

Converting Enzyme Activity and Angiotensin Metabolism in the Dog Brainstem

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SUMMARY The concentrations of angiotensin converting enzyme (ACE) activity, norepinephrine, and serotonin were measured in microdissected regions of the dog's brainstem and spinal cord. In addition, we determined the *in vitro* metabolism of ^{125}I -angiotensin I (Ang I) in homogenates of the same brain punch regions. High ACE-specific activity was found in the monoamine-containing regions of the brainstem and in the intermediolateral column of the spinal cord. In brainstem homogenates ^{125}I -Ang I was metabolized to angiotensin II (Ang-[1-8]) and the N-terminal heptapeptide Ang-(1-7). In the presence of MK 422 (50 μM), Ang-(1-7) was still generated, while the production of Ang-(1-8) was inhibited. This study revealed the presence of high ACE activity in monoamine regions of dog brainstem and spinal cord, and showed that the metabolite Ang-(1-7) is the major product generated from Ang I in the presence and absence of ACE inhibition. (Hypertension 11 [Suppl 1]: I-153-I-157, 1988)

KEY WORDS • medulla oblongata • spinal cord • hypertension • converting enzyme inhibitors • angiotensin II • neuropeptides • angiotensin-(1-7) • angiotensin metabolism

OUR understanding of the role of angiotensin II (Ang II) in the central nervous system is limited by our lack of knowledge of the functional neurochemistry of the brain renin-angiotensin system (RAS) in regions that may produce the peptide for actions on neuronal circuits engaged in autonomic function.^{1,2} Because catecholamine neuronal groups of the dorsal and ventral brainstem and the spinal cord have a major role in blood pressure regulation and the evolution of some forms of experimental hypertension,³ we determined the distribution of the angiotensin converting enzyme (ACE) in these areas in the dog. We studied also the proteolytic hydrolysis of angiotensin I (Ang I) in brain punch homogenates obtained from these regions to evaluate the biochemical pathways that account for the generation and metabolism of angiotensins in the brain.

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Materials and Methods

Experiments were performed on 14 mongrel dogs weighing 16 ± 2 kg and anesthetized with sodium pentobarbital (30 mg/kg *i.v.*). Arterial and venous catheters (Tygon, Faultless Rubber Co., Ashland, OH, USA) were inserted in a femoral artery and vein for the removal of blood. Ten of the 14 dogs were given a lethal dose of sodium pentobarbital, and the brain and thoracic portion of the spinal cord (C6-T3) were excised immediately. The brain was removed from four other dogs 6 hours after a single injection of MK 422 (enalaprilat) at a dose of 10 mg/kg *i.v.*

Tissues were partially frozen on dry ice and placed on a prechilled tissue slicer, modified from that described by Palkovits and Brownstein.⁴ A microtome knife (A. H. Thomas, Philadelphia, PA, USA) was situated at the obex; additional blades (set 2 mm apart) provided uniform serial sections from 6 mm caudal to the obex to the level of the inferior colliculus. Coronal sections (2000 μm thick) from either the brainstem or the spinal cord were laid on a frozen Petri dish placed under an operating microscope. Plugs of tissue were removed using a punch technique⁴ (13-15 gauge needle) throughout the rostrocaudal extension of structures that were shown by fluorescent histochemistry⁵ to belong to the catecholamine neuronal groups described in the rat.⁶ Punches were obtained from 1) the A1 region in the ventrolateral medulla from the pyramidal de-

cussation to the rostral pole of the inferior olive; 2) the A2 region, which includes the dorsal motor nucleus of the vagus nerve (DMNX), the nucleus of the tractus solitarius (NTS), and the solitary tract, between -6 mm and $+10$ mm from the obex; 3) the A5 region, which is dorsal to the superior olive and medial to the seventh nerve, between $+14$ and $+16$ mm from the obex; and 4) the A6 region on the dorsolateral pontine tegmentum (locus ceruleus) from $+18$ mm to $+22$ mm from the obex. Punches from coronal sections of the intermediolateral column of the spinal cord were obtained between C6 and T3. Blocks of tissue were also taken from the area postrema at the obex, the neurohypophysis, and the choroid plexus in the fourth ventricle. Remaining coronal tissue sections were analyzed separately. All tissue samples were kept on dry ice and stored at -70°C until assayed.

The ACE activity and angiotensin metabolism were measured in tissue samples obtained from five control and four MK 422-treated dogs. Five other dogs were used to determine the content of norepinephrine (NE) and serotonin (5-HT) in the same regions used to characterize the activity of the ACE.

Tissue punches (1–5 mg wet weight) were homogenized using a glass homogenizer (Kontes Scientific Glassware, Vineland, NJ, USA) in $140\ \mu\text{l}$ of 50 mM sodium borate buffer, pH 8.3, containing 112 mM NaCl and 0.1% Triton X-100. Larger tissue samples were homogenized to a final concentration of 100 mg tissue/ml buffer. The homogenates were centrifuged at $18,000\ g$ for 2 minutes at 4°C . The ACE activity was determined by incubating $10\ \mu\text{l}$ of the supernatants with $500\ \mu\text{l}$ of an assay solution containing 5 mM Hip-His-Leu (Sigma Chemical, St. Louis, MO, USA) in 0.4 M sodium borate buffer, pH 8.3, and 0.9 M NaCl for 15 minutes at 37°C .⁷ The product His-Leu was measured fluorometrically (365 nm excitation, 495 emission; Aminco spectrofluorometer, American Instruments, Silver Springs, MD, USA). In punches obtained from the brainstem, neurohypophysis, and choroid plexus, we showed that the rate of the reaction was linear over a 20-minute period and with the volume of the sample assayed (5–15 μl). Under these conditions, hydrolysis of the product His-Leu (Sigma) was less than 6% as evaluated indirectly by fluorometry.⁷ The specificity of the hydrolysis of the substrate as a measure of ACE activity was demonstrated by a 98% inhibition of product released in the presence of either 10 μM MK 422 or 1 mM EDTA. Tissue ACE specific activity was expressed as units corresponding to 1 nmol/min/mg protein. The concentration of proteins in the homogenates was determined by the method of Lowry et al.⁸

To study the metabolism of angiotensin in brain punch homogenates, ^{125}I -labeled Ang I and Ang II (New England Nuclear, Boston, MA, USA) were purified initially to remove the peptidase inhibitor trasylol by reverse phase high performance liquid chromatography (HPLC) using a Nova-Pak C_{18} column (Waters Associates, Milford, MA, USA). Peptides were eluted isocratically with 33.6% acetonitrile (Burdick and

Jackson, American Scientific Products, Columbus, OH, USA) in 0.13% heptafluorobutyric acid (HFBA; Sequenal grade, Pierce, Rockford, IL, USA) at a flow rate of 1 ml/min. The effluent (0.25 ml/fraction) was collected in tubes precoated with bovine serum albumin (BSA; Pentex, Miles Scientific, Naperville, IL, USA). After evaporation in a vacuum centrifuge (Savant, Farmingdale, NY, USA), the purified peptides were redissolved in water.

The metabolism of ^{125}I -Ang I and ^{125}I -Ang II was studied by incubating $10\ \mu\text{l}$ of brain homogenates with $190\ \mu\text{l}$ of an assay solution containing 50 pM labeled standards in 20 mM Tris HCl buffer, pH 7.5, and 0.01% BSA for 2 to 15 minutes at 37°C . The reactions were stopped by the addition of $100\ \mu\text{l}$ of 32% acetonitrile containing 0.13% HFBA, followed by centrifugation at $18,000\ g$ for 2 minutes. Samples were then submitted to HPLC (Model 2150, LKB Instruments, Gaithersburg, MD, USA) using a gradient of 0.13% HFBA (vol/vol in water, Buffer A) and 80% acetonitrile containing 0.13% HFBA (Buffer B). The gradient conditions were 32% Buffer B, 5 minutes; 32 to 43%, 5 to 20 minutes; 43 to 48%, 20 to 25 minutes; and 48%, 25 to 30 minutes at a flow rate of 1 ml/min. The eluate was collected in 0.25-ml fractions.

Products of angiotensin metabolism were identified by comparison of their retention times with those of purified ^{125}I -Ang I and ^{125}I -Ang II, and with standards of ^{125}I -angiotensin fragments. Labeled standards were prepared by iodination of synthetic Ang-(2–10) and Ang-(1–7) or by enzymatic hydrolysis of labeled Ang I, Ang II, and Ang-(2–10) with carboxypeptidase Y (Pierce Chemicals, Rockford, IL, USA), chymotrypsin (AMERESCO, Solon, OH, USA), or trypsin-TPCK (Sigma). The incubation was made at 37°C in $150\ \mu\text{l}$ of 10 mM sodium phosphate buffer, pH 6.0, for carboxypeptidase Y (0.25 U) and 10 mM Tris HCl, pH 7.5, for trypsin and chymotrypsin (5 μg). Peptide standards obtained by more than one method were verified to elute at the same position but not to coelute with each other under the HPLC conditions reported here. The recovery after HPLC of ^{125}I as ^{125}I -Tyr or ^{125}I -labeled peptides was $96 \pm 5\%$ for the punch hydrolysate experiments.

Plasma ACE activity was measured fluorometrically,⁷ and plasma renin activity (PRA) and immunoreactive Ang II (irAng II) were measured by radioimmunoassay.¹ The concentrations of NE and 5-HT in brain punches were determined with an HPLC-electrochemical detection system.⁹ All values are reported as means \pm SE. Statistical differences were evaluated by analysis of variance followed by either the Newman-Keuls or the Dunnett's multiple-range test. Hormonal changes were analyzed with Student's *t* test (nonpaired method). Differences were considered significant at a *p* value below 0.05.

Results

The regional rostrocaudal distribution of ACE activity in relation to the content of NE and 5-HT in the dorsal and ventral regions of the dog brainstem are shown in Figure 1. Tissue punches from the dorso-

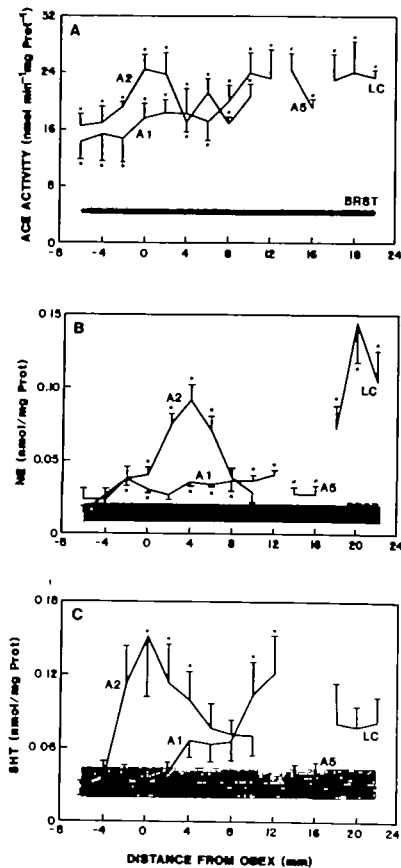


FIGURE 1. Data are means \pm SEM of rostrocaudal distribution of angiotensin converting enzyme activity (ACE; Panel A; $n = 4$); norepinephrine (NE; Panel B; $n = 5$) and serotonin (5-HT; Panel C; $n = 5$) in the ventral medulla (A1 region), dorsal medulla (A2 region), A5 area, and locus ceruleus (LC). Asterisks indicate significant difference ($p < 0.05$) when compared with the remaining brainstem tissue (BRST).

medial medulla in the A2 region rostral to the obex showed a high content of NE while punches obtained from the ventral medulla (A1) showed comparatively more uniform and lower levels than those measured in the dorsomedial medulla between +2 and +6 mm from the obex (see Figure 1B). A peak of 5-HT activity was located in the dorsomedial medulla immediate to the obex (see Figure 1C) and in the rostral aspects of the A1 region. As reported in other species,⁶ punches from the locus ceruleus region showed a high content of NE and appreciable quantities of 5-HT (see Figures 1B and 1C).

The ACE activity in all monoamine-containing regions was at least 3.75-fold higher than the values recorded in remaining coronal tissue sections of the brainstem (5.3 ± 0.8 U). A peak of ACE activity was found in the dorsomedial medulla adjacent to the obex, while the peak in the A1 region was located rostrally between +10 and +12 mm of the obex. Enzyme activity correlated only with 5-HT concentrations in both the A2 ($r = 0.74$) and A1 ($r = 0.90$) regions ($p < 0.02$ and $p < 0.0005$, respectively). Both the A5 and locus ceruleus regions contained high ACE activity comparable in magnitude to that found in the choroid

plexus (27.7 ± 3.5 U). In contrast, the levels of ACE activity in the area postrema (2.45 ± 1.47 U) and the neurohypophysis (1.28 ± 0.12 U) were similar to those measured in remaining tissues of the brainstem (see Figure 1A). In the intermediolateral column of the spinal cord, ACE activity averaged 10.6 ± 2.5 U, a value twofold higher than that measured in remaining spinal cord tissue (5.5 ± 1.1 U).

The finding of high ACE activity in brainstem structures involved in autonomic function persuaded us to study the effect of i.v. injection of MK 422 on the activity of the enzyme in the dog brain. In dogs given MK 422 6 hours earlier, ACE activity in the A2 region (between -2 and +4 mm of the obex) fell to 16.7 ± 1.7 U compared with 22.5 ± 1.6 U ($p < 0.05$) in untreated controls. In contrast, ACE activity in other regions of the brainstem did not change. The level of ACE activity in homogenates from the choroid plexus of dogs given MK 422 was reduced to 14.9 ± 0.6 U, 46% lower than the levels found in untreated dogs. Comparative measurements of ACE, PRA, and irAng II in the plasma 6 hours after injection of MK 422 confirmed that blockade of plasma ACE activity (2.1 ± 0.2 vs 17.7 ± 1.6 nmol/ml/min in untreated controls) was associated with a 175% increase in PRA and a significant suppression of plasma irAng II (8.4 ± 0.4 vs 23 ± 1.8 pg/ml in control animals; $p < 0.005$).

To evaluate the significance of the high ACE activity in monoamine-containing regions of dog brainstem, we measured the metabolism of labeled Ang I and Ang II. Figure 2 (left) shows the results obtained in punches from the A2 region at the level of the obex. Hydrolysis of radiolabeled Ang I was evidenced by the rapid metabolism of the decapeptide into Ang-(1-8) and progressive accumulation of the N-terminal fragment Ang-(1-7). Only trace amounts of other hydrolytic products, including the heptapeptide Ang-(2-8), were identified. Similar patterns of Ang I hydrolysis were obtained in other ACE-enriched regions of the brainstem, including the A1 (+10 mm), A5 (+14 mm), and A6 (+20 mm) regions. In the presence of 50 μ M MK 422 the generation of Ang-(1-8), but not Ang-(1-7), was reduced to undetectable amounts (see Figure 2, right). Experiments in which ¹²⁵I-Ang II was incubated with brain punch homogenates revealed that Ang-(1-7) was the primary metabolic product (data not shown).

Discussion

These experiments provide two important findings. First, we documented the existence of high ACE activity in the regions of the dog brainstem and pons corresponding to the NE neuronal groups first described in the rat.⁶ Second, we found that incubation of brainstem homogenates with labeled Ang I results in rapid accumulation of the N-terminal heptapeptide Ang-(1-7), both before and after blockade of ACE activity by MK 422. The unexpected finding that Ang-(1-7) is generated in the presence of ACE inhibition suggests an alternative route, independent of Ang

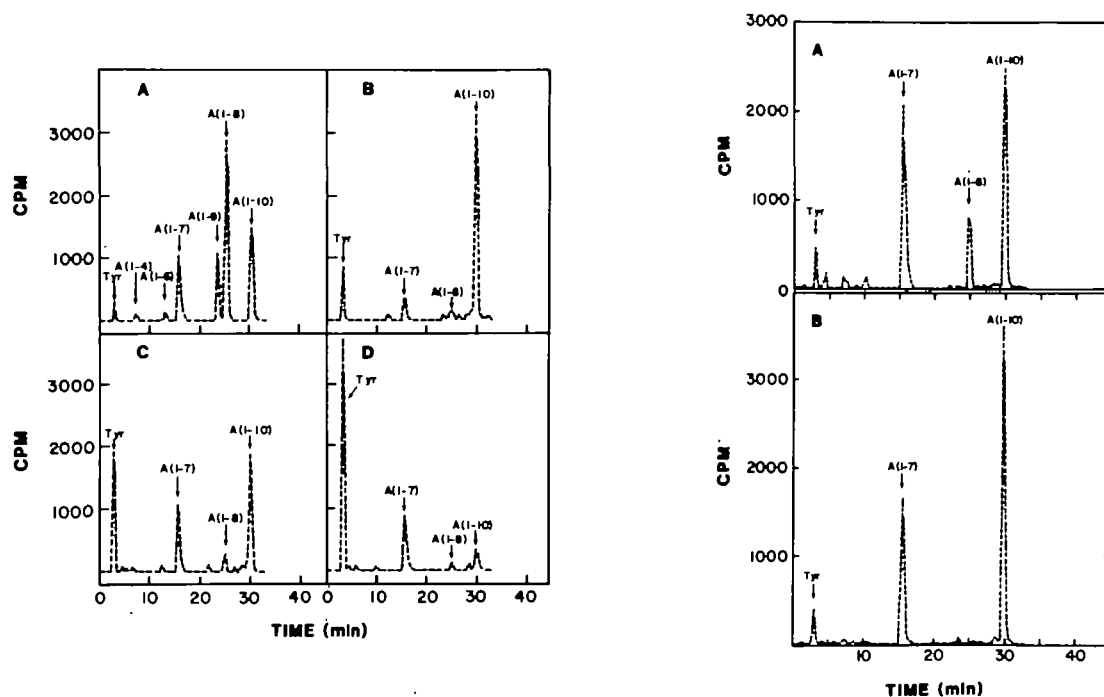


FIGURE 2. *Left: HPLC profiles of ^{125}I -labeled products produced from labeled Ang I incubated with a brain punch homogenate from the A2 region (obex level). Panel A represents the HPLC elution profile of ^{125}I -labeled angiotensin peptides (standards). Panels B, C, and D illustrate products obtained from the incubation of ^{125}I -Ang I with the brain punch homogenate at 2, 5, and 15 minutes, respectively. Similar patterns were observed when ^{125}I -Ang I was incubated with brain punches from the A1 (+10 mm), A5 (+14 mm), and A6 areas (+20 mm). Right: Effect of ACE inhibition on the metabolism of ^{125}I -Ang I by a brain punch homogenate from the A2 region (obex level). ^{125}I -Ang I was incubated with the brain punch homogenate for 5 minutes in the absence (A) or presence (B) of 50 μM MK 422.*

II, for the production of the heptapeptide Ang-(1-7).

The present study illustrates for the first time the regional distribution of NE, 5-HT, and ACE activity in the brainstem nuclei of the dog. This neurochemical analysis complements previous histochemical studies of the catecholamine-containing regions of dog brain.^{5, 10} The general pattern of neurotransmitter concentrations in dog brainstem is comparable to that described in rats⁶ and also agrees with the observations by Ishikawa et al.¹⁰ of the A5 and A6 regions in the dog. The highest concentration of NE in the dorsomedial medulla was found in the rostral DMNX-NTS (A2) region, while peak levels of 5-HT in the NE-enriched areas were located at the obex. In the ventral medulla the concentration of NE was relatively uniform, while levels of 5-HT increased progressively from the caudal to the rostral boundaries of the region. High levels of ACE activity, similar in magnitude to those measured in the choroid plexus of the fourth ventricle, were found in all regions containing NE and 5-HT; however, only 5-HT concentrations correlated with ACE activity in brainstem regions. This finding is of considerable interest, since previous studies suggested that brain monoamines influence the local generation of Ang II.^{1, 2} The presence of high ACE activity in the A1 and A2 regions provides a neurochemical basis for functional studies from our laboratory^{1, 11, 12} that showed an important *in situ* role for either blood or locally formed Ang II in the regulation of arterial pressure and

baroreceptor function. Furthermore, the measure of a selective inhibition of ACE activity in the A2 region after systemic administration of MK 422 suggests new insights into the mechanism of action of ACE inhibitors. These conclusions do not exclude the possibility that ACE may be involved also in the metabolism of other neuropeptides (e.g., substance P, bradykinin, and enkephalins)¹³ present in these regions.

The finding of high concentrations of ACE activity in regions of the brainstem and the choroid plexus agrees with previous reports in the rat.¹⁴ However, the levels of enzyme activity in the area postrema and the neurohypophysis are markedly different between the two species. In our experiments, the specific activity of ACE in the neurohypophysis and the area postrema was 15 to 20 times lower than that measured in the A2 region or the choroid plexus; in rats the area postrema and the neurohypophysis contain high levels of enzyme activity. Species differences may account for these contrasting findings. This interpretation is supported by studies showing that the density of Ang II binding sites in the area postrema of the rat¹⁵ is greater than that found in the area postrema of the dog.^{11, 16} Similarly, the specific activity of rat plasma ACE is five times higher than that measured in the dog.⁷

Peptide products generated in brainstem homogenates from ^{125}I -Ang I were determined to evaluate the functional significance of ACE activity in the brainstem. Picomolar concentrations of ^{125}I -labeled peptides

were used to avoid possible saturation of high-affinity-low-turnover peptidases without compromising the sensitivity for the detection of angiotensin metabolites. Previous studies showed that the metabolism of iodinated angiotensins does not differ significantly from those of the unlabeled peptides.¹⁷ Conversion of ¹²⁵I-Ang I to ¹²⁵I-Ang II was observed in all brainstem homogenates having high Hip-His-Leu-hydrolyzing activity. Furthermore, the proteolytic conversion of Ang I to Ang II was not detected in the homogenates containing MK 422. Together, these data emphasize that hydrolysis of the synthetic substrate Hip-His-Leu reflects the main Ang II-generating activity of brain tissue. It was surprising that the primary ¹²⁵I-labeled peptide generated from either Ang I or Ang II was the N-terminal heptapeptide Ang-(1-7). Only trace amounts of C-terminal fragments including angiotensin III were observed. Recently, Tonnaer *et al.*¹⁸ reported evidence for Ang-(1-7) generation in a rat synaptosomal fraction from whole brain. In their study, however, the primary products of Ang I and Ang II metabolism at pH 7.4 were the C-terminal fragments. It is unclear whether 1) angiotensin metabolism in dog brain differs from that in the rat,¹⁸ 2) regionally specific processing of angiotensin occurs, or 3) substrate concentration can affect the *in vitro* metabolism of angiotensins.

The observation that Ang-(1-7) was produced from labeled Ang I after ACE inhibition by MK 422 suggests the existence of an alternative pathway for the generation of this heptapeptide. Enzymes responsible for the generation of Ang-(1-7) from Ang I or Ang II may include carboxypeptidases¹⁹ and prolyl endopeptidases.²⁰ Under our incubation conditions (pH 7.5), the generation of Ang-(1-7) is not likely to involve carboxypeptidases, which usually have acidic pH optima.¹⁹ Several prolyl endopeptidases capable of hydrolyzing the Pro-X peptide bond at a neutral pH have been isolated from nervous tissue of several species.^{20, 21} Prolyl endopeptidase has been shown to hydrolyze the Pro-Phe bond of both Ang I and Ang II.^{20, 22} The further observation that its activity was shown to be resistant to captopril²¹ and enalapril²² implicates this enzyme as a likely candidate for the generation of Ang-(1-7). Since in our experiments Ang-(1-7) was the major product of labeled Ang I metabolism in brain punch hydrolysates, the data raise questions about its possible biological functions. Although Ang-(1-7) has weak agonistic pressor and drinking effects,¹⁸ the spectrum of biological actions of angiotensins is certainly not limited to either body fluid or cardiovascular regulation. Further studies are necessary to investigate the possible biological actions of this peptide.

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