Pergamon

Biochemical Pharmacology, Vol. 50, No. 9, pp. 1451–1459, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(95)02049-I

METABOLISM OF ANGIOTENSIN I IN ISOLATED RAT HEARTS

EFFECT OF ANGIOTENSIN CONVERTING ENZYME INHIBITORS

LIOMAR A. A. NEVES,*† ALVAIR P. ALMEIDA,‡ MAHESH C. KHOSLA§ and ROBSON A. S. SANTOS*

*Laboratorio de Hippertensão, Departamento de Fisiologia e Biofisica, ICB-UFMG, Belo Horizonte, MG, Brazil; ‡Departamento de Farmacologia, ICB-UFMG, Belo Horizonte, MG, Brazil; and §Cleveland Clinic Foundation, Cleveland, OH, U.S.A.

(*Received* 4 *January* 1995; *accepted* 24 *May* 1995)

Abstract—In this study, the formation of biologically active angiotensins from angiotensin I (Ang I) in isolated rat hearts was evaluated. The role of angiotensin converting enzyme (ACE) in Ang I metabolism was also investigated. HPLC analysis of heart perfusate showed that ^{125}I-Ang I was metabolized extensively (single passage) in the rat coronary circulation *in vitro* leading to the formation of the biologically active angiotensins: angiotensin II (Ang II), Ang-(2-8), Ang-(3-8) and Ang-(1-7). Ang II was the major product identified in HPLC fractions, corresponding to 7.8 ± 0.89% of the total radioactivity recovered. A similar profile was observed when single-passage metabolism of non-isotopic Ang I was evaluated by HPLC, followed by radioimmunoassay of the eluate fractions. When ^{125}I-Ang I was perfused in the presence of ACE inhibitors (enalaprilat, ramiprilat) in concentrations up to 130 μM, the formation of Ang II was only partially inhibited (approximately 50%). A similar tendency was observed for Ang-(2-8), Ang-(3-8) and Ang-(2-7). The formation of Ang-(1-7) and its related fragments Ang-(3-7) and Ang-(4-7) was not changed significantly by ACE inhibitors, although a slight increase in formation of these fragments was observed. No significant changes were observed for the carboxyl-terminal fragments of Ang I: Ang-(2-10), Ang-(3-10), and Ang-(4-10). The fractional metabolism of Ang I was not modified by ACE inhibition. These findings suggest that biologically active angiotensins can be formed from Ang I in the rat coronary circulation. These locally generated peptides may contribute to the actions of the renin-angiotensin system in the heart.

Key words: angiotensin-(1-7); angiotensin IV; angiotensin III; renin-angiotensin system; hypertension; blood pressure

The concept that the RAS‖ is a circulating system with a single biologically active end-product, Ang II, has changed in the past few years to a new concept in which the RAS is considered to be both local and circulating [1]. In addition to Ang II, other biologically active products can be formed by the limited-proteolysis processes involved in RAS activation [2, 3]. Several studies have provided evidence that at least three other angiotensins fulfill the requirements necessary to be considered mediators of the RAS; angiotensin-(2-8) (Ang III) [4, 5], angiotensin-(3-8) (Ang IV) [6–8], and angiotensin-(1-7) (Ang-(1-7)) [4, 9].

Ang III has been shown to be an endogenous angiotensin in plasma and tissues [3, 4] with important central and peripheral actions including stimulation of aldosterone release [10], neuronal stimulation [4, 5] and positive inotropic effect [11]. Ang-(3-8) is present in plasma and tissues [2, 12] and has been shown to increase renal cortical blood flow [13] and to produce an endothelium-dependent vasodilatation in rabbit cerebral arterioles [7]. In addition, Ang-(3-8) has been reported to enhance memory retrieval [6]. Specific binding sites for Ang-(3-8) have been demonstrated in the rabbit heart and guinea pig heart [14]. The heptapeptide Ang-(1-7) is also present in plasma and tissues [2, 3, 12] and exerts important central and peripheral effects related to cardiovascular function [3, 15–18] and hydroelectrolyte balance [19–21]. In contrast to Ang II, however, Ang-(1-7) does not possess significant myotropic, pressor or dipsogenic action [see Ref. 3].

The RAS participates in changes in chronotropism and inotropism, arrhythmogenesis, and cellular growth in the heart [11, 22]. These actions have been attributed to the interaction of Ang II with angiotensin receptor subtype AT_1 [11]. However, other peptides of the RAS could also be involved in the mediation of RAS effects, particularly in situations where the formation or actions of Ang II is (are) altered [3, 18, 23, 24]. Several studies have documented the formation of Ang III, Ang-(3-8) or Ang-(1-7) from Ang I or Ang II *in vivo* and *in vitro* [3, 4, 25], yet there are no data regarding the formation of these angiotensins in the heart circulation. Only a few studies have attempted to study the metabolism of Ang I in isolated heart preparations [1], and in these studies only Ang II formation was investigated. In the present study, we evaluated the formation of biologically active angiotensins from Ang I in isolated rat hearts, using non-isotopic and ^{125}I-labeled Ang I. The effect of ACE (EC 3.4.15.1) inhibitors on the single-pass metabolism of ^{125}I-Ang I was also determined.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 200–300 g were used. The animals were obtained from the "Centro de Bioterismo

† Corresponding author: Dr. Liomar A. A. Neves, Department of Biology, The University of Akron, Akron, OH 44325-3908, U.S.A. Tel. (216) 972-7155; FAX (216) 972-8445.

‖ Abbreviations: RAS, renin-angiotensin system; Ang, angiotensin; ACE, angiotensin converting enzyme; RIA, radioimmunoassay; TFA, trifluoroacetic acid; and HFBA, heptafluorobutyric acid.

do ICB-UFMG" (CEBIO, Federal University of Minas Gerais, Belo Horizonte, Brazil).

Drugs

BSA, *p*-hydroxymercury benzoate, 1,10-phenanthroline, pepstatin A and chymostatin were obtained from the Sigma Chemical Co. (St. Louis, MO). EDTA, chloramine T and HPLC grade solvents were obtained from Merck (E. Merck, Darmstadt, Germany). Enalaprilat (MK 422) was obtained from MerckSharp & Dohme (MerckSharp & Dohme, U.S.A.). $Na^{125}I$ was obtained from Amersham (Amersham International, U.K.). Neomycin was from Gibco (Life Technologies LTD. Paisley, U.K.). Phenylmethylsulfonyl fluoride (PMSF) was from Calbiochem (Calbiochem-Behring Co., La Jolla, CA, U.S.A.). Ramiprilat, synthesized at Hoechst (Germany), was provided by Dr. B. Shölkens and Dr. K. Wirth. Ang-(1-7) and Ang II antibodies were provided by Dr. C. M. Ferrario (Cleveland Clinic Foundation, U.S.A.) and Ang I antibody was provided by Prof. J. L. Pesquero (Federal University of Minas Gerais). Angiotensin peptides were synthesized by Dr. M. C. Khosla (Cleveland Clinic Foundation). All other reagents were commercial products of the highest available grade of purity.

Isolated rat heart preparation

Male Wistar rats were decapitated 15 min after an i.p. injection of 200 IU heparin. The thorax was opened, and the heart was carefully dissected from its connections and perfused with Krebs–Ringer solution through a 1.0 ± 0.3 cm aortic stump by the Langendorff technique. The perfusion fluid was kept at 37°, with a pressure of 65 mm Hg and constant oxygenation (5% CO_2 and 95% O_2). The composition of the Krebs–Ringer solution was as follows: 118.6 mM NaCl, 4.75 mM KCl, 2.52 mM $CaCl_2$, 2.52 mM KH_2PO_4, 1.17 mM $MgSO_4 \cdot 7H_2O$, 25.0 mM $NaHCO_3$ and 11.0 mM glucose.

^{125}I-Ang I metabolism in isolated rat heart

Ang I and its fragments (Ang II, Ang-(1-7), Ang III, Ang-(2-7), Ang-(2-10), Ang-(3-7), Ang-(3-8), Ang-(3-10), Ang-(4-7) and Ang-(4-10)) were radioiodinated (^{125}I) by the chloramine T method. Immediately after iodination, the peptides were partially purified by adsorption onto Bond-Elute cartridges as described below. Monoiodinated peptides (sp. act. 2176 Ci/mmol) were obtained by HPLC using an HFBA/acetonitrile gradient (see below). Amounts corresponding to 1–5 μCi were purified in each HPLC run.

^{125}I-Ang I dissolved in Krebs–Ringer solution containing 1 μg/L protease-free BSA was infused in isolated rat hearts with a peristaltic pump at a rate of 10,000 cpm/70 μL/min for 10 min. During the 10 min of infusion, the coronary sinus effluent (averaging 9.3 ± 0.8 mL/min, N = 35) was collected into an Erlenmeyer flask rinsed with 0.1% BSA and containing 1 mM *p*-hydroxymercuribenzoate, 9.1 mM 1,10- phenanthroline, 1 mM PMSF, 0.25 g/L neomycin (10 μL/mL perfusate), 0.5 mM pepstatin A (20 μL/mL perfusate) and 0.2 M EDTA (50 μL/mL perfusate). The effluent was kept on ice.

After collection of the basal metabolism sample, the isolated rat heart was perfused for 20 min with Krebs–Ringer solution or Krebs–Ringer containing 2.6 μM (N = 8) or 130 μM (N = 4), enalaprilat (MK 422), 2.6 μM enalaprilat plus 5 μM chymostatin (N = 2) or 2.6 μM ramiprilat (N = 2). Immediately after the equilibration period with enzymatic inhibitors, ^{125}I-Ang I (10,000 cpm/70 μL/min), dissolved in Krebs–Ringer solution containing protease-free BSA (1 μg/mL) and enzymatic inhibitors, was infused again over a 10-min period, and the perfusate was collected. The stability of the ^{125}I-Ang I metabolism over the duration of the experiments was documented in preliminary experiments in which ^{125}I-Ang I, dissolved in Krebs–Ringer solution containing protease-free BSA, was infused over a 10-min period through isolated rat hearts, within 40 and 70 min of the beginning of the perfusion. With the exception of an increase of Ang-(4-7) formation (56%) and a reduction of Ang-(2-7) formation (31%), only minor changes were observed in the profile obtained with 40 and 70 min of perfusion. The equilibration time of 20 min was based on preliminary experiments showing that 20 min after the end of the perfusion with ^{125}I-Ang I, the radioactivity in the heart effluent returned to background levels.

Samples were extracted by adsorption onto 500 mg Bond-Elute phenylsilica cartridges (Analytichem International, Harbor City, CA, U.S.A.). The columns were activated by sequential washes with 10 mL of 80% methanol/0.1% TFA, 10 mL of 0.1% TFA and 3 mL of 0.1% TFA containing 0.1% BSA. After application of the samples, the columns were washed with 40 mL of 0.1% TFA, and the adsorbed peptides were eluted with 3 mL of 80% methanol/0.1% TFA into polypropylene tubes containing 50 μL of 1% protease-free BSA.

Using this procedure, the recovery of radiolabeled standards and samples was 95 ± 0.4 and 35 ± 3.4%, respectively. The low recovery of the samples as compared with that of the radiolabeled standards was due to the loss of ^{125}I-Tyr and small fragments not identified with our standards, as determined by HPLC analyses of the Bond-Elute effluents collected using sample application and washing with 0.1% TFA. Therefore, all results regarding ^{125}I-Ang I metabolism were not reported as absolute percentages but as percentages of the recovered peptides. The samples were evaporated using a filtered (0.2 μm) air stream. After evaporation, the samples were analyzed using a Shimadzu HPLC System (Shimadzu Co., Kyoto, Japan) equipped with two LC-9A pumps controlled by an SCL-6B System Controller. Reverse-phase separation was carried out using a Nova Pak C_{18} column (3.9 × 150 mm, particle size 4 μm, Millipore, U.S.A.) coupled to a μBondapakTM C_{18} Waters pre-column (Millipore, U.S.A.). Gradient elution was obtained with aqueous 0.13% HFBA (mobile phase A) and 0.13% HFBA/80% acetonitrile in water (mobile phase B). Gradient conditions were: 31 to 36% mobile phase B in 20 min, concave gradient (Curve No. 10, SLC-6B); 36 to 41% mobile phase B from 20 to 40 min, concave gradient (Curve No. 10, SLC-6B), and 41 to 70% mobile phase B from 40 to 60 min, concave gradient (Curve No. 8, SLC-6B). The flow rate was 1.0 mL/min; 0.5-mL fractions were collected. The fractions were counted in a gamma counter. Products of the metabolism of ^{125}I-Ang I were identified by comparing their retention times with those of radiolabeled angiotensin standards. The system was calibrated by injecting approximately 10,000 cpm ^{125}I-labeled angiotensins, and monitoring each sample with a gamma counter. We verified that the recovery of ^{125}I-Ang I, either as Ang I or as ^{125}I-labeled peptides, was consistently greater than

95% after HPLC separation. Table 1 and Fig. 1 show the retention time of ^{125}I-Ang I peptides in the chromatographic conditions used in our study.

Stability of ^{125}I-Ang I during the experimental procedure was determined in all experiments at the beginning of the experiment by collecting the heart perfusate for 10 min into flasks containing approximately 100,000 cpm ^{125}I-Ang I and the inhibitors (see above). This sample was then processed with the experimental samples. Stability of ^{125}I-Ang I also was determined in the presence of protease-free BSA: ^{125}I-Ang I was incubated with 50 µL of 1% protease-free BSA at 37° for 25 min. After extraction into Bond Elute cartridges, the samples were analyzed by HPLC.

Ang I metabolism in isolated rat hearts

Non-isotopic Ang I was infused using the same procedure described for infusion of ^{125}I-Ang I, at a rate of 10 ng/70 µL/min. The perfusion time was also 10 min. With these conditions we evaluated basal metabolism. The presence of endogenous angiotensins in the heart effluent was evaluated by collecting the effluent during 10 min before infusion of non-isotopic Ang I.

The samples were extracted by adsorption onto C_{18} Sep Pak cartridges (Waters Associates, Millipore, U.S.A.). Columns were activated by sequential washes with 5 mL of methanol, 5 mL of tetrahydrofuran, 5 mL of hexane, 10 mL of methanol and 10 mL of water. After application of the samples, columns were washed with 30 mL of water and 30 mL of 4% acetic acid, and the adsorbed peptides were eluted with 3 mL of 90% ethanol/4% acetic acid/6% water into polypropylene tubes rinsed with 0.1% fat-free BSA. The sample was evaporated in a vacuum-centrifuge (Heto, U.S.A.) at 45°. After evaporation, the samples were analyzed using a Shimadzu HPLC System as described previously [20]. Briefly, reverse-phase separation was carried out using a Lichrospher 100 RP_{18} column (4 × 125 mm, particle size 5 µm, E. Merck) coupled to a Lichrocart 4-4 pre-column (E. Merck). Gradient elution was obtained with aqueous 0.13% HFBA (mobile phase A) and 0.13% HFBA/80% acetonitrile in water (mobile phase B). Gradient conditions were: 30 to 45% mobile phase B in 40 min and 45 to 60% mobile phase B from 40 to 50 min. The flow rate was 1.0 mL/min; 0.5-mL fractions were collected. The fractions were evaporated using a vacuum centrifuge at 45° and assayed by RIA (see below). Calibration of the system was achieved by injection of a mixture of standard angiotensins [Ang II, Ang-(1-7), Ang III, Ang-(2-10), Ang-(3-8), Ang-(3-10), and Ang-(4-10), 400 pg of each].

Table 1. Retention times of angiotensin peptides on HPLC conditions used for characterization of metabolites of ^{125}I-Ang I in isolated rat heart

Angiotensins	Retention time (min)
^{125}I-Ang-(4-7)	7.0
^{125}I-Ang-(3-7)	9.0
^{125}I-Ang-(1-7)	15.5
^{125}I-Ang-(2-7)	16.5
^{125}I-Ang-(3-8)	27.0
^{125}I-Ang-(1-8)	33.5
^{125}I-Ang-(2-8)	35.5
^{125}I-Ang-(4-10)	45.0
^{125}I-Ang-(3-10)	47.0
^{125}I-Ang-(1-10)	50.5
^{125}I-Ang-(2-10)	52.5

Gradient conditions are described in Materials and Methods.

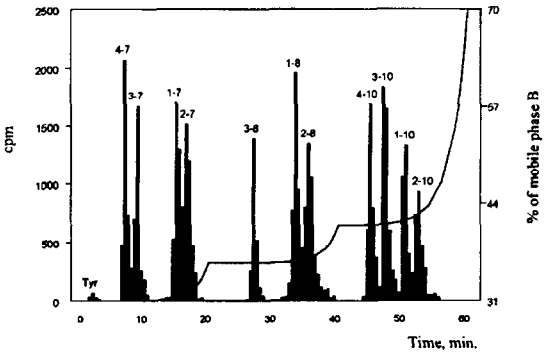

Fig. 1. Chromatography profiles of ^{125}I-labeled angiotensin peptides (standards). The standards were processed by HPLC using a Shimadzu HPLC system equipped with a SLC-6B gradient system and with a Nova Pack C_{18} column (3.9 × 150 mm, particle size 4 µm). Experimental conditions: mobile phase A, 0.13% HFBA; mobile phase B, 0.13% HFBA/80% acetonitrile; flow rate, 1.0 mL/min. Angiotensin metabolites were separated with a 20-min concave gradient (Curve No. 10, SLC-6B) of 31 to 36% mobile phase B, followed from 20 to 40 min by a concave gradient (Curve No. 10, SLC-6B) of 36 to 41% mobile phase B, and from 40 to 60 min by a concave gradient (Curve No. 8, SLC-6B) of 41 to 70% mobile phase B. Fractions of 0.5 mL were collected. Key: 1-10 (^{125}I-Ang I); 1-8 (^{125}I-Ang II); 1-7 (^{125}I-Ang-(1-7)); 2-8 (^{125}I-Ang III); 3-7 (^{125}I-Ang-(3-7)); 4-7 (^{125}I-Ang-(4-7)); 2-10 (^{125}I-Ang-(2-10)); 2-7 (^{125}I-Ang-(2-7)); 3-10 (^{125}I-Ang-(3-10)); 4-10 (^{125}I-Ang-(4-10)); 3-8 (^{125}I-Ang-(3-8)); and Tyr (tyrosine).

After evaporation, fractions were resuspended in 200 µL of an aqueous solution containing 0.1% BSA, 0.9% NaCl and 0.03% acetic acid. Concentrations of Ang I, Ang II and Ang-(1-7) in the fractions were determined by RIA using three different antibodies [20]. Ang I, Ang II and Ang-(1-7) were radioiodinated (^{125}I) by the chloramine T method (see above). Fractions 1–30 were processed by RIA for Ang-(1-7) as described previously [20]. The polyclonal Ang-(1-7) antibody cross-reacts less than 0.001% with Ang-(2-7), less than 0.5% with Ang-(3-7) and Ang-(4-7), and 0.005% with Ang-(1-5). Cross-reactivity with Ang I, Ang II, and other amino-terminal fragments is less than 0.001%. Fractions 31–64 were processed by RIA for Ang II as described previously [20]. The polyclonal Ang II antibody cross-reacts 100% with the angiotensin fragments, Ang-(2-8), Ang-(3-8) and Ang-(4-8). Cross-reactivity of less than 0.001% was observed with Ang I and Ang-(1-7), and of 0.18% with Ang-(1-9). Fractions 64–85 were processed by RIA for Ang I as described previously [20]. The polyclonal Ang I antibody cross-reacts less than 0.001% with Ang-(1-7) and Ang II. Ang I antibody cross-reacts 100% with the carboxyl-terminal fragments Ang-(2-10), Ang-(3-10) and Ang-(4-10). Products of the metabolism of Ang I were identified by comparison of their retention times with those of angiotensin standards.

Statistical analysis

All values are reported as means ± SEM. Differences between basal and samples collected in the presence of

enzymatic inhibitors were analyzed by a paired Student's t-test. The criterion for statistical significance was set at $P < 0.05$.

RESULTS

Basal metabolism

Infusion of ^{125}I-Ang I through isolated rat hearts led to the formation of several products in the coronary sinus effluent, including ^{125}I-Ang II, ^{125}I-Ang-(1-7), ^{125}I-Ang III, ^{125}I-Ang-(2-7), ^{125}I-Ang-(2-10), ^{125}I-Ang-(3-7), ^{125}I-Ang-(3-8), ^{125}I-Ang-(3-10), ^{125}I-Ang-(4-7) and ^{125}I-Ang-(4-10) (Fig. 2A). Other fragments were also formed (Fig. 2A); however, they could not to be identified due to the limitation of angiotensin standards (Fig. 1). As shown in Table 2, Ang II was the major peptide product of ^{125}I-Ang I metabolism (7.8 ± 0.89% of the total recovered), followed by carboxyl-terminal fragments of ^{125}I-Ang I, ^{125}I-Ang-(1-7) (1.15 ± 0.16% of the total recovered) and ^{125}I-Ang III (1.04 ± 0.25% of the total recovered). For the other identified fragments the percentage was less than 1% of the total recovered. Data presented in Table 2 also show that the percentage of metabolites formed as a result of N-terminal cleavage (i.e. missing Asp^1), 11%, was essentially the same as the percentage of metabolites formed by C-terminal cleavage (missing Phe-His-Leu or His-Leu) (12%), emphasizing the importance of aminopeptidase activity for the ^{125}I-Ang I metabolism under our experimental conditions.

The stability of ^{125}I-Ang I during the experimental procedures was 97.8 ± 0.22%, indicating that only 2% of ^{125}I-Ang I infused was metabolized during sample processing (Fig. 2C). HPLC analysis showed that the products formed were ^{125}I-Ang-(2-10), ^{125}I-Ang-(3-10) and ^{125}I-Ang-(4-10). Metabolism was not observed when ^{125}I-Ang I was incubated with protease-free BSA at 37° for 25 min.

The formation of Ang-(1-7) and other angiotensins from Ang I was confirmed by RIA, after infusion of non-isotopic Ang I through the isolated rat heart. As shown in Fig. 3, infusion of non-labeled Ang I led to the generation of Ang II, Ang-(1-7), Ang-(3-8), Ang-(2-10), Ang-(3-10) and Ang-(4-10) as determined by RIA. In addition, two other immunoreactive metabolites of Ang II were formed. The first (A-X) was eluted in the position expected for Ang-(4-8). The other one (A-Y) may be a transformed form of Ang-(4-8), as described by De Silva [26]. We also observed two unidentified immunoreactive metabolites of Ang-(1-7) representing less than 2% of the total Ang-(1-7) immunoreactivity. These results were apparently not influenced by endogenous angiotensins. When the presence of endogenous angiotensins in the heart effluent was evaluated, only traces of immunoreactivity were detected, comparable to the background levels.

Metabolism of Ang I in the presence of ACE inhibition

Dependence of Ang I metabolism on the activity of ACE was initially investigated by pretreatment of isolated rat hearts with enalaprilat (2.6 μM). In the presence of ACE inhibition, formation of ^{125}I-Ang II was still observed, although an average 43% reduction was seen (Fig. 4). In addition, production of ^{125}I-Ang III and ^{125}I-Ang-(2-7) was reduced by 59 and 36%, respectively (Fig. 4). A tendency to decrease was also observed for Ang-(3-8). The formation of ^{125}I-Ang-(2-10) and ^{125}I-Ang-(4-10) increased by 22 and 30%, respectively (Fig. 4). An increase of 47% in the formation of the peptides that could not be identified based on our standards was also observed. The generation of ^{125}I-Ang-(1-7) and metabolites [Ang-(3-7), Ang-(4-7) and Ang-(3-10)] was not affected significantly by blockade of ACE (Fig. 4), even when formation of Ang II was reduced more noticeably (Fig. 2B). Overall, the hydrolysis rate of ^{125}I-Ang I was not affected by blockade of ACE. Before ACE treatment, the rate of hydrolysis of ^{125}I-Ang I corresponded to 20.7 ± 1.84% of the total radioactivity recovered; after ACE treatment it corresponded to 19.3 ± 4.52% of the total radioactivity recovered.

In additional experiments, ACE inhibitor concentration was increased to 130 μM. Under these conditions, a similar inhibition of ^{125}I-Ang II formation occurred (1.97 ± 0.59 vs 3.82 ± 1.08% before enalaprilat), and the formation of the other peptides was not different from that observed with the lower concentration of the ACE inhibitor. The hydrolysis rate of ^{125}I-Ang I also was not changed significantly in the presence of 130 μM enalaprilat (^{125}I-Ang I was 83.9 ± 0.85% of the total recovered before treatment and 83.7 ± 1.49% in the presence of enalaprilat). Formation of ^{125}I-Ang II observed in the presence of 2.6 μM ramiprilat, a second ACE inhibitor, was not different from that observed in the presence of 2.6 μM enalaprilat (5.34 ± 1.74 vs 8.28 ± 1.95% in the ramiprilat-treated hearts). Formation of Ang-(1-7) also was unchanged.

To evaluate whether a chymotrypsin-like enzyme could be involved in the generation of ^{125}I-Ang II from ^{125}I-Ang I in enalaprilat-perfused hearts, the metabolism of ^{125}I-Ang I was examined in two preparations perfused with 2.6 μM enalaprilat containing 5 μM chymostatin. No changes in ^{125}I-Ang II formation were observed (8.46 ± 0.56% vs 5.34 ± 1.74% in enalaprilat-treated hearts), whereas the formation of Ang-(1-7) increased (2.78 ± 0.3 vs 1.43 ± 0.34% in enalaprilat-treated hearts). Increases in Ang-(4-7) and Ang-(3-8) fragments were also observed (96 and 162%, respectively). In contrast, the formation of Ang-(2-8) and Ang-(3-10) was reduced in enalaprilat-chymostatin-perfused hearts (41 and 73%, respectively). Formation of Ang-(2-7) in enalaprilat-chymostatin-treated hearts was undetectable.

DISCUSSION

In this study, we observed that Ang I was metabolized extensively during a single pass through isolated rat hearts, leading to the formation of several biologically active angiotensins [Ang II, Ang III, Ang-(3-8) and Ang-(1-7)]. The HPLC conditions used in our study allowed the separation of eleven ^{125}I-labeled angiotensins: ^{125}I-Ang I, ^{125}I-Ang II, ^{125}I-Ang-(1-7), ^{125}I-Ang III, ^{125}I-Ang-(2-7), ^{125}I-Ang-(2-10), ^{125}I-Ang-(3-7), ^{125}I-Ang-(3-8), ^{125}I-Ang-(3-10), ^{125}I-Ang-(4-7) and ^{125}I-Ang-(4-10). We observed, however, that the angiotensin metabolism was complex in the isolated rat hearts, leading to the formation of several unidentified products as well.

Theoretically, twenty-eight iodinated metabolites can be formed from ^{125}I-Ang I, and twenty-one of these metabolites are expected to elute before ^{125}I-Ang II. Thus, under our HPLC conditions some metabolites with retention times smaller than ^{125}I-Ang II could co-elute.

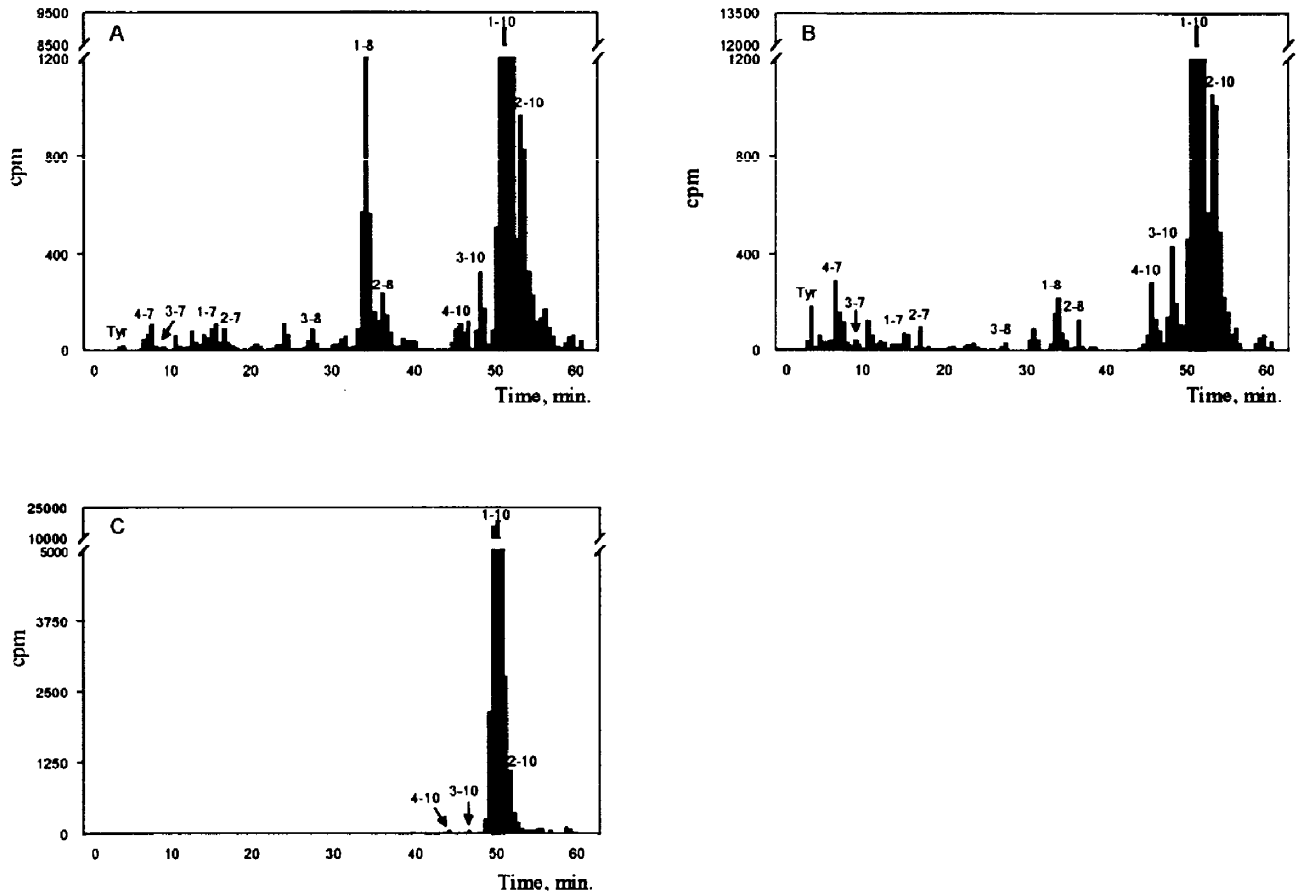

Fig. 2. Chromatographic profiles of angiotensin metabolites after infusion of ^{125}I-angiotensin I in isolated rat hearts. Panel A illustrates products obtained in basal conditions. Panel B illustrates products obtained after perfusion with the angiotensin converting enzyme inhibitor enalaprilat (MK 422, 2.6 μM). Panel C illustrates the profile of radioactivity obtained when the heart eluate was collected into flasks containing ^{125}I-Ang I (≅100,000 cpm). The samples were processed by HPLC as described in the legend of Fig. 1. Key: 1-10 (^{125}I-Ang I); 1-8 (^{125}I-Ang II); 1-7 (^{125}I-Ang-(1-7)); 2-8 (^{125}I-Ang III); 3-7 (^{125}I-Ang-(3-7)); 4-7 (^{125}I-Ang-(4-7)); 2-10 (^{125}I-Ang-(2-10)); 2-7 (^{125}I-Ang-(2-7)); 3-10 (^{125}I-Ang-(3-10)); 4-10 (^{125}I-Ang-(4-10)); 3-8 (^{125}I-Ang-(3-8)); and Tyr (tyrosine).

Table 2. Angiotensin metabolites formed during a single passage of ^{125}I-Ang I through Krebs–Ringer perfused isolated rat hearts: Basal conditions

Angiotensins	N	Radioactivity (% of the total recovered)
^{125}I-Ang-(4-7)	14	0.50 ± 0.12
^{125}I-Ang-(3-7)	14	0.17 ± 0.05
^{125}I-Ang-(1-7)	19	1.15 ± 0.16
^{125}I-Ang-(2-7)	19	0.38 ± 0.07
^{125}I-Ang-(3-8)	19	0.62 ± 0.19
^{125}I-Ang-(1-8)	19	7.80 ± 0.89
^{125}I-Ang-(2-8)	14	1.04 ± 0.25
^{125}I-Ang-(4-10)	14	1.95 ± 0.19
^{125}I-Ang-(3-10)	14	1.50 ± 0.15
^{125}I-Ang-(1-10)	19	79.30 ± 0.19
^{125}I-Ang-(2-10)	14	4.76 ± 0.38

Values are means ± SEM; N = number of determinations. Total recovered = 35,000 ± 3,400 cpm.

Therefore, to confirm the identity of the biologically active angiotensins, Ang-(1-7) and Ang-(3-8), metabolic studies using non-isotopic Ang I and HPLC-RIA analysis of the heart perfusate were also performed. In these experiments, the identities of Ang II, Ang-(1-7), Ang III and Ang-(3-8) were further confirmed. Other immunoreactive angiotensins were also detected, including carboxyl-terminal fragments of Ang I, Ang-(2-10), Ang-(3-10) and Ang-(4-10). Substantial differences were observed in the relative amount of peptides formed from Ang I or ^{125}I-Ang I. For example, the Ang II/Ang-(1-7) ratio was nearly 1 using unlabeled Ang I and approximately 7 using ^{125}I-Ang I. This finding is likely due to the differences in substrate concentration, which was much higher when non-isotopic Ang I was used. In addition, we cannot exclude the effects of iodination of Tyr^4 on the affinity of ^{125}I-Ang I or its iodinated metabolites for angiotensin- metabolizing enzymes.

The profiles of angiotensin peptides formed from ^{125}I-Ang I or non-isotopic Ang I indicate the presence of high aminopeptidase activity in the Wistar rat coronary circulation. It is well known that the Asp^1-Arg^2 of Ang I, and of Ang II as well, is hydrolyzed by the enzyme aminopeptidase A (EC 3.4.11.7), which is present in the vascular endothelium [27], in addition to its wide distribution in the kidney. Sequential removal of N-terminal amino acids from Ang-(2-10), Ang III and Ang-(1-7) by additional aminopeptidases such as aminopeptidase B (EC 3.4.11.6) or M (EC 3.4.11.2) could be involved in the formation of the other carboxyl-terminal fragments including Ang-(3-8). Dipeptidyl peptidases such as DAP III (EC 3.4.14.4), which remove N-terminal dipeptides from angiotensins [25], may also contribute to the generation of carboxyl-terminal fragments of Ang I, Ang II and, probably, Ang-(1-7). Further studies using specific aminopeptidase inhibitors are necessary to identify the major aminopeptidases involved in angiotensin metabolism in isolated rat hearts.

It should be noted that the pattern of angiotensin metabolism observed in the present study, showing high aminopeptidase activity, differs from our previous studies in cultured endothelial cells (human, bovine and pig), where formation of des-[Asp]^1-angiotensin peptides was below the level of detection [28]. Another important difference was the fractional conversion of Ang I to Ang II and Ang-(1-7). In cultured endothelial cells, Ang-(1-7) was the major angiotensin peptide formed, whereas in our present study Ang I was preferentially converted to Ang II. These findings are likely due to significant differences in the cell-surface enzymes [28].

The fractional conversion of Ang I to Ang II found in the present study (7.80 ± 0.87%) is in good agreement with the values found by Lindpaintner and Ganten [1] in a similar preparation (6–9%). We also found that Ang II formation was inhibited by ACE inhibitors. However, in our study inhibition was partial, averaging approximately 50%. This appears to be related to species differences or to the technique used to quantitate Ang I conversion. Other investigators have reported conversion of Ang I to Ang II in isolated heart preparations that is independent of ACE [29]. Our results are more in accordance with the growing concept that generation of circulating or tissue Ang II is only partially dependent upon ACE. In humans, for example, the major Ang II forming enzyme in the heart appears to be chymase (EC 3.4.21.39) [29].

No further reduction of Ang II formation was ob-

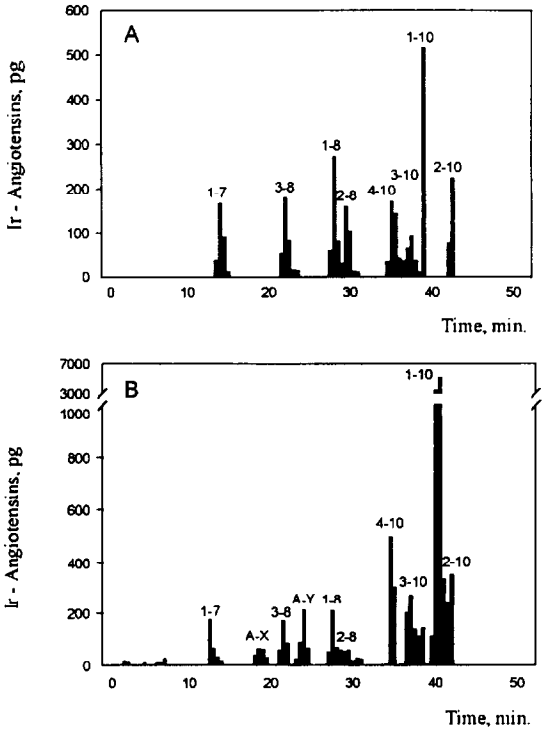

Fig. 3. Angiotensin metabolites obtained after infusion of angiotensin I in isolated rat heart. Panel A represents the HPLC elution profile of angiotensin peptides (standards). Panel B illustrates products obtained under basal conditions. Samples were processed by HPLC with a Lichrospher 100 RP_{18} column (4 × 125 mm, particle size 5 μm). Experimental conditions: mobile phase A, 0.13% HFBA; mobile phase B—0.13% HFBA/80% acetonitrile; flow rate, 1.0 mL/min. Angiotensin metabolites were separated with a 40-min linear gradient, 30 to 45% mobile phase B, followed by a linear gradient from 40 to 50 min, 45 to 60% mobile phase B. Fractions (0.5 mL) were collected and processed by three different radioimmunoassays: fractions 1–30, Ang-(1-7); fractions 31–64, Ang II; fractions 64–85, Ang I. Key: 1-10 (Ang I); 1-8 (Ang II); 1-7 (Ang-(1-7)); 2-8 (Ang III); 2-10 (Ang-(2-10)); 3-10 (Ang-(3-10)); 4-10 (Ang-(4-10)); 3-8 (Ang-(3-8)); Ir (immunoreactivity).

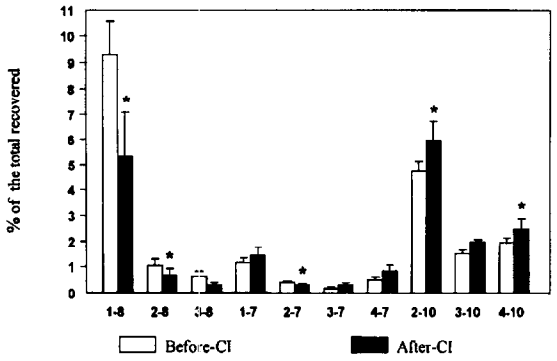

Fig. 4. Angiotensin metabolites obtained after infusion of ^{125}I-Ang I into isolated rat hearts under basal conditions and after perfusion with the angiotensin converting enzyme inhibitor enalaprilat (MK 422, 2.6 μM, N = 8). Data are reported as a percentage (means ± SEM) of the total recovered (35,000 ± 3,400 cpm). Key: * $P < 0.05$ (Student's t-test). CI—angiotensin converting enzyme inhibitor.

served in hearts perfused with enalaprilat-chymostatin, suggesting that enzymes of the chymotrypsin family do not participate in the conversion of Ang I to Ang II in enalaprilat-treated hearts. Enzymes such as chymase [29] or cathepsin G (EC 3.4.21.20) [30] can form Ang II from Ang I by cleavage at the Phe^8-His^9 peptide bond of Ang I [31]. Other enzymes including kallikrein (EC 3.4.21.34), tonin (EC 3.4.21) [31], or tonin-like enzymes could be involved in the generation of Ang II under our conditions. However, the contribution of non-ACE enzymes in the conversion of Ang I to Ang II *in vivo* is probably limited because these enzymes can be inhibited by circulating inhibitors as demonstrated for tonin [30] and cathepsin G [31].

Although a major role of chymotrypsin-like enzyme in the formation of Ang II during ACE inhibition can apparently be excluded, a partial role cannot be ruled out. Partial inhibition of Ang II formation by chymostatin could be masked by an expected parallel reduction of Ang II hydrolysis at the Tyr^4-Ile^5 bond [9]. Indeed, a slight increase in fractional formation of Ang II was observed in enalaprilat-chymostatin-perfused hearts. A more pronounced increase was observed for Ang-(1-7), which can also be metabolized by chymotrypsin-like enzymes by hydrolysis at the same peptide bond as Ang II. An intriguing finding in the chymostatin-treated hearts was the reduction in the formation of Ang-(2-8), Ang-(2-7) and Ang-(3-10), suggesting participation of chymostatin-sensitive enzymes in their formation. Further studies directed to elucidating the role of chymostatin-sensitive enzymes in angiotensin metabolism in rat hearts are needed to confirm these preliminary observations.

As observed for Ang II, formation of Ang-(2-8) and Ang-(3-8) was partially dependent upon ACE activity. The increases observed in Ang-(2-10) and Ang-(3-10) in the presence of ACE inhibition also indicated that sequential cleavage of Ang I by aminopeptidases and ACE is one of the enzymatic pathways for the generation of Ang III and Ang-(3-8). Previous studies in dogs [2] suggested a similar pathway for the formation of Ang-(3-8) in the pulmonary vascular bed. However, the Ang III and Ang-(3-8) forming pathways probably also involve cleavage of Ang II by aminopeptidases. Formation of Ang-(3-8) in the heart circulation may be particularly relevant in view of the demonstration of binding sites and biological actions of this angiotensin in the heart [14].

We also observed that formation of Ang-(1-7) was not changed significantly by ACE inhibition. Formation of Ang-(1-7) has been reported to be independent of ACE in several preparations, including dog brain homogenates [9], endothelial cell cultures [28], and NG108-15 neuroblastoma × glioma hybrid cells [32]. Studies *in vivo* have confirmed that Ang-(1-7) formation is independent of ACE activity [2, 12, 33, 34]. It should be mentioned, however, that based on its sequence Ang-(1-7) can also be a substrate for ACE. Thus, participation of a pathway involving Ang II hydrolysis by carboxypeptidases [2, 4, 35] or prolyl-endopeptidase (EC 3.4.21.26) [36] may be masked by a reduction in the metabolism of Ang-(1-7) during ACE inhibition. It is interesting that formation of the Ang-(2-7) fragment was reduced in ACE inhibition, suggesting that formation of this angiotensin peptide could be more dependent upon the Ang II pathway. In this regard, Abhold and Harding [37] have observed that Ang-(2-7) formation is a major route of Ang II and Ang III metabolism in synaptic membrane preparations. The increase we observed in radioactive peaks corresponding to the elution position of Ang-(3-7) and Ang-(4-7) suggests that either these angiotensins are derived mainly from Ang-(1-7) or these peptides are more sensitive than Ang-(2-7) to hydrolysis by ACE.

Formation of Ang-(1-7) from Ang I has been attributed to prolyl-endopeptidase [28, 32, 36] or to endopeptidase 24.11 (EC 3.4.24.11) [28, 34]. Early suggestions that prolyl-endopeptidase is involved in the formation of Ang-(1-7) were based on the observed reduction of Ang-(1-7) generation in the presence of Z-Pro-prolinal [28], reportedly a specific inhibitor of prolyl-endopeptidase [38]. However, it has been reported recently that Z-Pro-prolinal also blocks the actions of the prolylendopeptidase-related enzyme, prolylcarboxypeptidase [35]. Prolylcarboxypeptidase can form Ang-(1-7) from Ang II generated by hydrolysis of Ang I by ACE, or from Ang II formed by the sequential release of Leu^{10} and His^9 from Ang I by other enzymes such as carboxypeptidases or deamidase [39]. However, in the rat an enzyme more likely involved in the generation of Ang-(1-7) from Ang I is endopeptidase 24.11 [34].

Formation of Ang-(1-7) in the coronary circulation may have important implications. It has been shown recently that this angiotensin produces a potent vasodilatation in the coronary vessels of pigs [23]. We have also obtained evidence that Ang-(1-7) is active in rat coronary circulation.* However, in the rat, Ang-(1-7) acts as a vasoconstrictor, as it does in hamsters [40]. These contrasting observations may be related to species differences. In this regard, the vasodilatory effect of Ang-(1-7) on the feline hindquarter vascular bed is mainly dependent upon release of NO [41], whereas in pithed rats Ang-(1-7) produces systemic vasodilatation dependent upon prostaglandin release [5].

In summary, we have shown that Ang I is metabolized

* Neves LAA, Almeida AP, Carvalho AC, Nogueira PC and Santos RAS, Angiotensin-(1-7) enhances reperfusion arrhythmias in isolated rat hearts. *Proceedings of the 13th International Symposium on Kinins*, Brazil, 1993.

in the rat coronary circulation, leading to the appearance of several biologically active angiotensins: Ang II, Ang III, Ang-(3-8) and Ang-(1-7). Formation of Ang II and its carboxyl terminal fragments is partially dependent upon ACE, while formation of Ang-(1-7) is not changed significantly by ACE inhibitors. Generation of biologically active angiotensins from circulating Ang I in the coronary circulation probably contributes to the cardiac actions of the RAS.

Acknowledgements—This work was supported by FAPEMIG (Fundação de Amparo a Pesquisa de Minas Gerais) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico). L. A. A. Neves was the recipient of a CNPq fellowship. We thank Dr. Amy Milsted for review of the manuscript, and Paulo Cesar Nogueira and Soraia Silveria Silva for technical assistance.

REFERENCES

1. Lindpaintner K and Ganten D, The cardiac renin-angiotensin system: An appraisal of present experimental and clinical evidence. *Circ Res* **68:** 905–921, 1991.
2. Santos RAS, Brum JM, Brosnihan KB and Ferrario CM, The renin-angiotensin system during acute myocardial ischemia in dogs. *Hypertension* **15:** I121–I127, 1990.
3. Santos RAS and Campagnole-Santos MJ, Central and peripheral actions of angiotensin-(1-7). *Braz J Med Biol Res* **27:** 1033–1047, 1994.
4. Brosnihan KB, Santos RAS, Block CH, Schiavone MT, Welches WR, Chappell MC, Khosla MC, Greene LJ and Ferrario CM, Biotransformation of angiotensin in the central nervous system. *Ther Res* **9:** 48–59, 1988.
5. Wright JW and Harding JW, Regulatory role of brain angiotensins in the control of physiological and behavioral responses. *Brain Res Rev* **17:** 227–262, 1992.
6. Braszko JJ, Wlasienko J, Koziolkiewicz W, Janecka A and Wisniewski K, The 3-7 fragment of angiotensin II is probably responsible for its psychoactive properties. *Brain Res* **554:** 49–54, 1991.
7. Harbel RL, Decker PJ and Einhaupl KM, Angiotensin degradation products mediate endothelium-dependent dilatation of rabbit brain arterioles. *Circ Res* **68:** 1621–1627, 1991.
8. Harding JW, Cook VI, Miller-Winger AV, Hanesworth JM, Sardinia MF, Hall KL, Stobb JW, Swanson GN, Coleman JKM, Wright JW and Harding EC, Identification of an AII(3-8) [AIV] binding site in guinea pig hippocampus. *Brain Res* **583:** 340–343, 1992.
9. Santos RAS, Brosnihan KB, Chappell MC, Pesquero JL, Chernicky CL, Greene LJ and Ferrario CM, Converting enzyme activity and angiotensin metabolism in dog brainstem. *Hypertension* **11:** I153–I157, 1988.
10. Goodfriend TL and Peach MJ, Angiotensin III: Evidence and speculation for its role as an important agonist in the renin-angiotensin system. *Circ Res* **36/37**[Suppl I]: 38–40, 1975.
11. Baker KM, Booz GW and Dostal DE, Cardiac actions of angiotensin II: Role of an intracardiac renin-angiotensin system. *Annu Rev Physiol* **54:** 227–241, 1992.
12. Campbell DJ, Lawrence AC, Towrie A, Kladis A and Valentijn AJ, Differential regulation of angiotensin peptide levels in plasma and kidney of the rat. *Hypertension* **18:** 763–773, 1991.
13. Coleman JKM, Ong B, Sardinia MF, Harding JW and Wright JW, Changes in renal blood flow due to infusions of angiotensin II(3-8) [AIV] in normotensive rats. *FASEB J* **6:** A981, 1992.
14. Hanesworth JM, Sardinia MF, Krebs LT, Hall KL and Harding JW, Elucidation of a specific binding site for angiotensin II(3-8), angiotensin IV, in mammalian heart membranes. *J Pharmacol Exp Ther* **266:** 1036–1042, 1993.
15. Campagnole-Santos MJ, Diz DI, Santos RAS, Khosla MC and Ferrario CM, Cardiovascular actions of angiotensin-(1-7) microinjected into the dorsal medial medulla. *Am J Physiol* **257:** H324–H329, 1989.
16. Campagnole-Santos MJ, Heringer SB, Batista EN, Khosla MC and Santos RAS, Differential baroreceptor reflex modulation by centrally infused angiotensin peptides. *Am J Physiol* **263:** R89–R94, 1992.
17. Silva LCS, Fontes MAP, Campagnole-Santos MJ, Khosla MC, Campos RR Jr, Guertzenstein PG and Santos RAS, Cardiovascular effects produced by microinjection of angiotensin-(1-7) on vasopressor and vasodepressor sites of the ventrolateral medulla. *Brain Res* **613:** 321–325, 1993.
18. Fontes MAP, Silva LCS, Campagnole-Santos MJ, Khosla MC, Guertzenstein PG and Santos RAS, Evidence that angiotensin-(1-7) plays a role in the central control of blood pressure at the ventro-lateral medulla acting through specific receptors. *Brain Res* **665:** 175–180, 1994.
19. Santos RAS and Baracho NC, Angiotensin-(1-7) is a potent antidiuretic peptide in rats. *Braz J Med Biol Res* **25:** 651–654, 1992.
20. Botelho LM, Block CH, Khosla MC and Santos RAS, Plasma angiotensin-(1-7) immunoreactivity is increased by salt load, water deprivation, and hemorrhage. *Peptides* **15:** 723–729, 1994.
21. Schiavone MT, Santos RAS, Brosnihan KB, Khosla MC and Ferrario CM, Release of vasopressin from the hypothalamo-neurohypophysial system by angiotensin-(1-7) heptapeptide. *Proc Natl Acad Sci USA* **85:** 4095–4098, 1988.
22. Grinstead WC and Young JB, The myocardial renin-angiotensin system: Existence, importance, and clinical implications. *Am Heart J* **123:** 1039–1045, 1992.
23. Pörsti I, Bara AT, Busse R and Hecker M, Release of nitric oxide by angiotensin-(1-7) from porcine coronary endothelium: Implications for a novel angiotensin receptor. *Br J Pharmacol* **111:** 652–654, 1994.
24. Santos RAS, Campagnole-Santos MJ, Baracho NCV, Fontes MAP, Silva LCS, Neves LAA, Oliveira DR, Caligiorne SM, Rodrigues ARV, Gropen C Jr, Carvalho WS, Simões e Silva AC and Khosla MC, Characterization of a new angiotensin antagonist selective for angiotensin-(1-7): Evidence that the actions of angiotensin-(1-7) are mediated by specific angiotensin receptors. *Brain Res Bull* **35:** 293–298, 1994.
25. Erdös EG and Skidgel RA, Renal metabolism of angiotensin I and II. *Kidney Int* **38:** 24–27, 1990.
26. De Silva EPNPS, Angiotensin II immunoreactivity in rat tissues. *Ph.D. Thesis,* Cleveland State University, Cleveland, OH, 1988.
27. Johnson AR and Erdös EG, Metabolism of vasoactive peptides by human endothelial cells in culture: Angiotensin I converting enzyme (kininase II) and angiotensinase. *J Clin Invest* **59:** 684–695, 1977.
28. Santos RAS, Brosnihan KB, Jacobsen DW, DiCorleto PE and Ferrario CM, Production of angiotensin-(1-7) by human vascular endothelium. *Hypertension* **19** (Suppl II): II56–II61, 1992.
29. Urata H, Kinoshita A, Misono KS, Bumpus FM and Husain A, Identification of a highly specific chymase as the major angiotensin II-formating enzyme in the human heart. *J Biol Chem* **265:** 22348–22357, 1990.
30. Boucher R, Asselin J and Genest J, A new enzyme leading to the direct formation of angiotensin II. *Circ Res* **34/35**(Suppl II): I203–I212, 1974.
31. Reilly CF, Tewksbury DA, Schechta NM and Travis J, Rapid conversion of angiotensin I to angiotensin II by neutrophil and mast cell proteinases. *J Biol Chem* **257:** 8619–8622, 1982.
32. Chappell MC, Tallant EA, Brosnihan KB and Ferrario CM, Processing of angiotensin peptides by NG108-15 neuro-

blastoma x glioma hybrid cell line. *Peptides* **11:** 375–380, 1990.
33. Kohara K, Brosnihan KB, Chappell MC, Khosla MC and Ferrario CM, Angiotensin-(1-7): A member of circulating angiotensin peptides. *Hypertension* **17:** 131–138, 1991.
34. Yamamoto K, Chappell K, Brosnihan B and Ferrario CM, *In vivo* metabolism of angiotensin I by neutral endopeptidase (EC 3.4.24.11) in spontaneously hypertensive rats. *Hypertension* **19:** 1201–1211, 1992.
35. Tan F, Morris PW, Skidgel RA and Erdös EG, Sequencing and cloning of human prolylcarboxypeptidase (angiotensinase C): Similarity to both serine carboxypeptidase and prolylendopeptidase families. *J Biol Chem* **268:** 16631–16638, 1993.
36. Greene LJ, Spadaro ACC, Martin AR, Perussi de Jesus WD and Camargo ACM, Brain endooligopeptidase B: A post-proline cleaving enzyme that inactivates angiotensin I and II. *Hypertension* **4:** 178–184, 1982.
37. Abhold RH and Harding JW, Metabolism of angiotensin II and angiotensin III by membrane bounded peptidases from rat brain. *J Pharmacol Exp Ther* **245:** 171–177, 1988.
38. Friedman TC, Orlowski M and Wilk S, Prolyl endopeptidase: Inhibition *in vivo* by *N*-benzyloxycarbonyl-prolyl-prolinal. *J Neurochem* **42:** 237–241, 1984.
39. Jackman HL, Tan F, Tamei H, Beurling-Harbury C, Li X, Skidgel RA and Erdös EG, A peptidase in human platelets that deamidases tachykinins: Probable identity with the lysosomal "protective protein." *J Biol Chem* **265:** 11265–11272, 1990.
40. Kumagai H, Khosla M, Ferrario C and Fouad-Tarazi FM, Biological activity of angiotensin-(1-7) heptapeptide in the hamster heart. *Hypertension* **15:** I29–I33, 1990.
41. Osei SY, Ahima RS, Minkes RK, Weaver JP, Khosla MC and Kadowitz PJ, Differential responses to angiotensin-(1-7) in the feline mesenteric and hindquarters vascular beds. *J Pharmacol* **234:** 35–42, 1993.