Evidence for the Existence of a Family of Biologically Active Angiotensin I-Like Peptides in the Dog Central Nervous System

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SUMMARY. A family of angiotensin I-like peptides has been derived from endogenous precursors present in dog cerebrospinal fluid after incubation with species homologous renin. These peptides are immunologically and pharmacologically similar to [Leu5]angiotensin 1, and have molecular weights ranging between 1300 and 2200 daltons. The presence of precursors in the cerebrospinal fluid able to generate various biologically active angiotensin I-like peptides dissimilar to plasma angiotensin I supports the concept of a local angiotensin I-forming system in the brain. (Circ Res 52: 460-464, 1983)

IT IS well established that angiotensin II (Ang II) has a variety of effects upon the sympathetic control mechanism, some of which originate from an action of the peptide in the central nervous system (Phillips, 1978). The nature of the physiological mechanism by which Ang II acts upon the sympathetic nervous system to raise blood pressure and upon the body fluid regulatory mechanisms to elicit thirst, sodium appetite, and natriuresis is not fully understood. It appears that blood Ang II can gain access into brain receptive regions via the discontinuities in the blood-brain barrier that are characteristics of brain circumventricular organs (Buckely and Ferrario, 1977). Because the various proteins composing the cascade of the biochemical pathways that leads to the formation of the active peptide have also been found in brain tissue (Fischer-Ferraro et al., 1971; Ganten et al., 1971; Yang and Neff, 1972; Reid and Ramsay, 1975; Printz and Lewicki, 1977; Hirose et al., 1978; Osman et al., 1979), it has been suggested that Ang II can be formed locally in the brain.

An important step toward understanding the role of Ang II in the brain could be facilitated by identifying both the peptide and its precursors from either neuronal tissue or cerebrospinal fluid (CSF). This study is of significance in part because conflicting evidence has been obtained regarding the possible nature of the peptide purported to elicit a local action in the brain. For example, Hermann et al. (1982) have reported recently that brain angiotensin I (Ang I)- and Ang II-like immunoreactivities in the rat is indistinguishable from blood [Ile5]Ang I and [Ile5]Ang II, respectively. However, other investigators (Finkielman et al., 1972; Hutchinson et al., 1978; Semple et al., 1980; Meyer et al., 1982) have suggested that the brain Ang I- and Ang II-like immunoreactivities are dissimilar to that obtained from plasma. Further confusion has been added by the study by Red et al. (1977), who concluded that the Ang I-like immunoreactivity in the brain may occur as a result of an immunoassay artifact. To shed light on the problem, we have partially purified and pharmacologically characterized the Ang I-like peptides (Ang I-LP) generated from angiotensinogen present in dog CSF following an incubation with species homologous renin. CSF was chosen as the source of angiotensinogen because this fluid has been shown to be rich in Ang I precursor (Printz et al., 1978).

**Methods**

CSF was collected by cisternal puncture from 40 mongrel dogs anesthetized with pentobarbital sodium (20 mg/kg). Only CSF samples showing a negative reaction for hemoglobin by Hemastix (Ames Company) were used. CSF stored at –20°C for no longer than 6 months was thawed and dialyzed for 36 hours at 4°C against 0.1 M ammonium acetate buffer, pH 6.5, containing 1% sodium chloride and three angiotensinase inhibitors: ethylenediaminetetraacetic acid (10 mM), N-ethylmaleimide (5 mM), and phenylmethylsulfonylfluoride (0.5 mM). The dialyzed CSF (130 ml) then was incubated for 1 hour at 30°C with 2 mg of dog kidney renin [specific activity: 40 μg Ang I/hr per mg; purified by the method of Dzau et al. (1979)] in the presence of moist Dowex 50W X8 resin (NH4+ form) preequilibrated with the dialysis buffer. The Dowex cation exchange resin was included to ensure a rapid capture of the generated Ang I-LP (Boucher et al., 1967). The absorbed Ang I-LP were eluted from the resin at an alkaline pH and evaporated to dryness under reduced pressure. The generated Ang I-LP were purified further by ion-exchange chromatography on SP Sephadex (Pharmacia), by reverse phase high pressure...
Radioimmunoassays

The Ang I–l, prepared by the modified Boucher's procedure (Boucher et al., 1967), was first deproteinized on a Sep-Pak (Waters Assoc.). The Ang I–l were absorbed on a Sep-Pak activated by sequential washing with 10 ml ethanol, 10 ml tetrahydrofuran, 10 ml methanol, and 13 ml water; then eluted with 3 ml ethanol:water:acetic acid (80:20:4). After lyophilization, the deproteinized residue was dissolved in 0.05 M sodium phosphate buffer, pH 3, and applied to an SP Sephadex C-25 column (1.6 x 57 ml) preequilibrated with the same buffer. The column was developed with a linear gradient between 225 ml 0.05 M sodium phosphate, pH 3.0, and 225 ml 0.15 M sodium phosphate, pH 9.15. Fractions 70 to 73 and 88 to 94 were combined to form pools I and II, respectively. Each pool was further characterized on reverse phase HPLC.

Reverse Phase HPLC

The Ang I–l in pools I and II were individually desalted using Sep-Pak prior to application on reverse phase HPLC columns. Reverse phase HPLC separations were performed on a C-18 microBondapak column using two isotropic elution steps: 55 minutes with methanol:water:trifluoroacetic acid (400:600:1) followed by 30 minutes with ethanol:water:acetic acid (80:20:4), at a flow rate of 1 ml/min. Samples were applied to the column in methanol:water:trifluoroacetic acid (400:600:1). To determine the retention time of synthetic [Ile5]Ang II, [Ile6]Ang I, and [des-Asp1, Ile5]Ang I, 1 µg of each peptide was dissolved in methanol:water:trifluoroacetic acid (400:600:1) and applied to the same reverse phase column under identical elution conditions.

Gel Filtration Chromatography

The HPLC fractions which contained Ang I–l were concentrated by lyophilization and filtered through a column (0.9 x 60 ml) of Bio-Gel P6 (200 to 400 mesh) equilibrated with 75 mm sodium phosphate, pH 7.0. This column was calibrated using blue dextran, ACTH, [Ile6]Ang I, [Ile6]Ang II, and [125I]Ang I.

Angiotensin Assays

Radioimmunoassays

Ang I immunoreactivity was assessed by means of an Ang I radioimmunoassay (RIA) kit (New England Nuclear). Ang II–like immunoreactivity was assessed using a RIA for Ang II developed by P. desilva et al. in our laboratories (unpublished observations).

Bioassays

Pressor bioassays of Ang I–l were carried out in bilaterally vagotomized, ganglion-blocked (hexamethonium chloride, 25 mg/kg, ip) anesthetized (sodium amytal, 100 mg/kg, ip) rats. Synthetic [Ile6]Ang I and Ang II were used as standards. Pharmacological specificity of the pressor effects of the derived Ang I–l was determined by pretreating rats with an inhibitor of Ang I converting enzyme (captopril, 400 µg/kg per min, iv) or an Ang II receptor antagonist (I Sar1, Ile8]Ang II, 250 µg/kg per min, iv). These doses were sufficient to antagonize completely the pressor effects of synthetic [Ile6]Ang I and Ang II, respectively.

Results

After desorption from the Dowex resin and lyophilization, the residue obtained from the renin-incubated CSF contained a total of 7.5 µg of Ang I equivalents, based upon measurement by RIA, but no detectable Ang II–like immunoreactivity. No Ang I–like immunoreactivity was detected in incubated dialyzed CSF or in dialyzed CSF incubated for 2 hours at 37°C in the absence of renin. This indicated that all of the Ang I–l in the residue were generated during the incubation of the CSF with renin. In the rat, intravenous administration of the residue in saline produced a pressor response that was completely blocked by the Ang II receptor antagonist [Sar1, Ile8]Ang II. The peptides in the residue appeared to require conversion to a smaller molecule to attain biological activity, based on subsequent experiments which indicated that the pressor activity of similarly derived material was greatly diminished (by ~94%) in the presence of the Ang I converting enzyme inhibitor, captopril.

The Ang I–l present in the residue were fractionated into several peaks, by SP Sephadex ion exchange chromatography (Fig. 1). Peak fractions containing Ang I–l were divided into two pools (I and II), as shown in Figure 1. Pool I elicited a more prolonged pressor response than that produced by either pool II or pure synthetic [Ile6]Ang I (Fig. 2A). However, the pressor activity of either pool was blocked by [Sar1, Ile8]Ang II (Fig. 2B). Pools I and II contained the majority of the Ang I–l (Fig. 1) and were fractionated further by means of reverse phase HPLC. More than 80% of the Ang I–like immunoreactivity of pool II applied to the column was eluted, by the solvent system described, during an 85-minute run (Fig. 3A). Only one major peak eluting with a retention time identical to that of synthetic [Ile6]Ang I was observed. The Ang I–like biological activity of this peak, as determined in the rat pressor bioassay, was identical to the amount of Ang I equivalents estimated in this peak by RIA for Ang I. Since pool II contained approximately 60% of the Ang I–like immunoreactivity of the total present in pools I and II, the HPLC data suggest, but do not prove, that approximately 60% of the Ang I–l derived from CSF is [Ile6]Ang I.

In contrast to pool II, pool I yielded four peaks of Ang I–like immunoreactivity (HPLC peaks a-d; Fig. 3B) during a 55-minute run. In addition, a fifth peak (HPLC peak e; Fig. 3B) was obtained when the more hydrophobic solvent system was used. More than 67% of the Ang I–like immunoreactivity from pool I applied to the column was eluted by the two solvent systems. HPLC peaks b–e contained most of the...
recovered Ang I-like immunoreactivity (>95%), and did not correspond with either the retention time of synthetic [Ile5]Ang I, those of other known angiotensins, or their fragments. The smallest peak (HPLC peak a) contained less than 5% of the Ang I-like immunoreactivity recovered, and displayed the same retention time as that of [Ile5]Ang I. Further purification by gel permeation chromatography was performed to estimate the molecular weight of the peptides in the various HPLC peaks of pool I. Since the amount of the Ang I-Ip in the HPLC peaks a and d was not sufficient to obtain reliable estimates, molecular weight determinations were made for HPLC peaks b, c, and e only. HPLC peak e, which contained the greatest amount of Ang I-like immunoreactivity of pool I, yielded four Ang I-Ip peaks. Apparent molecular weights of these Ang I-Ips were estimated to be 1300, 1450, 1650, and 2200 daltons. All four peptide peaks showed significant pressor activity (of the type displayed by pool I in Fig. 2) when tested in the rat bioassay. Because of the low resolution of Ang I-Ip between HPLC peaks b and c, they were combined to estimate molecular weights. After gel filtration, two peptide peaks were obtained with estimated molecular weights of 1350 and 1800 daltons; the scarcity of the material obtained prevented their full quantification in the rat bioassay.

The effect of the purification procedures on the biochemical characteristics of CSF-derived Ang I-Ip was examined indirectly. Ang I derived from angiotensinogen in plasma was subjected to the same purification procedures utilized for the purification of CSF Ang I-Ip. As expected, over 95% of the Ang I-like immunoreactivity obtained from the incubated

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**FIGURE 1.** Separation of Ang I-Ip generated from dog CSF by ion exchange chromatography. Crude Ang I-Ip were prepared by the modified Boucher et al. (1967) procedure (see text for details). Six-microgram equivalents of Ang I-like immunoreactivity dissolved in 0.05 M sodium phosphate buffer, pH 3, were applied to a SP-Sephadex C25 column (1.6 x 57 cm) preequilibrated with the same buffer. The column was developed with a linear gradient between 225 ml 0.05 M sodium phosphate, pH 3, and 225 ml 0.15 M sodium phosphate, pH 9.15. Fractions 70 to 73 and 88 to 94 were combined to form pools I and II, respectively. More than 50% of the Ang I-like immunoreactivity and Ang I pressor activity applied to the column was recovered in the two pools. Synthetic [Ile5]Ang I was recovered between fractions 88 and 94, using an identical gradient elution.

**FIGURE 2.** Pressor responses to Ang I, Ang II and Ang I-Ip in the rat. Pressor activities of 5.4 ng Ang I (1), pool II from Figure 1 (2), 3.6 ng Ang II (3), and pool I from Figure 1 (4) in the anesthetized rat in the absence (panel A) and presence (panel B) of the Ang II receptor antagonist [Ser5, Ile8]Ang II infusion (250 ng/kg per min).

**FIGURE 3.** Profiles obtained by adding Ang I-Ip in pool II (panel A) and pool I (panel B) from Figure 1 to a reverse phase HPLC system and measuring aliquots of each fraction by an Ang I RIA. Separations were performed on a C18Bondapak HPLC column (Waters Assoc.) using two isocratic elutions: 55 minutes with methanol:water:trifluoroacetic acid (400:600:1) followed by 30 minutes with ethanol:water:acetic acid (80:20:4) at a flow rate of 1 ml/min. Samples were applied to the column in methanol:water:trifluoroacetic acid (400:600:1). Fraction volume was 1 ml. UV absorbance was monitored at 210 nm. Since no further Ang I-like immunoreactivity was obtained after 62 minutes, no trace is shown after this time. More than 65% and 80% of the Ang I-like immunoreactivity in pool I and pool II, respectively, applied onto the HPLC column was recovered. Under identical elution conditions, synthetic [Ile5]Ang II, [Ile5]Ang I, and [des-Asp7, Ile8]Ang I displayed retention times of 10.9, 22.5, and 24.0 minutes, respectively.
plasma was indistinguishable from synthetic [Ile\textsuperscript{6}]Ang I on SP Sephadex ion exchange chromatography and reverse phase HPLC.

**Discussion**

A family of Ang I-Ip has been derived from endogenous precursors present in dog CSF after incubation with species homologous kidney renin. These peptides are immunologically and pharmacologically similar to [Ile\textsuperscript{6}]Ang I (molecular weight 1300 daltons) and have molecular weights ranging between 1300 and 2200 daltons. Since integrity of the carboxy terminal structure of Ang I is necessary for recognition by the Ang I antibody (Vallotton, 1974) and by the receptors of the resultant Ang II (Khosla et al., 1974), these observations suggest that the CSF-derived Ang I-Ip are similar in their carboxy terminal structures to Ang I. Furthermore, since many of the Ang I-Ip appear to have molecular weights greater than that of Ang I, it is likely that these Ang I-Ip represent analogues of Ang I that contain amino terminal extensions. Incubation of dog plasma, containing angiotensinogen of peripheral origin, with purified dog kidney renin, generated Ang I-like immunoreactivity, most (>95%) of which was chromatographically indistinguishable from [Ile\textsuperscript{6}]Ang I. Since a similar purification procedure was used to purify plasma-derived Ang I as was used for CSF-derived Ang I-Ip, these results indicate that the high molecular weight Ang I-Ip derived from CSF were not artifacts related to the purification procedure.

It is possible that the physiological significance of the present experiments is limited, since dog kidney renin and not brain isorenin has been used. Although Hirose et al. (1978) have demonstrated the presence of a renin-like neutral protease in rat brain similar to kidney renin, our studies in dogs indicate the presence in the brain of a neutral protease with renin-like activity (brain isorenin) biochemically and immunologically distinct from kidney renin (Osman et al., 1979). We believe, however, that the use of kidney renin in these experiments does not detract from the conclusion. Renin is one of the most specific proteolytic enzymes known (Skeggs et al., 1968), and the possibility that brain renin and kidney renin would rapidly split any bond other than the leu-leu bond is highly unlikely.

Our preliminary data show that Ang I-like immunoreactivity is generated following the incubation of CSF with a partially purified preparation of dog brain isorenin. Although the material generated as a result of this incubation shows significant pressor activity when injected intravenously into an anesthetized rat blood pressure preparation, the small quantities of the generated pressor material have prevented further quantification by pharmacological and chromatographic techniques. This finding may be of physiological significance, since we have shown previously that measurable quantities of brain isorenin are present in the CSF of dogs (Brosnihan et al., 1982).

We wish to thank C. Lakios and D. Wilk for their expert technical assistance.

This work was supported in part by Grants HL-6835, HL-27164 and Biomedical Research Support Grant 5674.

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INDEX TERMS: Angiotensin I-like peptides · Cerebrospinal fluid · Neuropeptide · Hypertension

Circulation Research/Vol. 52, No. 4, April 1983

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Circ Res. 1983;52:460-464
doi: 10.1161/01.RES.52.4.460

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