60 mg/kg IP) and then laparotomized. After the administration of heparin (1000 U), the vena cava and abdominal aorta were cannulated with PE-100 tubing, the animal was transected above the cannulas, and perfusion was initiated with a Krebs’ bicarbonate buffer containing dextrose and 2% Ficoll 70 (Sigma Chemical Co), pH 7.4, at 37°C. Flow was adjusted (8 to 10 mL/min) with a Rainin peristaltic pump to maintain a perfusion pressure of ~60 mm Hg. Pressure was monitored through a side arm of the
aortic cannula with a transducer (Uniflow Pressure Transducer; Baxter Healthcare Corp). The electronic signal was directed to an analog-to-digital converter for analysis of perfusion pressure as previously described.1,2

**Release Experiments**

After a 60-minute washout period to remove blood contaminants, the venous effluent was collected directly into C18 SepPak columns (Waters) connected to a vacuum manifold system to facilitate the immediate absorption of Ang peptides. For each 15-minute collection period, the columns were replaced and washed with 10 mL of 0.1% trifluoroacetic acid (Pierce Chemical Co), and the Ang peptides were eluted with 5 mL of 80% methanol/0.1% trifluoroacetic acid. Aliquots of the eluent were completely evaporated in a Savant vacuum centrifuge and subjected to radioimmunoassay (RIA) for Ang-(1-7). The Ang-(1-7) RIA recognizes Ang-(1-7) and Ang-(2-7) equally but cross-reacts <0.1% with Ang-(3-7), Ang II, Ang I, or their amino terminal fragments. Sensitivity of the assay is 2.5 fmol/tube. To characterize immunoreactive Ang-(1-7), pooled extracts were evaporated, diluted in 0.5 mL of 20% acetonitrile/0.1% heptfluorobutyric acid (HFBA), and applied to a NovaPak C18 column (2.1x150 mm; Waters). Mobile phase A was 0.1% HFBA, and mobile phase B was 80% acetonitrile/0.1% HFBA. The gradient consisted of 2.5 minutes linear of 20% B, 20 minutes linear of 20% to 40% B, and 10 minutes isocratic of 40% B with a flow rate of 0.35 mL/min. Fractions were collected at 1-minute intervals, evaporated, and analyzed with the use of the Ang-(1-7) RIA. To determine the influence of ACE on the release of Ang-(1-7), lisinopril (1 μmol/L final concentration) was added to the perfusion buffer after the second collection period, and peptide release was determined for 2 additional periods. To characterize the Ang-(1-7)-forming enzymes in the hindlimb, the neprilysin inhibitor SCH 39370 (10 μmol/L) was added to the lisinopril perfusion buffer after the second collection period. Blank immunoreactive levels were determined from the extracted perfusion buffer (alone, lisinopril, or lisinopril/SCH 39370) collected for 15 minutes directly from the pump and then subtracted from the hindlimb samples.

**Peptide Metabolism**

Peptidase activity in the nonextracted perfusate was determined on the basis of the metabolism of 125I-Ang I. Briefly, the iodinated peptide (0.2 pmol) was incubated with 0.2 mL of the perfusate in the absence or presence of inhibitors for 60 minutes at 37°C. Samples were diluted in 0.5% phosphoric acid, and the metabolites analyzed with the use of HPLC with a 0.1% phosphoric acid/acetonitrile solvent system.13

**Statistical Analysis**

Statistical differences in peptide release resulting from experimental manipulations were evaluated with 1-way ANOVA followed by Newman-Keuls post hoc test. For the metabolism study, the effects of peptidase inhibitors were assessed with the use of paired Student’s t test. Data analysis was performed with the statistical and graphics program Prism (GraphPAD Software). All data are presented as mean±SEM, and the criterion for statistical significant was set at P<0.05.

**Results**

In Figure 1, we show the time course for the release of Ang-(1-7) from the perfused hindlimb in buffer alone (top) or after the addition of the ACE inhibitor lisinopril (bottom). The release of Ang-(1-7) ranged from 24 to 45 fmol over the four 15-minute collection periods (n=4). The addition of the ACE inhibitor lisinopril (1 μmol/L) after the second collection period (buffer alone) caused an immediate increase in the recovery of Ang-(1-7) from the perfusate. In the presence of lisinopril, the rate of Ang-(1-7) release increased ~5-fold (35±13 fmol for period 1 versus 163±35 fmol for period 4,

![Figure 1](http://hyper.ahajournals.org/)

![Figure 2](http://hyper.ahajournals.org/)

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Figure 1. Time course for release of Ang-(1-7) from perfused hindlimb in absence (top) or presence (bottom) of ACE inhibitor lisinopril. Arrow indicates start of perfusion with buffer containing 1 μmol/L lisinopril. Values are mean±SEM (n=4 for control and n=8 for lisinopril experiments). *P<0.05 compared with preceding periods 1 or 2.

To begin to characterize the identity of the enzymes that form Ang-(1-7) in the hindlimb, enzyme inhibitors were
added to the perfusion buffer containing lisinopril (1 μmol/L) after the second collection period. In Figure 3 (top), constant perfusion with the ACE inhibitor maintained an elevated level of Ang-(1-7) throughout the 60-minute collection period (210±25 fmol for period 1 versus 165±47 fmol for period 4, n=4). We and others have previously reported that neprilysin inhibitors, including SCH 39370, reduce (>60%) circulating levels of Ang-(1-7).1,2,4 As shown in Figure 3 (bottom), treatment with the neprilysin inhibitor SCH 39370 (10 μmol/L) diminished but did not abolish the release of Ang-(1-7) (177±32 fmol for period 1 versus 87±23 fmol for period 4, n=6).

We also assessed the ex vivo metabolism of radiolabeled Ang I in control (no inhibitors) or lisinopril buffer (1 μmol/L) collected directly from the hindlimb. As shown in Figure 4, chromatographic analysis revealed the conversion of Ang I to Ang-(1-7) after a 60-minute incubation period. The rate of Ang-(1-7) formation in the perfusate was low, averaging 15 fmol·mL⁻¹·h⁻¹, and represented 10% to 12% conversion of Ang I. Characterization of the Ang-(1-7)-forming activity was determined by the addition of selective endopeptidase inhibitors (10 μmol/L each) against neprilysin (SCH 39370), thimet oligopeptidase (c-phenylpropyl-alanine-alaninoephylalanine-p-aminobenzoate [AAF]), or prolyl oligopeptidase (z-prolyl-prolinal). Although all 3 endopeptidases hydrolyze Ang I directly to Ang-(1-7), only the thimet oligopeptidase agent AAF inhibited Ang-(1-7) formation (>85%, Figure 3 and inset, n=3). Consistent with inhibitory effects of AAF and sensitivity to thiol inhibitors,25 p-chloromercuriophenylsulfonate (0.5 mmol/L) also effectively abolished Ang-(1-7) formation (>90%, n=3). Although not shown, metabolism of Ang I in the perfusate containing lisinopril was essentially identical to that of the perfusate alone. Furthermore, we did not detect the formation of Ang II from Ang I, suggesting the absence of ACE (or other Ang II–forming enzymes) in the collected perfusate without lisinopril or containing the 2 inhibitors that blocked Ang-(1-7) formation (Figure 3).

### Discussion

In the present study, we characterized the release of Ang peptides from the rat hindlimb and demonstrated that Ang-(1-7) is a component of a local Ang system that may consist of both vascular and extravascular sites. Our findings also demonstrate that the recovery of Ang-(1-7) release in the hindlimb is augmented by the addition of an ACE inhibitor to the perfusion buffer. These results imply that ACE has a significant influence on the regulation of Ang-(1-7) release, most likely through the direct metabolism of the peptide. Chappell et al11 and Deddish et al26 demonstrated that Ang-(1-7) exhibits a high affinity for ACE and is cleaved to the pentapeptide Ang-(1-5). Moreover, chronic ACE inhibition increases the half-life of circulating Ang-(1-7) by >6-fold and augments endogenous levels of the peptide.14 Recent studies have also reported the release of kinins from the perfused hindlimb; the addition of captopril and other peptidase inhibitors substantially augmented kinin release.23,27 Vascular accumulation of both bradykinin and Ang-(1-7), as well as the reduction in Ang II after ACE inhibition, may contribute to the beneficial effects of this therapeutic agent. That ACE inhibition augments both Ang-(1-7) and bradykinin is of particular interest given the recent observations that these peptides exhibit synergistic actions to induce vasodilation or lower blood pressure.28-31
The recovery of Ang-(1-7) from the hindlimb perfused with buffer lacking the precursor components renin and angiotensinogen is consistent with previous studies demonstrating vascular release of Ang II and Ang I. Although the rate of Ang-(1-7) release is significantly less than that of Ang II and Ang I originally reported by Mizuno et al. for the rat hindlimb, our findings are more comparable to the recent data of Hilgers et al. for both Ang I and Ang II. In addition, Hilgers et al. demonstrated a 10-fold increase in Ang I release to ~800 fmol with the chelating agent phenanthroline. This agent had minimal effects on the clearance of exogenous Ang I or Ang II, suggesting a greater inhibitory action on tissue versus luminal peptidases. In view of the present results, the actions of phenanthroline to augment Ang I in the study by Hilgers et al. may include inhibition of Ang II as well as that of Ang-(1-7) formation; ACE, neprilysin, and thimet oligopeptidase are metalloendopeptidases and are sensitive to chelating agents. In addition, Mizuno et al. report that acute ACE inhibition alone increased hindlimb release of Ang I to ~880 pg (680 fmol) and that chronic administration augmented levels to 1700 pg (1300 fmol). Although we did not determine Ang I levels, the results of these previous studies suggest that the increased levels of the decapeptide after ACE inhibition may well contribute to Ang-(1-7) release. The inhibition of Ang-(1-7) release with SCH 39370 is consistent with several studies that demonstrate a reduction in circulating levels of Ang-(1-7) with various neprilysin inhibitors. Although the kidney contains the highest concentration of neprilysin localized primarily to the proximal tubules, the ectopeptidase is expressed on the luminal endothelium. Thus, neprilysin is appropriately situated to contribute to the hydrolysis of Ang I within the vasculature. The analysis of the hindlimb perfusate for hydrolysis of radiolabeled Ang I did not reveal the formation of Ang II. These data suggest the absence of ACE or other Ang II–forming enzymes that may be released during the perfusion experiments. The metabolism studies did reveal in vitro generation of Ang-(1-7) from Ang I in the collected perfusate. Not surprisingly, SCH 39370 failed to inhibit Ang-(1-7) formation. Neprilysin is anchored to the plasma membrane, and very low levels of enzyme activity are evident in the circulation. In contrast, both thimet oligopeptidase and thiol peptidase inhibitors essentially abolished Ang-(1-7) generation. Thimet oligopeptidase inhibitors were reported to lower blood pressure through a reduction in kinin metabolism, but subsequent studies suggest that the antihypertensive actions are more likely due to metabolism of the peptide inhibitor and blockade of ACE. We previously found that thimet oligopeptidase was responsible for the direct formation of Ang-(1-7) from Ang I in vascular smooth muscle cells from spontaneously hypertensive and Wistar-Kyoto rats. Lasdun et al. reported significant levels of serum thimet oligopeptidase and that the peptidase inhibitor AAF increased the half-life of circulating luteinizing hormone-release hormone. Although the presence of thimet oligopeptidase in the perfusate may reflect leakage from cellular damage, active secretion of the enzyme was recently demonstrated. It is not known whether this endopeptidase contributes to Ang-(1-7) in the perfusate after neprilysin inhibition in the hindlimb or to circulating levels of Ang-(1-7) in the plasma. Moreover, the involvement of thimet oligopeptidase raises the issue that extravascular or interstitial sites contribute to the processing and release of Ang-(1-7). Additional studies with the thimet oligopeptidase inhibitor are required to determine whether the enzyme participates in the vascular release of Ang-(1-7).

In conclusion, the present study demonstrates the recovery of endogenous Ang-(1-7) from the perfused hindlimb. The release of Ang-(1-7) is significantly influenced by inhibition of ACE, which may reflect increased substrate (Ang I) levels, and reduced metabolism of the peptide. Our study provides additional insight into the local generation of Ang peptides and the potential contributions of Ang-(1-7) to the antihypertensive and antiproliferative mechanisms of ACE inhibition.

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References


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