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Original Contributions

Angiotensin-(1–7)
A Member of Circulating Angiotensin Peptides

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We measured the concentrations of three principal products of the renin-angiotensin system and seven of their metabolites in the plasma of anesthetized normal dogs and in dogs 24 hours after bilateral nephrectomy. The levels of the angiotensin peptides were measured by high-performance liquid chromatography combined with radioimmunoassay using three specific antibodies that recognized different epitopes in the sequences of angiotensin I, angiotensin II, and angiotensin-(1–7). The analysis revealed that angiotensin-(1–7) is present in the plasma of intact (4.9±2.2 fmol/ml) and nephrectomized (0.5±0.5 fmol/ml) dogs. An intravenous injection of purified hog renin (0.01 Goldblatt unit/kg) increased plasma levels of angiotensin I, angiotensin II, and angiotensin-(1–7) both before and after nephrectomy. These changes were associated with parallel increases in the concentrations of fragments of the three parent peptides. Administration of MK-422 led to the disappearance of circulating angiotensin II and its fragments both before and after a second injection of the same dose of renin. In contrast, MK-422 augmented the plasma levels of both angiotensin I and angiotensin-(1–7). The concentrations of these two peptides, but not the blood pressure, were again augmented by a second injection of renin given after blockade of converting enzyme. These effects were observed both before and after bilateral nephrectomy. These findings show that angiotensin-(1–7) circulates in the blood of normal and nephrectomized dogs. In addition, we found that angiotensin-(1–7) is generated in the blood from the cleavage of angiotensin I through a pathway independent of converting enzyme (EC 3.4.15.1). (Hypertension 1991;17:131–138)

Our laboratory has shown that the amino terminal heptapeptide angiotensin-(1–7) or des-[Phe8]-angiotensin II [Ang-(1–7)] is a bioactive component of the renin-angiotensin system. This finding suggests that angiotensin receptors may bind congeners of angiotensin II (Ang II) that lack an amino acid in the eight position. Ang-(1–7) was first shown to display the same potency as Ang II as a stimulus for the secretion of vasopressin. Furthermore, this amino terminal heptapeptide stimulated the activity of neurons both in vivo and in vitro. Immunocytochemical studies showed that Ang-(1–7) is localized to regions of the rat hypothalamus and neurohypophysis that participate in the synthesis and secretion of vasopressin. In this species, we also found that the endogenous content of Ang-(1–7) in hypothalamic tissue was as high as that measured for either angiotensin I (Ang I) or Ang II. In additional studies, Chappell et al found that at least one neuronal cell line processed Ang I into Ang-(1–7) in a preferential manner. Preliminary evidence for the presence of Ang-(1–7) outside the central nervous system was obtained in both rats and dogs. In one study, the heptapeptide was identified in the venous blood that emerged from the coronary sinus of the dog. In another study, Ang-(1–7) was found in the blood obtained from the trunks of decapitated rats.

These data led us to investigate basic conditions that may influence the presence of Ang-(1–7) in the peripheral circulation. With this in mind, we evaluated the action of exogenous renin on the endogenous production of circulating Ang-(1–7) in dogs before and after blockade of angiotensin converting enzyme. Plasma levels of Ang-(1–7) and other angiotensin peptides were determined by a combined high-performance liquid chromatography (HPLC)
and radioimmunoassay (RIA) in normal dogs and in dogs 24 hours after bilateral nephrectomy (BNX).

Methods

Five male mongrel dogs (20–22 kg body wt) were housed individually for 3 consecutive weeks and were fed a solid diet (Purina Dog Chow, Fetter Brother, Bedford, Ohio) that provided a daily intake of 65 meq sodium and 65 meq potassium. Water was given ad libitum.

Experimental Protocols

Experiment 1. Dogs were anesthetized with sodium pentobarbital (25 mg/kg i.v.); respiration was assured mechanically via a tube placed into the trachea. Under sterile conditions, plastic catheters (16 gauge Angiocaths, Deseret Medical Inc., Sandy, Utah) were inserted percutaneously into a femoral artery and a forearm vein. After a 1-hour stabilization period, the dogs were injected with 0.01 Goldblatt unit/kg of purified hog renin (Sigma Chemical Co., St. Louis, Mo.). Serial samples of blood (12 ml) were obtained from the arterial cannula before and again at 10 and 60 minutes after the injection of renin. After this time, the dogs received a bolus injection of a converting enzyme inhibitor (Enalaprilat [MK-422] 10 mg/kg, Merck Co., West Point, Pa.). A second injection of renin was given 1 hour after blockade of angiotensin converting enzyme. Additional samples of arterial blood were obtained before and after these treatments. At the completion of the experiments, catheters were withdrawn and the animals were returned to the kennel. Antibiotics were given to prevent infections.

In each experiment, no more than 60 ml blood was removed from each dog. Amounts of blood drawn for assays were replaced with equivalent injections of saline. The dose of converting enzyme inhibitor used in these experiments was shown to inhibit the generation of Ang II, both in this and in previous experiments.1-9,10

Experiment 2. Six days after experiment 1, both kidneys were removed from dogs in which anesthesia was induced with thiomyal sodium (Surital, 10 mg/kg i.v., Parke-Davis, Morris Plains, N.J.) and maintained with 2% halothane. The procedure of bilateral nephrectomy was done via flank incisions as described elsewhere.11 The protocol described above was repeated 24 hours after performing the BNX.

Biochemical Assays

Blood samples were assayed for plasma renin activity (PRA) and plasma concentrations of angiotensinogen (Aogen), Ang I, Ang II, and Ang-(1–7). Samples for the assay of PRA and Aogen concentrations were collected in NH4EDTA (25 mM), o-phenanthroline (0.44 mM), and pepstatin A (0.12 mM final concentration, Sigma). In previous studies, we found that this cocktail prevents the in vitro metabolism of Ang I during manipulation of the sample.12 Blood was centrifuged at 3,000g for 30 minutes at 4°C; the plasma was then stored at −20°C. As reported in a previous experiment,9 the plasma was extracted on a Sep-Pak C18 column (Waters Instruments, Milford, Mass.). The efficiency of the extraction procedure was evaluated by addition of 5–30×103 cpm of either 125I-Ang I, 125I-Ang II, or 125I-Ang-(1–7) to plasma samples before extraction. Average recovery values after HPLC from eight experiments are 74±4% for 125I-Ang I, 84±4% for 125I-Ang II, and 79±3% for 125I-Ang-(1–7). In previous studies,7,13,14 we showed that iodinated angiotensin peptides showed recoveries that are similar to those obtained with unlabeled angiotensins as determined by RIA after extraction and HPLC separation.

Details of the technique that allows analysis of 10 angiotensin peptides using three separate antisera are published.7 The procedure developed in our laboratory,7,13 entails separation of angiotensin peptides by HPLC and subsequent quantification by RIA. Angiotensin peptides were eluted by a modified reversed-phase HPLC method. Peptides were fractionated on an LKB HPLC system (Pharmacia LKB Biotechnology Inc., Piscataway, N.J.) with a Nova-Pak C18 column (3.9×150 mm, Waters). The mobile phase comprised 0.13% heptafluorobutyric acid (HFBA) (Sequanol Grade, Pierce Chemical Co., Rockford, Ill.) vol/vol in water (Buffer A) and 80% acetonitrile (Burdick and Jackson, Muskegon, Mich.) in 0.13% HFBA (Buffer B). The column was eluted with a 32–50% convex gradient of Buffer B (curve 13 of the LKB Programmable Gradient Controller) for 30 minutes at a flow rate of 1 ml/min. Samples were collected every 12 seconds and were evaporated in a vacuum centrifuge (Savant Instruments, Inc., Farmingdale, N.Y.) before RIA. Standard retention times for synthetic peptides were established with angiotensins provided by either M.C. Khosla (Cleveland Clinic Foundation, Cleveland, Ohio) or purchased from Peninsula Laboratories, Belmont, Calif. HPLC fractions between 4 and 9 minutes (fractions 20–45), 9 and 16 minutes (fractions 46–80), and 16 and 23 minutes (fractions 81–115) were assayed for immunoreactive levels of Ang-(1–7), Ang II, and Ang I, respectively. Figure 1 illustrates the profile of angiotensin peptides present in dog plasma after an intravenous injection of renin and after blockade of angiotensin converting enzyme.

Three separate antisera that recognize a different epitope of the angiotensin molecule were used as described elsewhere.7,9 For measurements of Ang I and Ang II immunoreactivity, evaporated HPLC fractions were reconstituted in RIA buffer (100 mM Tris, pH 7.4, 0.15 mM EDTA, 0.1% lysozyme) and were assayed as described previously.7 The Ang I antibody (New England Nuclear, Boston, Mass.) has a 100% cross-reactivity with Ang I, Ang-(2–10), and
Ang-(3–10), but the cross-reactivity of this antibody with either Ang II or Ang-(1–7) averaged less than 0.1%. In these experiments, sensitivity of the Ang I RIA was 5 fmol/tube. The Ang II antibody (CDI-4) was obtained in the laboratory. This antibody cross-reacts 100% with Ang II and its C-terminal fragments but showed less than 0.01% binding with either Ang I or Ang-(1–7) fragments. The sensitivity of the Ang II RIA averages 0.5 fmol/tube.

Samples for Ang-(1–7) RIA were reconstituted in 600 μl of a 25 mM Tris buffer solution (pH 7.4) that contained 50 mM NaCl and 0.1% bovine serum albumin. 125I-Ang-(1–7) was diluted with Tris buffer to a final concentration of 6,000 cpm/100 μl. The antibody for Ang-(1–7) was obtained from New Zealand White rabbit (rabbit D, bleed 11.07.88) in the laboratory. The antiserum was diluted 1:64,000 in 25 mM Tris-buffer, pH 7.4. The assay solution consisted of either 600 μl of standard Ang-(1–7) in Tris buffer (0–2,000 fmol) or 600 μl of unknown reconstituted sample, 100 μl of 125I-Ang-(1–7), and 100 μl of diluted antibody. The solution was incubated for 20 hours at 4°C. Separation of the bound and free radioligand was done by adding 1 ml dextran-coated charcoal (250 mg charcoal, 25 mg dextran in 100 ml of 0.1 M Tris buffer, pH 7.4). After a 20-minute centrifugation at 4°C, the supernatant was separated for counting. The Ang-(1–7) RIA showed a 100% cross-reactivity with Ang-(1–7), Ang-(2–7), and Ang-(3–7), whereas it showed less than 0.01% cross-reactivity with either Ang I or Ang II. The sensitivity of this assay is 4 fmol/tube.

Data Analysis

Data are mean±1 SEM. Differences resulting from drug treatments were evaluated by one-way analysis of variance followed by Duncan’s multiple range test. Comparison between experimental conditions were done by two-way analysis of variance. The Student’s t test for paired data was applied when applicable. The criterion for statistical significance is p<0.05.

Results

Effects of Renin and MK-422 on Blood Pressure and Heart Rate

Baseline values of mean arterial pressure and heart rate were comparable in intact and 24-hour BNX dogs (Figure 2). An injection of renin elevated mean arterial pressure more in BNX than in intact dogs. The pressor response was accompanied by significant bradycardia only after BNX (p<0.01). Administration of MK-422 decreased mean arterial pressure both before and after BNX (Figure 2). Although tachycardia of a transient nature accompanied the fall in arterial pressure in animals with intact kidneys, cardiac rate did not change in BNX dogs. A second injection of renin administered 60 minutes after treatment with MK-422 caused no changes in either mean arterial pressure or heart rate in either intact or BNX dogs (Figure 2).

Effects of Renin and MK-422 on Endogenous Levels of Renin Activity and Angiotensin Peptides

Circulating levels of PRA rose to similar values in intact and BNX dogs after an injection of renin.
Effects of Renin and MK-422 on Circulating Levels of Angiotensin Fragments

Plasma from intact but not BNX dogs contained C-terminal fragments of Ang I and Ang II (Figure 4). Two Ang-(1–7) fragments, Ang-(2–7) and Ang-(3–7), were found in the plasma of BNX dogs at concentrations that did not differ from those measured in the same animals before removal of the kidneys. Ten minutes after the first injection of renin, circulating levels of Ang I and Ang II fragments were significantly elevated in both intact and BNX dogs (Figure 4). However, the injection of renin did not significantly augment production of Ang-(1–7) fragments in the plasma of either normal or BNX dogs (Figure 4).

Blockade of angiotensin converting enzyme resulted in the disappearance of Ang II fragments in the blood of both normal and BNX dogs. No further
TABLE 1. Baseline Plasma Concentrations of Angiotensin Peptides

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>Before MK-422</th>
<th>After MK-422</th>
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</thead>
<tbody>
<tr>
<td>Plasma renin activity</td>
<td></td>
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<tr>
<td>Intact</td>
<td>7.5±2.6</td>
<td>6.7±1.5</td>
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<td>5.9±1.5</td>
<td>2.2±0.3*</td>
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<td>p&lt;</td>
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<td>NS</td>
<td>0.01</td>
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<td>Plasma angiotensinogen</td>
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<tr>
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<td>350±25</td>
<td>344±35</td>
<td>346±30</td>
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<tr>
<td>Nephrectomy</td>
<td>509±38</td>
<td>514±23</td>
<td>457±29*</td>
</tr>
<tr>
<td>p&lt;</td>
<td>0.01</td>
<td>0.01</td>
<td>0.05</td>
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<tr>
<td>Plasma Ang I (fmol/ml)</td>
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<tr>
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<td>133±30</td>
<td>122±38</td>
<td>177±49*</td>
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<tr>
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<td>3±2</td>
<td>86±8</td>
<td>40±5*</td>
</tr>
<tr>
<td>p&lt;</td>
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<td>NS</td>
<td>NS</td>
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<tr>
<td>Plasma Ang II (fmol/ml)</td>
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<td>Intact</td>
<td>63±25</td>
<td>82±35</td>
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<tr>
<td>Nephrectomy</td>
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<td>66±31</td>
<td>0.8±0.8</td>
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<tr>
<td>p&lt;</td>
<td>0.05</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Plasma Ang-(1-7) (fmol/ml)</td>
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<td></td>
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</tr>
<tr>
<td>Intact</td>
<td>4.9±2.2</td>
<td>8.5±4.2</td>
<td>23.5±7.4*</td>
</tr>
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<td>Nephrectomy</td>
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<td>NS</td>
<td>0.05</td>
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<td>Plasma Ang III (fmol/ml)</td>
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<tr>
<td>Nephrectomy</td>
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<td>1.5±0.7</td>
<td>ND</td>
</tr>
<tr>
<td>p&lt;</td>
<td>NS</td>
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<td>Plasma Ang-(2-7) (fmol/ml)</td>
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<td>Nephrectomy</td>
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<td>1.0±0.6</td>
</tr>
<tr>
<td>p&lt;</td>
<td>NS</td>
<td>NS</td>
<td>0.05</td>
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Values are mean±SEM of baseline determination in five intact and nephrectomized dogs. Control values are averages obtained before any injection of renin. Before MK-422 values are measurements obtained 60 minutes after the injection of renin and just before administration of the angiotensin converting enzyme inhibitor. After MK-422 values are averages obtained 60 minutes after the injection of the blocker and just before a second injection of renin. Probability values shown in the table denote statistical significance of differences between intact and nephrectomized animals as determined by two-way analysis of variance for repeated measures. ND, not detectable; NS, not statistically significant.

changes were elicited by a second injection of renin 60 minutes after MK-422 administration (Figure 4). In contrast, MK-422 did not alter the concentrations of Ang-(2-7) and Ang-(3-7), whereas a second renin injection augmented the concentration of Ang-(3-7) (Figure 4). The levels of Ang-(1-7) fragments generated by a second injection of renin were of a comparable magnitude both before and after removal of the kidneys.

Discussion

We have used an in vivo "renin bioassay" and precise and sensitive RIA procedures combined with HPLC to readdress the question of what are the products of Ang I that are formed by the endogenous metabolism of Angogen by renin in the dog. In agreement with previous studies,9 we confirmed that the combination of HPLC with RIA makes it possible to identify femtomole quantities of 10 angiotensin peptides in the plasma. In addition, we found Ang-(1-7) and two of its congeners in the plasma obtained from either normal or BNX dogs. Injection of renin stimulated the generation of Ang-(1-7) both before and after administration of MK-422. This finding agrees with previous studies,18,10 which showed that Ang-(1-7) may be produced through an enzymatic pathway that is independent of angiotensin converting enzyme.

The combination of HPLC separation with three kinds of RIAs is a necessary strategy for the accurate measurement of plasma and tissue concentrations of angiotensin peptides. Both we13 and Nussberger et al15 have called attention to the problems that arise from the insufficient specificity of the direct RIA. Cross-reactivity of the antibodies with small angiotensin fragments overestimates concentrations of the
parent peptides. This limitation is especially important when enzymatic inhibitors are used to assess specific blockade of bioactive products that result from the hydrolysis of Ang I. We have shown before that the procedures used in these experiments measure authentic concentrations of angiotensin fragments.7,13 These procedures allowed the determination of the relative proportions of the fragments in relation to their respective parent peptides. We also took the precaution of collecting the blood in the presence of a cocktail of inhibitors that included pepstatin. From current studies that include measurements of the in vitro metabolism of labeled Ang I, we learned that the chelating agents NH4-EDTA and o-phenanthroline, alone or in combination, do not inhibit a time-dependent metabolism of Ang I during blood withdrawal and sample preparation (unpublished observations from our laboratory). In addition, we found that collection of blood in the presence of pepstatin prevents induction of values of Ang I higher than those found in the circulation.12

Our studies demonstrated that Ang-(1-7) circulates in the plasma of both normal and BNX dogs although at low concentrations. These findings agree with a previous report by Santos et al,9 who first detected the presence of immunoreactive Ang-(1-7) across the coronary circulation of the canine heart. But tissues rather than blood may be the major source of Ang-(1-7). For example, in the brain of rats Chappell et al7 showed that the endogenous levels of Ang-(1-7) were as high as those of Ang I and Ang II. From other studies, we learned that Ang-(1-7) is also produced in large concentrations during incubation of labeled Ang I with a neuroblastoma×glioma hybrid cell line.8 The differences in the relative concentrations among Ang-(1-7), Ang I, and Ang II in both tissue and plasma indicate that the circulatory system is not the sole source for the generation of Ang-(1-7). This interpretation is compatible with a previous hypothesis, which suggested that Ang-(1-7) functions as a paracrine hormone of the renin-angiotensin system.1

We confirmed that Ang I and Ang II are the major circulating components of the renin-angiotensin sys-
tem. In the intact dogs, the concentrations of Ang II averaged 47% of the prevailing levels of Ang I. In contrast, Ang-(1–7) circulated in concentrations that amounted to 4% of those measured for Ang I. According to Nussberger et al,\textsuperscript{15} the ratio between the concentrations of the prohormone (Ang I) and peptide product provides an estimate of processing. In normal animals, we calculated that the Ang I/Ang-(1–7) ratio averaged 27:1 (measured in femtomoles). After nephrectomy, this ratio decreased to 6:1. In other words, the small but detectable amounts of Ang-(1–7) circulating in the plasma of the nephrectomized dogs now represented a larger fraction of the prevailing concentrations of the parent substrate Ang I. Corresponding values for the Ang I/Ang II ratio were 2:1 in intact dogs and less than 1:1 after nephrectomy. Blockade of converting enzyme increased the concentrations of Ang I and Ang-(1–7), and it suppressed the formation of Ang II. In this condition, the Ang I/Ang-(1–7) ratio decreased from 14:1 to 7.5:1 in normal dogs and from 22:1 to 16.7:1 in BNX dogs. These data suggest that blockade of converting enzyme increases conversion of Ang I into Ang-(1–7). These conclusions agree with studies that showed that Ang-(1–7) is produced directly from Ang I.\textsuperscript{8,10}

Intravenous injections of renin were used to stimulate the endogenous production of Ang I and to evaluate whether these changes were associated with increased formation of Ang-(1–7). The data clearly demonstrate that Ang-(1–7) may be formed endogenously by conditions that accelerate production of Ang I and Ang II, both in the presence and absence of the kidneys. As expected, the pressor response to renin was augmented in animals without kidneys.\textsuperscript{16} This was associated with higher circulating levels of Ang II and its C-terminal congeners fell to levels that were at or below the detectable level of the RIA. This finding confirms that renal renin contributes to the production of angiotensin peptides in the blood.\textsuperscript{15} However, among other factors the disappearance of angiotensin peptides from the circulation may be a time-dependent phenomena. Other studies showed that Ang II may accumulate in the blood of animals 48 hours after removal of both kidneys.\textsuperscript{11} The studies also showed that nephrectomy had a smaller effect on the plasma concentrations of Ang-(1–7) and its fragments Ang-(2–7) and Ang-(3–7). This finding suggests, but obviously does not demonstrate, that tissues other than the kidneys may be important sites for the generation and processing of amino terminal–derived angiotensin peptides.

A second challenge with renin in the presence of angiotensin converting enzyme inhibition augmented the production of both Ang I and Ang-(1–7). However, it did not cause the reappearance of Ang II or its C-terminal fragments in plasma. Therefore, at least two distinct enzyme activities are involved in the production of Ang II and Ang-(1–7). Our experiments confirm that angiotensin converting enzyme is directly involved in the generation of Ang II, but we now also show that Ang-(1–7) is produced in vivo in the presence of MK-422. A similar finding was reported by Santos et al\textsuperscript{10} in homogenates of the canine medulla oblongata. Enzymes capable of cleaving the Pro\textsuperscript{3}–Phe\textsuperscript{4} bond of Ang I include prolylendopeptidase (E.C. 3.4.21.26),\textsuperscript{18} neutral endopeptidase 24.11 (E.C. 3.4.24.11),\textsuperscript{19} and membrane-bound proline endopeptidase.\textsuperscript{20} Angiotensinase C\textsuperscript{21} and prolyl endopeptidase also hydrolyze the Pro\textsuperscript{3}–Phe\textsuperscript{4} bond of Ang II. Z-pro-prolinal is a specific inhibitor of the activity of prolyl-endopeptidase,\textsuperscript{22} an enzyme that is found in high concentrations in the brain and kidneys.\textsuperscript{23} Welches et al\textsuperscript{24} and Chappell et al\textsuperscript{8} found that Z-pro-prolinal inhibited the conversion of Ang I to Ang-(1–7) in both canine hypothalamic homogenates and a neuronal cell line, respectively. In the more complete studies of Chappell et al,\textsuperscript{8} we found that the formation of Ang-(1–7) from labeled Ang I was only partially prevented by the addition of Z-pro-prolinal to NG108-15 neuroglioma cells in culture. The formation of Ang-(1–7) in this preparation was abolished in the presence of p-chloromercuriphenylsulfonate, an inhibitor of sulfhydryl containing proteases.\textsuperscript{8} These data suggest the existence of more than one enzymatic activity in the processing of Ang I into Ang-(1–7). Because blockade of angiotensin converting enzyme did not decrease the circulating levels of Ang-(1–7), either before or after stimulation of Aogen metabolism by renin, the data confirm that the enzymatic pathway that accounts for the production of Ang-(1–7) does not require prior formation of Ang II. Indeed, the increases in the concentrations of Ang-(1–7) that occurred after the injection of MK-422 suggest the existence of alternate processing pathways for the production of angiotensin peptides. A series of studies from our laboratories\textsuperscript{1} has determined the biological importance of Ang-(1–7) in the control of autonomic function and vasopressin secretion. Evidence for the presence of an angiotensin-type receptor that displays a high affinity for Ang-(1–7) was found in the adrenal cortex and in the nuclei of the dorsal vagal-solitarii complex that mediate changes in the sensitivity of the baroreceptor reflexes.\textsuperscript{25} In keeping with this observation, microinjections of Ang-(1–7) into the same brain region elicited depressor responses of a magnitude comparable with those obtained with Ang II.\textsuperscript{26} In addition, recent studies demonstrated that Ang-(1–7) is a potent stimulus for the release of prostaglandins in the rabbit vas deferens\textsuperscript{27} and rat C-6 glioma cells\textsuperscript{28} at concentrations that are comparable with those of Ang II. These findings established the basis for further investigations of the nature of conditions that account for the production of Ang-(1–7) in both blood and tissues.

In summary, the present experiments show that Ang-(1–7) is an endogenous component of the circulating renin-angiotensin system in the dog. The existence of the peptide in concentrations that are not
suppressed by blockade of angiotensin converting enzyme indicates alternative routes for the production and processing of Ang I. These data add credence to a revised concept of the renin-angiotensin system in which the processing of Ang I into a family of bioactive angiotensin peptides occurs by parallel cleaving activities of converting enzyme and prolylendopeptidase.1

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