ANGIOTENSIN AND RENIN IN RAT AND DOG BRAIN

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Since the discovery of the angiotensins a link was sought in vain between these polypeptides and the physiological mechanism that regulates the systemic blood pressure (1). This search, however, was rich in surprises as soon as the amazing diversity of pharmacological actions of angiotensin II was established. Besides being the most powerful vasopressor molecule known, the polypeptide is the hormone for aldosterone release (2) and has several effects on the autonomic nervous system: (a) it inhibits the uptake of catecholamines by sympathetic nerve endings (3); (b) it has a direct action on cholinergic ganglionic cells (4); (c) it accelerates the rate of biosynthesis of norepinephrine (5); and (d) it acts directly or indirectly on central sympathetic structures inducing a prolonged increase in blood pressure and heart rate (6). Even if this versatility shattered the early ideas concerning the function of the polypeptide, the classical scheme that accounted for angiotensin I production was not challenged until very recently. The liberation of the polypeptide was supposed to be restricted to the plasma, where a circulating alpha-2 globulin synthetized by the liver, angiotensinogen, is split by a proteolytic enzyme, renin, synthesized by the granular cells of the juxtaglomerular apparatus of the kidney. This proteolytic step releases a 10-residue long polypeptide from angiotensinogen, denominated angiotensin I, which is itself the substrate for a specific, Cl-activated carboxipeptidase, the converting enzyme, giving rise to the octapeptide angiotensin II.

This scheme was first questioned after the finding that isolated rat renal glomeruli, devoid of plasma contamination and incubated in a simple salt medium, were able to secrete angiotensin I in great amounts (7-8). The release of the polypeptide was suppressed with protein-synthesis inhibitors.[1] The tissue synthesis of angiotensin I was strongly supported by the finding of a polypeptide in several tissues of the rat and the dog with pharmacological and

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[1] Goldstein, D. J., S. Finkielman, C. Fischer-Ferraro, and V. E. Nahmod. 1970. Regulation of the in vitro synthesis of angiotensin I. Submitted for publication.

chemical properties identical with those of angiotensin I (9). At the same time, renin-like activities and a new enzyme, pseudorenin, were demonstrated in different tissues (10–11). All these results cast some doubt upon the exclusive plasma origin of the polypeptide. We report here the identification of a polypeptide isolated from rat and dog brain as angiotensin I and the finding of an enzyme with the characteristics of classical renin from those tissues.

Materials and Methods

Animals.—Male Wistar rats weighing about 300 g each were killed by a blow on the head. After performing a thoracotomy, the aorta was cannulated and the vascular system thoroughly flushed with ice-cold Ringer solution until the brain was left bloodless. Mongrel dogs were anesthetized with sodium pentobarbital, 30 mg/kg, and anticoagulated with heparin. After bleeding them, a thoracotomy was performed and the aorta cannulated upstream and perfused with a sufficient amount of Ringer to render the brain completely devoid of blood. Four dogs were nephrectomized 36 hr before being submitted to these procedures.

Isolation of Angiotensin I.—Brain tissue was obtained through a craniotomy. Two methods for the isolation of angiotensin were used: (a) The tissues were homogenized in two volumes of phosphate buffer, 0.01 M, pH 7.5, containing ethylenediaminetetraacetic acid (EDTA), 0.003 M, in a glass homogenizer with a motor-driven glass pestle. The homogenate was boiled for 15 min and centrifuged for 10 min at 23.000 g. Four volumes of 95% ethanol were added to the supernatant and left for 12 hr at 4°C. After centrifugation the precipitate was discarded and the clear supernatant was concentrated in a Craig vacuum rotatory evaporator. Samples corresponding to 8–12 g of initial tissue were passed through a column containing Dowex 50W × 2 equilibrated with phosphate buffer, pH 7.4. The column size was 1 × 12 cm. The column was washed with phosphate buffer, pH 7.4, 0.1 M, until no more UV absorbing material appeared. It was then washed with water and the polypeptide was eluted with 7.5 ml of 0.1 N NaOH followed by 20 ml of water. The eluate was neutralized with HCl and 10 mg of polyvinylpyrrolidone were added. The solution was concentrated and passed through a Sephadex G-25 (fine) column using dextran blue 2000 and phenol red as markers. 1 ml fractions were collected and assayed for their pressor activity. Recoveries obtained when valyl-5-angiotensin II (Ciba Pharmaceutical Co., Summit, N. J.) were added in excess to brain tissue were about 90%. (b) The tissues were homogenized in 95% ethanol (1:4), and after removing the precipitate by centrifugation the supernatant was processed and assayed for angiotensin by the method of Scornik and Paladini (12).

Isolation of Renin from Brain Tissue. and Angiotensinogen—Renin and plasma angiotensinogen were isolated by the method of Haas et al. (13). Brain renin was assayed directly and indirectly, after incubation with plasma angiotensinogen. Renin concentrations were expressed in Goldblatt units (GU).[2] Protein was determined by Lowry's method (14).

Bioassay and Identification.—The pressor activity of the final samples was assayed in nephrectomized rats treated with pentolinium. Duplicate determination were used for each sample and repeated on at least two rats. Valyl-5-angiotensin II was used as standard.

Oxytocic activity was assayed on the isolated rat uterus; virgin rats weighing 150–200 g were used. One uterine horn was suspended in a 10 ml bath containing Jalon solution at 30°C. Contractile activity was assayed on the isolated guinea pig ileum. The organ was suspended in a 10 ml bath containing Tyrode's solution at 35°C. The recording was performed with the aid of an isotonic frontal writing lever with a load of 0.5 g on the uterus and 2.0 g on the ileum.

100 g of rat brain tissues were extracted as described for isolation of angiotensin (method a). This material was concentrated and subjected to countercurrent distribution. The solvent

[2] *Abbreviation used in this paper:* GU, Goldblatt units.

mixture consisted of equal volumes of 2-butanol and aqueous buffer composed of 0.1 M Na-phosphate and 3.0 M NaCl, pH 7. After 40 transfers all tubes were acidified by the addition of 0.15 ml of concentrated HCl. Time was allowed for reequilibration and the butanol phase removed and dried. Samples were diluted in saline and assayed for their pressor activity in nephrectomized rats treated with pentolinium.

RESULTS

Renin-Like Activity of Brain Extracts on Direct Assay.—Both brain and kidney extracts obtained by the method of Haas and Goldblatt showed a similar long-lasting pressor activity when assayed directly on the rat by intravenous injection. The duration of the pressor response was from 5 to 15 min according
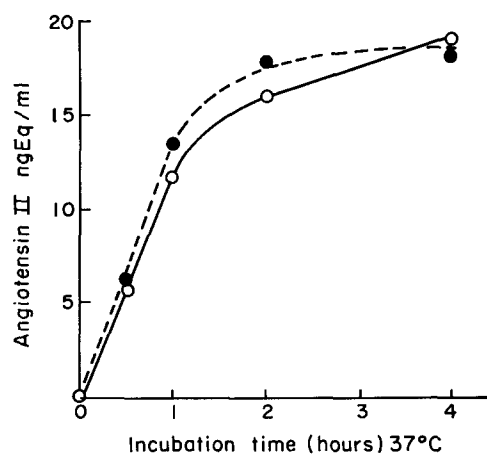

Fig. 1. Formation of angiotensin from 0.005 GU of dog brain and kidney tissue extracts with dog angiotensinogen (0.07 GU/ml). O———O Brain; ●-------● Kidney.

to its initial height. A dose-response curve could be obtained. Tachyphylaxia was observed when injections were repeated before blood pressure returned to control levels. The pressor activity was suppressed by heat coagulation.

Renin-Like Activity of Brain Extracts on Indirect Assay.—Brain extracts incubated with angiotensinogen produced a substrate bearing a short-lasting pressor activity that was identified as angiotensin. The effect of incubating 5×10^{-3} GU of brain extract with plasma renin substrate up to 4 hr can be seen in Fig. 1. There was no difference in activity when brain extracts from nephrectomized animals were employed. In all cases the reaction was conducted at 37°C in phosphate buffer, pH 7.5. The rate of angiotensin formation by the incubation of equivalent amounts of brain and kidney renin were nearly the same. The results of the direct and indirect assay of brain and kidney renin are compared in Table I. Fig. 2 shows the effect of increasing amounts of brain extract on the formation of angiotensin.

Effect of pH on the Yield of Angiotensin from Tissue Extracts and Angio-

tensinogen.—The optimal pH for the kidney and brain renin was found to be between 7 and 7.5. Below and above these pH values the activity began to decline (Fig. 3). However, at pH 5 kidney renin showed a peak.

Angiotensin-Like Activity in Brain Extracts.—A substance with short lasting pressor activity on the rat blood pressure was isolated from brain by two dif-

TABLE I

Renin Concentration in Dog Brain and Kidney Extract

Tissue	Direct method	Indirect method	Ratio indirect: direct	Specific activity GU/g of protein
	GU/g of tissue	GU/g of tissue per hr*		
Brain	0.0034	0.04	11.8	52.4
Kidney	0.61	10.50	17.0	74.3

Comparison of the direct and the indirect methods for the assay of brain and kidney renin. Each value is the average of three determinations.

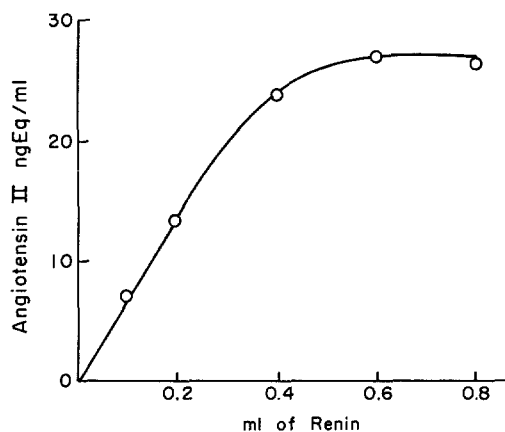* Angiotensinogen employed had a concentration of 0.2 GU/ml.

FIG. 2. Formation of angiotensin with various concentrations of dog brain tissue extracts, after 30 min incubation at 37°C with dog angiotensinogen (0.1 GU/ml). Renin activity was 0.03 GU/ml.

ferent methods. Similar results were obtained in both cases. This activity had the same characteristics as standard angiotensin and remained after boiling. The results of the experiments on Sephadex indicated that the molecular weight of the pressor substance was below 2000. In order to define chemically the active material, it was incubated with trypsin. The angiotensin-like pressor activity was completely destroyed, as was that of angiotensin II used as standard control. The angiotensin-like substance obtained from the action of brain renin-like material on plasma renin substrate behaved similarly.

Effect of Angiotensin-Like Extracts on Isolated Organ Preparation.—Two isolated organ preparations, the guinea pig ileum and the rat uterus, were used. Brain extracts contracted actively the guinea pig ileum but the response on the rat uterus was scarce. However, upon the addition of whole rat plasma or α-amilase (15), devoid by themselves of any contractile activity, to the pressor material and its incubation for 5 min at 37°C, a strong contraction was elicited on the uterus preparation. Trypsinization abolished the contractile activity. The same action on isolated organ preparations was observed when the short-lasting pressor substance, obtained from incubation of brain extracts with renin-like activity and plasma angiotensinogen, was assayed.
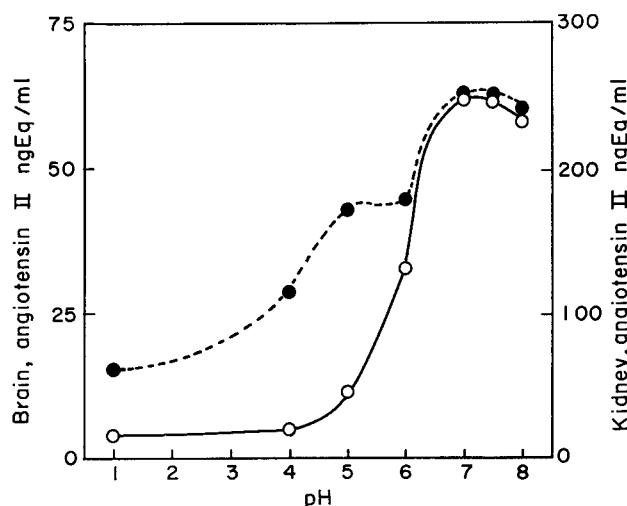

Fig. 3. Effect of pH on the formation of angiotensin with dog brain and kidney tissue extracts, after 60 min incubation at 37°C. O——O Brain; ●--● Kidney.

Countercurrent Distribution of the Short-Lasting Pressor Material Isolated from Brain. Two pressor peaks, A and B (Fig. 1), were observed on a countercurrent distribution of the pressor material obtained from rat brain by the method employed for the isolation of angiotensin. Their respective distribution coefficients were k = 0.17 for peak A and k = 0.74 for B. The pressor activity was lost upon incubation with trypsin. The material of peaks A and B caused a strong contraction on the isolated guinea pig ileum preparation. Samples from peak A contracted the rat uterus but those from peak B did not elicit any response, unless doses 20 times larger than those from peak A were used. Nevertheless, after incubation with fresh plasma devoid of pressor activity, samples of peak B were almost as active as those of peak A on the isolated rat uterus preparation.

In order to corroborate that peak B corresponds to angiotensin I a new

countercurrent distribution was performed and samples from tubes 14–24 were pooled and incubated with fresh rat plasma and 0.003 M of NaEDTA for 10 min at 37°C. On a second countercurrent distribution almost all the pressor activity of the original peak B was found in the new peak A, which is characteristic of angiotensin II.
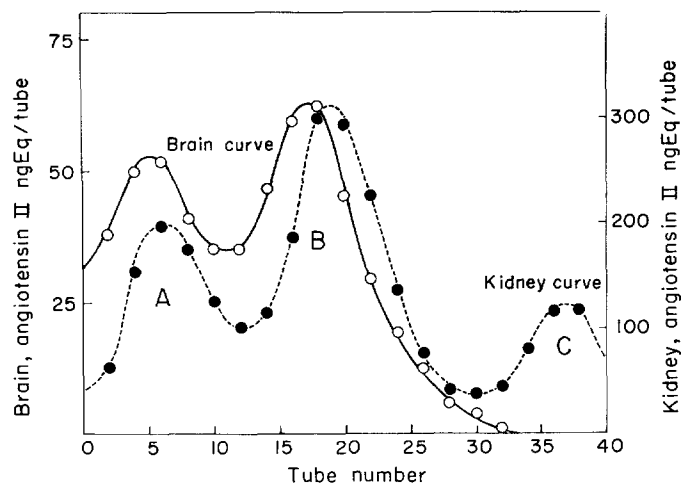

Fig. 4. Countercurrent distribution of brain tissue extracts of rats with angiotensin-like activity (O——O) compared with that of the supernatant of incubated renal glomeruli (●---●).

TABLE II

Concentration of angiotensin and norepinephrine* in different segments of dog brain

Tissue	Angiotensin‡ (ng/g tissue)	Norepinephrine (μg/g tissue)
Total brain	4.86	0.16
Cerebellum	0.00	0.06
Basal nuclei	3.00	0.09
Cerebrum§	3.62	0.12
Brain stem	9.84	0.39
Hypothalamus	85.30	0.76

Dogs were nephrectomized 36 hr before they were killed.
* Data according to Carlsson (16).
‡ Average of three to five determinations.
§ Cerebrum without basal nuclei.

In Fig. 4 two countercurrent distribution curves are compared, one corresponding to rat brain material and the other one to the supernatant of incubated glomeruli. The correspondence of peaks A and B in either distribution is remarkable. Peak C, a still unidentified pressor substance, was only found in the kidney.

Concentration of Renin and Angiotensin in Brain of Rats and Dogs.—Concentration of the enzyme and the polypeptides is higher in rat brain (4.86 ng per g of tissue in dog brain, 11.20 ng per g of tissue in rat brain). From countercurrent distribution experiments it seems clear that the main pressor activity is due to the presence of angiotensin I, about 62%, and that our brain extracts also contain angiotensin II. Bilateral nephrectomy did not affect renin and angiotensin concentration in the brain.

Distribution of Angiotensin in Dog Brain.—Concentrations of angiotensin are markedly different in several portions of the encephalon (Table II). The hypothalamus showed the highest levels, whereas no angiotensin was present in the cerebellum. A remarkable correlation was found between the angiotensin content of various segments of brain and that of norepinephrine, as reported in the literature (16, Table II).

DISCUSSION

The presence in brain extracts of nephrectomized rats and dogs of a vasoactive substance with long-lasting pressor activity which is thermolabile, nondialyzable, and forms a vasoconstrictive material when incubated with renin plasma substrate at pH 7, defines this substance as renin, with almost identical characteristics to classical kidney renin. Renin-like activity was found in various tissues from different species (11); this is the first report of such a finding in the nervous system. Gould, Skeggs, and Kahn failed to demonstrate renin in hog brain (17) and pseudo-renin (10), a new angiotensin-forming enzyme, was not investigated.

Since a polypeptide similar to angiotensin I was pharmacologically and chemically detected in several fresh, nonincubated tissues of the rat, even in brain tissue (9), our search was focused on a closer identification of the polypeptide and its distribution in different portions of the encephalon. From the effect elicited by this material on (*a*) the rat blood pressure, on (*b*) the guinea pig ileum and on (*c*) the rat uterus, where its activation by isologous blood plasma and α-amilase was demonstrated, and (*d*) by its inactivation when incubated with trypsin, it was concluded that the pressor substance present was angiotensin I or a closely related polypeptide.

Countercurrent distribution confirmed that the main pressor polypeptide present in brain extracts was actually angiotensin I, though significant amounts of angiotensin II were also demonstrated. The presence of angiotensin II may be related to the reported finding of a converting enzyme in brain tissue (18). Contamination with minute amounts of plasmatic converting enzyme seems far less probable. The particular distribution of such a molecule in brain and its striking correlation with the norepinephrine content of different portions of the encephalon may be significant, if it is considered that angiotensin II accelerates the biosynthesis of catecholamines (5).

Angiotensin II has an unequivocal effect on arterial blood pressure when

injected either continuously during long periods in the blood stream (19) or intraventricularly in the brain (6). The available pharmacological evidence suggests that angiotensin may increase sympathetic activity when the peptide comes in contact with structures reached from ventricular cavities. The hypothalmic region, close to the third ventricule, shows the highest level of angiotensin found, and about one-third of it is angiotensin II. This may have important physiological and physiopathological consequences in areas related to the biosynthesis of catecholamines, the regulation of the cardiovascular system (20), the mechanism of thirst (21), and the over-all behavior of the autonomic nervous system.

The finding of renin and angiotensin in brain tissue is an important clue to its local formation. Brain renin substrate still remains to be studied. Preliminary investigations suggest that the renin substrate pool is small.[1]

SUMMARY

An enzyme with the characteristics of classical renin was isolated from brain extracts of nephrectomized dogs. This enzyme is thermolabile, nondialyzable, and forms a vasoconstrictive material when incubated with renin plasma substrate at pH 7.

A short lasting pressor activity was also found in brain extracts of dogs and rats. This activity was due to a substance identified by chemical and pharmacological tests as angiotensin. Countercurrent distribution of brain extracts of rats showed that 38% of the pressor activity corresponded to angiotensin II and the remainder to angiotensin I. A remarkable correlation was found between angiotensin and norepinphrine concentrations in different portions of the encephalon of the dog.

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