In Vivo Metabolism of Angiotensin I by Neutral Endopeptidase (EC 3.4.24.11) in Spontaneously Hypertensive Rats

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We investigated the processing enzymes involved in the formation of circulating angiotensin-(1-7) after intravenous administration of angiotensin I to conscious spontaneously hypertensive and Wistar-Kyoto rats. Immunoreactive products, including angiotensin I, angiotensin II, and angiotensin-(1-7), were measured in arterial blood by three specific radioimmunoassays. Angiotensin I infusion (2 nmol) induced a rapid increase in immunoreactive angiotensin II and angiotensin-(1-7). Pretreatment with the angiotensin converting enzyme inhibitor enalaprilat (2 mg/kg) eliminated angiotensin II formation and augmented circulating levels of angiotensin I and angiotensin-(1-7) in spontaneously hypertensive and Wistar-Kyoto rats. The elevated levels of angiotensin-(1-7) in enalaprilat-treated rats were blocked by concurrent treatment with the neutral endopeptidase (EC 3.4.24.11) inhibitor SCH 39,370 (15 mg/kg) in both strains. Administration of SCH 39,370 alone decreased angiotensin-(1-7) levels in spontaneously hypertensive rats not given any of the enzyme inhibitors. In addition, levels of angiotensin I were higher after administration of SCH 39,370 in hypertensive rats. These novel findings reveal that neutral endopeptidase EC 3.4.24.11 participates in the conversion of angiotensin I to angiotensin-(1-7) and in the metabolism of angiotensin II in the circulation of both spontaneously hypertensive and Wistar-Kyoto rats. Our results suggest that neutral endopeptidase EC 3.4.24.11 is a major enzymatic constituent of the circulating renin-angiotensin system. (Hypertension 1992;19:692-696)

KEY WORDS • angiotensin I • angiotensin II • angiotensin converting enzyme • enalaprilat • enzyme inhibitors • peptide peptidohydrolases • spontaneously hypertensive rats

Angiotensin-(1-7) [Ang-(1-7)] is the first member of the angiotensin peptide family to cause cells to secrete hormones and release autacoids without eliciting accompanying changes in blood pressure, water intake, and aldosterone secretion. Incubation of hypothalamic explants with Ang-(1-7) stimulates a dose-dependent release of vasopressin. Addition of Ang-(1-7) to human astrocytes and vascular endothelial cells promotes production of prostanoids by activation of a Ca2+-independent second messenger system. Angiotensin I (Ang I) forms Ang-(1-7) in brain homogenates, vascular endothelium, and neuronal cells in culture. These effects of Ang-(1-7) have prompted us to suggest a regulatory role for this peptide as a paracrine hormone.

Inhibition of angiotensin converting enzyme (ACE) augments the concentration of Ang-(1-7) in plasma. These data suggest that alternate enzymatic pathways exist for the processing of biologically active Angiotensin peptides. In searching for peptides that hydrolyze the Pro7-Phe8 bond of Ang I or angiotensin II (Ang II), we found that prolyl endopeptidase (EC 3.4.21.26) is an Ang-(1-7)-forming enzyme in neuroblastoma glioma cells and brain tissue. However, inhibition of prolyl endopeptidase did not prevent the generation of Ang-(1-7) in other tissues. Therefore, we have now investigated whether neutral endopeptidase EC 3.4.24.11 (NEP 24.11) is involved in the metabolism of Ang I into Ang-(1-7). Studies were done in conscious spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) controls in which Ang I was injected intravenously. Studies were done in this species because we found that circulating levels of Ang-(1-7) were significantly increased in the plasma of rats with spontaneous hypertension.

Methods

Experimental Protocol

All experiments were carried out in strict adherence to the principles sponsored by the American Physiological Society. Male 16-week-old SHR (319±4 g) and WKY (315±4 g) controls (Charles River Breeding Laboratories, Wilmington, Mass.) were housed in rooms maintained on a 12-hour light/dark cycle. Animals ate a solid diet of rat chow (Purina, Bedford, Ohio)
and had free access to water. Cannulas (PE-50 and PE-10, Clay Adams Becton Dickinson, Parsippany, N.J.) were implanted into a femoral artery and vein by using aseptic conditions in rats anesthetized with 1% halothane 24 hours beforehand. The free ends of the cannulas were tunneled under the skin and externalized at the back of the neck.

Conscious rats were divided into five groups. Group 1 (n=4 SHR and 4 WKY controls) received an intravenous injection of 0.9% NaCl. Group 2 (n=4 SHR and 4 WKY controls) was given an intravenous injection of enalaprilat (MK-422, 2 mg/kg). Group 3 (n=4 SHR and 4 WKY controls) was injected with N-[N-[1-(S)-car-boxy-3-phenylpropyl]-L-(S)-phenylalanyl]-L-(S)-isoserine (SCH 39,370) at a dose of 15 mg/kg. This compound is a specific and potent inhibitor of NEP 24.11 but does not inhibit ACE or carboxypeptidase A.12 Group 4 (n=4 SHR and 4 WKY controls) received both MK-422 (2 mg/kg) and SCH 39,370 (15 mg/kg). Group 5 (n=3 SHR and 3 WKY) was given MK-422 (2 mg/kg) and Z-prolyl-prolinal (5 mg/kg), a specific inhibitor of prolyl endopeptidase.13 Either saline (vehicle) or the enzyme inhibitors were administered 20 minutes before an intravenous injection of 0.2 ml Ang I (2 nmol) in heparinized saline. Serial samples of arterial blood (0.2 ml) were rapidly collected into ice-chilled tubes containing 5 ml of 80% ethanol/0.1N HCl every 15–30 seconds before and after injection of Ang I. The amount of blood removed in each sample was replaced with an equal volume of saline. The dead space (0.08 ml) of the femoral artery catheter was cleared of any remaining blood or blood before the samples were obtained.

**Peptide Extraction and Analyses**

Samples were centrifuged at 5,000g for 20 minutes; the supernatant was stored for 12 hours at −20°C. After a second centrifugation step (10,000g for 20 minutes) the supernatant was diluted 1:1 (vol/vol) with 1% heptfluorobutyric acid (HFBA), stored for 4–6 hours at 4°C, and centrifuged again at 15,000g. This supernatant was further diluted 1:4 with 0.2% HFBA and concentrated on a Sep-Pak Vac cartridge (Millipore Corp., Milford, Mass.) activated beforehand with 3 ml of 60% acetonitrile/0.2% HFBA, 3 ml of 0.1% bovine serum albumin in 0.2% HFBA, and 5 ml HFBA. After application of the supernatant, the column was washed with 10 ml of 0.2% HFBA. Angiotensin peptides were eluted with 8 ml of 60% acetonitrile/0.2% HFBA. Fractions were evaporated to dryness in a vacuum centrifuge (Savant Instrument Inc., Hicksville, N.Y.) for measurements by radioimmunoassay. Different aliquots of the samples were assayed to ensure linearity. Peptide recovery for Ang I added to the acid ethanol solution before blood sample averaged 85±4% (n=6, mean±SEM); peptide values were not corrected for recovery. Moreover, we did not observe any in vitro conversion of Ang I into either Ang-(1–7) or Ang II.

The radioimmunoassays used to determine concentrations of Ang I, Ang II, and Ang-(1–7) are described in detail elsewhere.6,9 The Ang I antibody (New England Nuclear, Boston, Mass.) shows a 100% cross-reactivity with Ang I, Ang-(2–10), and Ang-(3–10) but does not cross-react with either Ang II or Ang-(1–7). The sensitivity of the Ang I assay (defined as twice the minimum detection level) averaged 5 fmol per tube. The Ang II antibody was produced in our laboratory. The antibody cross-reacts 100% with Ang II and its C-terminal fragments Ang-(2–8), Ang-(3–8), and Ang-(4–8) but showed less than 0.01% binding with either Ang I or Ang-(1–7) fragments. The sensitivity of the Ang II radioimmunoassay is 0.5 fmol per tube. The antibody of Ang-(1–7) (core 1) was obtained in our laboratory.6,9 It shows 100% cross-reactivity with Ang-(1–7) and Ang-(2–7) but does not recognize either Ang I or Ang II. The sensitivity of this assay is 4 fmol per tube. We verified the identity of the angiotensin peptides by high-performance liquid chromatography of a pool sample of rat blood, as described previously.6,9 For data analysis, nondetectable values are assigned the value of the sensitivity of the assay.

**Materials**

SCH 39,370 was kindly provided by Dr. E.J. Sybertz (Scherering-Plough Research, Bloomfield, N.J.). The inhibitor was dissolved in a mixture of 2.0N NaOH and heparinized saline. MK-422 was a gift from Merck, Inc., Rahway, N.J. Z-prolyl-prolinal was made and kindly provided to us by Dr. S. Wilk (Mount Sinai University, New York, N.Y.). Ang I was purchased from Bachem, Inc., Torrance, Calif.

**Statistical Analysis**

Differences resulting from drug treatments were evaluated by one-way analysis of variance followed by Duncan's multiple range test. Comparison among the five groups over all time points was done by two-way analysis of variance with repeated measures. All data are mean±SEM. The criterion for statistical significance is p<0.05.

**Results**

Baseline levels of Ang I were not different between WKY (0.11±0.04 pmol/ml) and SHR (0.08±0.02 pmol/ml). At baseline, Ang II and Ang-(1–7) were near or below the detectable level of the assay because of the small volume of blood withdrawn. The pattern of clearance of Ang I in blood of control rats is shown in Figure 1. Corresponding changes in blood levels of Ang II and Ang-(1–7) are illustrated in Figures 2 and 3, respectively. In all experiments peak levels of angiotensin peptide concentrations were observed in the first sample taken 15 seconds after injection of Ang I. In control rats peak increases in the concentration of Ang I were 3.8±0.5 pmol/ml in WKY and 4.9±0.9 pmol/ml in SHR. This difference was not statistically significant (p>0.05). Fifteen seconds after Ang I injection, levels of Ang II averaged 11.5±2.1 pmol/ml in WKY and 13.1±2.2 pmol/ml in SHR (p>0.05). In contrast, levels of Ang-(1–7) between 15 and 60 seconds after injection of Ang I were significantly greater in SHR as assessed by two-way analysis of variance (p<0.05). Plasma Ang-(1–7) at 15 seconds after Ang I injection averaged 0.57±0.10 pmol/ml in WKY and 0.83±0.10 pmol/ml in SHR. Peak levels of Ang-(1–7) represented 4.9% and 6.3% of Ang II concentrations in WKY and SHR, respectively. We verified that Ang I, Ang II, and Ang-(1–7) were the predominant forms of circulating an-
giotensins from analysis of plasma samples by using the combination of high-performance liquid chromatography followed by radioimmunoassay.

Pretreatment with MK-422 significantly elevated baseline levels of Ang I in both WKY (1.4±0.4 pmol/ml) and SHR (0.9±0.1 pmol/ml) and caused a significant upward shift of the time-concentration clearance of Ang I in both strains of rats (Figure 1). This was associated with a marked inhibition of Ang II formation, averaging 1% of values found in untreated WKY and SHR. In contrast, peak and time-concentration levels of Ang-(1-7) were significantly increased in rats given MK-422 as compared with vehicle (Figure 3). Peak levels of Ang-(1-7) averaged 1.8±0.2 pmol/ml in WKY and 1.9±0.1 pmol/ml in SHR (p<0.05). These data represent a twofold and threefold increase over values found in control rats (0.6±0.1 pmol/ml and 0.8±0.1 pmol/ml, p<0.05). In addition, these values are 10% and 13% of peak Ang I levels in WKY and SHR, respectively.

Inhibition of NEP 24.11 produced a pattern of Ang I and Ang II metabolism that did not differ from that obtained in control rats (Figures 1 and 2). Peak levels of Ang I were higher in SHR (6.2±0.4 pmol/ml) compared with WKY rats (4.3±0.4 pmol/ml, p<0.01). Baseline levels of Ang II were significantly higher after SCH 39,370 (WKY rats, 0.04±0.02 versus 0.12±0.04 pmol/ml, p<0.05; SHR, 0.02±0.02 versus 0.18±0.04 pmol/ml, p<0.05). Likewise, peak levels of Ang II were significantly higher than in control rats (p<0.05). Ang II averaged 21.8±5.4 pmol/ml in WKY and 27.2±3.8 pmol/ml in SHR (p>0.05). Figure 3 shows that SCH
39,370 inhibited the formation of Ang-(1-7). Differences in time concentrations of Ang-(1-7) between vehicle and SCH 39,370 groups were statistically significant in SHR (p<0.01) but not in WKY rats (p>0.05).

Combined blockade of ACE and NEP 24.11 decreased the rate of Ang I metabolism, prevented Ang II formation, and caused marked reductions in Ang-(1-7) levels in both WKY and SHR as compared with MK-422 treatment alone (Figures 1-3). Peak levels of Ang-(1-7) averaged 0.15±0.04 pmol/ml in WKY and 0.13±0.03 pmol/ml in SHR (p>0.05). These values represented 1.1% and 0.9% of peak Ang I levels in control WKY and SHR, respectively. In rats pretreated with a combination of MK-422 and an inhibitor of prolyl endopeptidase, the injection of Ang I produced a pattern of metabolism bearing similarities to that found in rats given MK-422 alone. However, this combination of inhibitors produced levels of Ang I (Figure 1) and Ang-(1-7) (Figure 3) that were significantly below those found in WKY and SHR given MK-422 only (p<0.05). Production of Ang II remained at levels that were close to or at the detectable level of the assay.

Discussion

The injection of Ang I into the circulation of WKY and SHR causes increased formation of Ang II and Ang-(1-7). Repeated sampling during the first 60 seconds after the injection of Ang I showed that levels of Ang-(1-7) were consistently greater in SHR compared with WKY controls. Conversion of Ang I into Ang II, indicating an active participation of this enzyme in the degradation of Ang II. The dual role of NEP 24.11 acting both as a processing and degrading enzyme may regulate plasma levels of angiotensin peptides. To our knowledge, this is the first in vivo demonstration of how an enzyme can affect levels of angiotensin peptides by cleaving the Pro7-Phe8 bond of Ang I and inactivating Ang II by hydrolysis at the Tyr5-Ile6 bond of the peptide.

The pattern of the metabolism of injected Ang I under the various conditions reveals that a dynamic equilibrium exists among angiotensin-forming enzymes. Strictly speaking, we see the process as a "yin-yang" mechanism. The proportional rate of conversion of Ang I into Ang-(1-7) was increased when the formation of Ang II was blocked by inhibition of ACE. At the peak of the time-concentration curve, Ang-(1-7) levels in MK-422-treated WKY rats increased 307% compared with vehicle-treated rats. Corresponding increases in SHR averaged 225%. At the same time the levels of Ang II were reduced by 99% in both WKY and SHR. Inhibition of NEP 24.11 reduced peak levels of Ang-(1-7) by 51% in WKY and 74% in SHR. Combined blockade of both ACE and NEP 24.11 produced significant reductions in Ang-(1-7) that averaged 73% and 84% below vehicle-treated WKY and SHR, respectively. Neither SCH 39,370 alone nor in combination with MK-422 blocked the formation of Ang-(1-7) in its entirety. In preliminary experiments, larger doses of SCH 39,370 caused no further inhibition of Ang-(1-7) production. In addition, we found no evidence for a contribution by prolyl endopeptidase. Therefore, another enzyme(s) may contribute to the formation of the heptapeptide in the circulation of WKY and SHR. These findings show that there are multiple metabolic pathways for Ang I.

NEP 24.11 degrades both atrial natriuretic peptide and kinins.14-15 Our findings suggest that this endopeptidase also influences the metabolism of Ang I by decreasing hydrolysis of Ang II and facilitating conversion of Ang I into Ang-(1-7). Inhibition of Ang II degradation appears to be an important action of SCH 39,370. In our experiments, SCH 39,370 caused decreased baseline and peak plasma levels of Ang II to double in both WKY and SHR. Gafford et al16 have reported that NEP 24.11 hydrolyzes Ang II at the Tyr4-Ile5 to yield Ang-(1-4). Therefore, the observation that SCH 39,370 did not reduce blood pressure in SHR may reflect the opposing effects that NEP 24.11 has on the various peptidergic systems that regulate vascular resistance.15
Ang-(1-7) is not dependent on an intermediate produc-

tion. We also confirmed that conversion of Ang I into
corticosterone acetate-salt hypertensive rats. 12 Because this
finding by Kohara et al,11 who found increased plasma
levels of Ang-(1-7). In addition, injection of Ang I elicited
significantly smaller increases in the peak and time-
concentration clearance of Ang I in SHR treated with
MK-422. These data suggest altered pathways of Ang I
metabolism in hypertensive animals. Because several
processes other than metabolism influence the rate of
removal of a peptide from the circulation, these data
suggest, but obviously do not prove, the existence of
altered pathways for the generation or degradation of
angiotensin peptides in the circulation of SHR.

In summary, NEP 24.11 has a major role in the in vivo
metabolism of Ang I in the circulation of WKY and

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