Ghera, G. Klein, *J. Natl. Cancer Inst.* **60**, 227 (1978).
21. S. Ohno *et al.*, *Cell* **18**, 1001 (1979).
22. G. L. C. Shen-Ong, E. J. Keath, S. P. Piccoli, M. D. Cole, *ibid.* **31**, 443 (1982); L. J. Harris, R. B. Long, K. B. Marcu, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 4175 (1982); J. M. Adams *et al.*, *ibid.*, p. 6966; J. F. Mushinski, S. R. Bauer, M. Potter, E. P. Reddy, *ibid.* **80**, 1073 (1983).
23. J. M. Adams, S. Gerondakis, E. Webb, L. M. Corcoran, S. Cory, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1982 (1983).
24. R. F. Doolittle *et al.*, *Science* **221**, 275 (1983); M. D. Waterfield *et al.*, *Nature (London)* **304**, 35 (1983).
25. M. D. Hoggan, C. E. Buckler, J. F. Sears, W. P. Rowe, M. A. Martin, *J. Virol.* **45**, 473 (1983).
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Angiotensin Synthesis in the Brain and Increased Turnover in Hypertensive Rats

Abstract. *The missing link in the evidence for an active endogenous renin angiotensin system in the brain has been the demonstration of local angiotensin synthesis in the central nervous system in vivo. In this report the extraction and characterization of angiotensin I and angiotensin II from the brain of rats is described. The accumulation of angiotensin I was enhanced in hypertensive rats when the conversion to angiotensin II was blocked in vivo by the converting enzyme inhibitor captopril.*

The renin angiotensin system (RAS) is still usually considered to be a circulating blood hormone system. Angiotensin II (ANG II) in the brain stimulates pituitary hormone release and sympathetic tone and thereby influences blood pressure and volume homeostasis (*1, 2*). These effects suggested that ANG II, like other peptide hormones, might also occur endogenously in the brain as a neuropeptide (*3*). Immunohistochemical evidence supported such a possibility (*4–8*), but biochemical data were controversial (*9, 10*) or negative (*11*).

We report here the presence of [Ile^5]ANG I, [Ile^5]ANG II, and [Ile^5]-ANG III in the brain of intact and bilaterally nephrectomized rats. We also studied the turnover of angiotensin in the brain and found that inhibition of converting enzyme (CE) led to an accumulation of ANG I in nephrectomized rats, thus proving that active endogenous synthesis of the peptide occurs in the brain. This effect was more marked in hypertensive than nonhypertensive rats, indicating that the brain RAS is stimulated in spontaneously hypertensive rats of the stroke prone strain (SHRSP). Our data thus show that ANG II is a neuropeptide that is locally synthesized in the brain.

Cerebrospinal fluid (CSF) was collected by puncture of the fourth brain ventricle (*12*) from one group of ten untreated rats and another group of rats that received, 15 minutes before the CSF sampling, an injection of 100 mU of hog kidney renin in the fourth ventricle. The generation of ANG I was measured in vitro in pooled CSF, that was incubated at 37°C for 4 hours with purified hog kidney renin and tris-maleate buffer, *p*H 5.5, containing angiotensinase inhibitors (*12*). ANG I and ANG II were measured in all samples by radioimmunoassay and the peptides were characterized by high-pressure liquid chromatography (HPLC).

Angiotensin was extracted from the brain of adult rats, each weighing 250 g. The animals were bilaterally nephrectomized and brain tissue was obtained 24 hours later. The brains were freed of blood by transcardiac perfusion with warm 0.9 percent saline containing 25 U of heparin per milliliter at 37°C and then quickly removed, dissected, and frozen on dry ice. For the extraction of angiotensin, tissue was homogenized in 0.1*N* HCl at a ratio of 1:10, weight to volume, boiled for 5 minutes, and centrifuged at 30,000*g* and 4°C for 30 minutes. The clear supernatant was collected and the pellet was reextracted with 0.1*N* HCl. The supernatants were pooled and purified on octadecasilyl-silica (ODS-silica) cartridges (Sep-Pak C 18, Waters Associates). In this procedure, the cartridges were carefully washed with 3 ml of methanol and 10 ml of a 1 percent trifluoracetic acid (TFA) solution in water. To minimize nonspecific adsorption, the cartridges were then coated with 1 ml of a 1 percent polypeptide solution (Serva, Heidelberg) and washed again with a

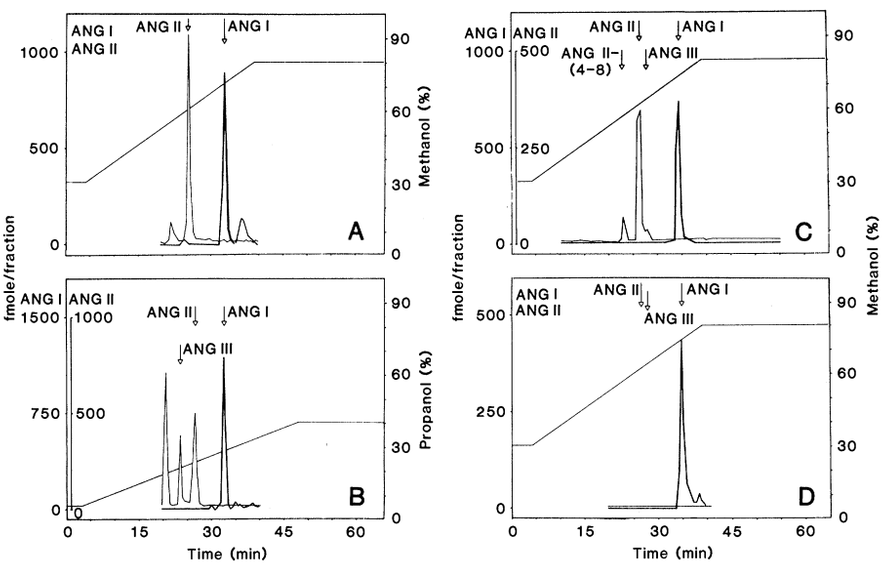

Fig. 1. (A and B) Characterization of ANG I and ANG II extracted from brain stem of rats. (C and D) ANG I and ANG II in CSF after reaction with renin in vivo (C) or in vitro (D). The HPLC system consisted of two pumps (model 6000A), a model 660 solvent programmer, and a U6K injection system coupled to a variable wavelength UV-detector (model 450, Waters Associates). All separations were done on ODS-silica reversed phase columns (Bondapak C18, 300 by 4 mm, Waters Associates) with a particle size of 10 μm and two different gradient elution systems. (A) For the methanol gradient, 10 m*M* ammonium acetate buffer was adjusted to *p*H 4.5 or *p*H 5.4 with acetic acid, and methanol was increased linearly from 30 to 80 volumes per 100 volumes within 35 minutes. (B) For the isopropanol gradient, 10 m*M* triethylammonium-phosphate (TEAP) was adjusted to *p*H 3.0 with concentrated phosphoric acid. In a linear gradient, isopropanol concentration was increased from 5 to 40 volumes per 100 volumes within 45 minutes. Sample injection volumes varied between 100 and 950 μl; flow rate was 1.0 ml/minute and fractions were collected for 30 to 60 seconds into polyethylene tubes and subjected directly to radioimmunoassay. Blank controls for Sep-Pak purification and HPLC comprised the complete procedure as described but without sample application. Angiotensin was measured in the fractions by radioimmunoassay as described (*12*). Note the baseline separation of angiotensin peptides on the HPLC systems and the identity of ANG cleaved in vivo (C) and in vitro (D) by renin from CSF angiotensinogen and extracted from the brain (A and B) with synthetic [Ile^5]ANG I and [Ile^5]ANG II (arrows). The first peak of immunoreactivity with the ANG II antibody (B) is unidentified but may correspond to the ANG II-(4–8) pentapeptide fragment.

mixture of methanol, water, and TFA (80:19:1, by volume) and with a 1 percent TFA solution. The brain extract was applied and the cartridges washed twice with 5 ml of a mixture of 1 percent TFA in 1 percent NaCl. The peptides were eluted twice with 1 ml of a mixture of methanol, water, and TFA (80:19:1, by volume). The organic phase of the eluant was removed under a flow of nitrogen and then taken to dryness in a vacuum box. The residue was dissolved in 1000 μl of 0.001*N* HCl and transferred into polyethylene tubes. This solution was applied to the HPLC column.

The angiotensin peptides in the eluate fractions were measured by specific radioimmunoassays (*12*). All methodological experiments were done in triplicate or more. The recovery of angiotensin was monitored by adding ^{125}I-labeled ANG I to the tissue immediately after sampling and counting the intact peptide after HPLC. Mean recovery of iodinated ANG I was 61.53 ± 2.1 percent. The same values were obtained if unlabeled synthetic ANG I or ANG II was added. Values are corrected for recovery. For angiotensin extraction from brain areas the tissue was pooled from ten rats. Five to seven groups, each with ten rats, were used. Results are given as means (± standard error of the mean). Significance of differences was assessed by Student's *t*-test or by analysis of variance.

For all angiotensin peptides baseline separation was achieved on the HPLC systems described in Fig. 1. The retention times on the methanol and isopropanol systems, respectively, were 33.85 and 31.85 minutes for [Ile^5]ANG I, 25.7 and 23.79 minutes for [Ile^5]ANG II, 26.9 and 26.0 minutes for [Ile^5]ANG III, and 23.4 and 20.3 minutes for [Val^5]ANG II. Each of the peptides could thus be separated and unequivocally identified on the HPLC systems. Coelution of the brain peptides with the synthetic standard angiotensins on two different HPLC systems and measurement by specific ANG I and ANG II radioimmunoassays was taken as evidence for identity and amino acid homology.

The data in Fig. 1 show that ANG I is cleaved from brain angiotensinogen in the CSF in vivo and in vitro. It is the same as ANG I extracted from brain and corresponds to [Ile^5]ANG I. ANG I is converted to [Ile^5]ANG II in the CSF in vivo but not under in vitro incubation conditions when CE is inhibited. The angiotensins extracted directly from brain tissue (Fig. 1, A and B) had the same retention times on two different HPLC systems and the same immunospecificity and are thus probably the same as CSF [Ile^5]ANG I and [Ile^5]ANG II and are identical with the synthetic standard peptides. These data demonstrate the presence of ANG I and ANG II in the brain. In addition, they show that the amino-terminal part of the high molecular weight precursor from which brain angiotensin is cleaved is the same as CSF angiotensinogen, though differences of the brain and plasma angiotensinogen molecules are still possible.

Table 1. Distribution of ANG I and ANG II in the brain. Tissue from ten rats was pooled. Values represent measurement in five pools for each brain area and are corrected for recovery. The data are expressed as femtomoles per gram of tissue (wet weight).

Brain area	ANG I	ANG II
Hypothalamus	121 ± 15	590 ± 106
Brain stem	94 ± 9	69 ± 6
Cerebellum	63 ± 9	57 ± 4
Cortex	45 ± 3	15 ± 1

Brain ANG I concentrations ranged between 45 and 121 fmole per gram of tissue and ANG II concentrations between 15 and 590 fmole. The ANG I concentrations in the hypothalamus were not significantly altered by nephrectomy; 121 ± 15 fmole/g in controls and 143 ± 8 fmole/g in nephrectomized rats (Table 1). For comparison, ANG II levels in the plasma were in the range of 20 to 800 fmole/ml depending on the pathophysiological conditions (*2*).

Since measurement of absolute levels of neuropeptides do not allow a precise quantitative assessment of their biological activity, we attempted to measure the turnover of ANG I to ANG II. This was achieved by blockade of CE activity in the brain of nephrectomized rats. Accumulation of ANG I and decrease of ANG II under these conditions would indicate an active turnover of the peptides. The CE inhibitor captopril (500 μg) was injected intracerebroventricularly three times at 60-minute intervals. Sixty minutes after the last drug administration, the brains were removed and ANG I and ANG II were extracted and measured as described above. In SHRSP's treated with CE inhibitor, we observed a statistically significant increase of 99 percent ($P < 0.05$) for ANG I and a decrease of 36 percent for ANG II (control: 102 ± 10 fmole/g for ANG I and 282 ± 54 fmole/g for ANG II; captopril treated: 203 ± 36, fmole/g for ANG I and 180 ± 31 fmole/g for ANG II). In normotensive Wistar Kyoto rats (WKY) treated with CE inhibitor, however, no statistically significant changes of ANG I and ANG II concentrations were observed in the hypothalamus (control: 224 ± 23 fmole/g for ANG I and 128 ± 40 fmole/g for ANG II; captopril treated: 270 ± 24 fmole/g for ANG I and 177 ± 31 fmole/g for ANG II) (Fig. 2). We have no explanation for this finding. The dose of captopril used was previously shown to completely block the blood pressure response to intracerebroventricularly injected ANG I (*13*). The data are probably best explained by a higher activity of the brain RAS in SHRSP and preferential blockade of CE at stimulated, blood pressure relevant, circumventricular sites. Incomplete CE blockade and a slower turnover must be assumed in other brain areas of SHRSP and in WKY.

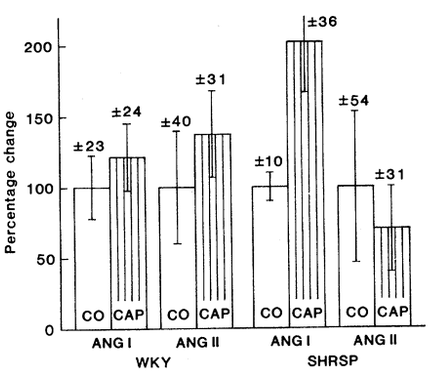

Fig. 2. Turnover of angiotensin peptides in the hypothalamus of nephrectomized normotensive Wistar Kyoto rats (*WKY*) and spontaneously hypertensive rats of the stroke prone strain (*SHRSP*). The biosynthetic pathway to ANG II was blocked by inhibition of converting enzyme in the brain with captopril (*CAP*). Accumulation of ANG I and decrease of ANG II indicates higher turnover in SHRSP compared to WKY. For experimental details and absolute values see Table 1 and text. Values for five to seven groups of ten rats each are expressed relative to controls (*CO*).

The clear identification of [Ile^5]ANG I and [Ile^5]ANG II in the brain, the identity of these angiotensins with the angiotensin cleaved from CSF angiotensinogen, the persistence of ANG I at or above control levels after nephrectomy and its accumulation after CE blockade in SHRSP not only prove the active synthesis of angiotensin in the brain, but also show that the turnover is more rapid in hypertensive rats. These data are in agreement with previous findings that renin activity is higher in cardiovascular control centers of SHRSP (*14*) and that blockade of the brain RAS in SHRSP leads to a lowering of blood pressure (*5, 13, 16*). There is also evidence that the brain RAS may be stimulated in patients with high blood pressure. The lowering of blood pressure in these subjects with CE inhibitors introduced as antihypertensive agents may, in part, be due to an inhibition of central ANG II (*13, 15, 16*). The possible interference of these drugs

with local angiotensin in the brain or other tissue is therefore of practical importance.

These data also have theoretical implications. ANG II is present in the blood, kidney (*17–19*), adrenal gland (*19, 20*), and brain. This adds evidence to the theory that the same substance may act as a circulating blood hormone, tissue factor, neurohormone, or neurotransmitter (*21*). The occurrence of angiotensin in the central nervous system, endocrine glands, and blood may thus explain its coordinating function in cardiovascular regulation, which includes direct control of peripheral resistance and blood volume as well as complex behavior such as thirst and salt appetite.

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References and Notes

1. W. B. Severs and A. E. Daniels-Severs, *Pharmacol. Rev.* **25**, 415 (1973).
2. D. Ganten and G. Stock, *Klin. Wochenschr.* **56**, (I), 31 (1978).
3. D. Ganten, M. P. Printz, M. I. Phillips, B. Schölkens, Eds., *Experimental Brain Research* (Suppl. 4) (Springer-Verlag, New York, 1982).
4. K. Fuxe, D. Ganten, T. Hökfelt, P. Bolme, *Neurosci. Lett.* **2**, 229 (1976).
5. D. Ganten *et al.*, *Front. Neuroendocrinol.* **5**, 61 (1978).
6. D. G. Changaris, L. M. Demers, L. C. Keil, W. B. Severs, *Central Actions of Angiotensin and Related Hormones*, J. P. Buckley and C. M. Ferrario, Eds. (Pergamon, New York, 1977), p. 233.
7. E. A. Zimmermann, in *Neurosecretion and Brain Peptides*, J. B. Martin, S. Reichlin, K. L. Bick, Eds. (Raven, New York, 1981), p. 63.
8. M. I. Phillips, J. Weyhenmeyer, D. Felix, D. Ganten, W. E. Hoffman, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38**, 2260 (1979).
9. J. A. Reid, *Circ. Res.* **41** (No. 2), 147 (1977).
10. D. Ganten, *ibid.* **42** (No. 5), 732 (1978).
11. D. K. Meyer, M. I. Phillips, L. J. Eiden, *J. Neurochem.* **38**, 816 (1982).
12. P. Schelling, U. Ganten, G. Sponer, Th. Unger, D. Ganten, *Neuroendocrinology* **31**, 297 (1980).
13. Th. Unger, I. Kaufmann-Bühler, B. Schölkens, D. Ganten, *Eur. J. Pharmacol.* **70**, 467 (1981).
14. P. Schelling *et al.*, *Neuropharmacology* **21**, 455 (1982).
15. J. F. E. Mann, M. I. Phillips, R. Dietz, H. Haebara, D. Ganten, *Am. J. Physiol.* **234**, H629 (1978).
16. W. J. McDonald, C. Wickre, S. Aumann, D. Ban, B. Moffitt, *Endocrinology* **107**, 1305 (1980).
17. R. Taugner *et al.*, *Klin. Wochenschr.* **60**, 1218 (1982).
18. F. A. O. Mendelsohn, *Clin. Sci. Mol. Med.* **57**, 173 (1979).
19. K. Hermann, R. Taugner, Th. Unger, R. E. Lang, D. Ganten, in preparation.
20. R. L. Aguilera, A. Schirar, A. Baukal, K. J. Catt, *Nature (London)* **289**, 507 (1981).
21. J. Roth *et al.*, *N. Engl. J. Med.* **306** (No. 9), 523 (1982).

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Morphine Tolerance in Genetically Selected Rats Induced by Chronically Elevated Saccharine Intake

Abstract. *Rats of line LC2-Hi that drank about 50 milliliters of a highly palatable saccharine solution daily for 28 consecutive days did not show morphine analgesia or an opioid form of stress-induced analgesia and were not responsive to naloxone. These findings support the idea that chronically elevated saccharine intake may cause increased release and utilization of endogenous opiates.*

There is evidence that the ingestion of sweet substances is, in part, under the control of endogenous opioids. Specifically, naloxone, an opioid antagonist, markedly reduces the ingestion of a sweet solution (*1, 2*) while having only moderate effects on drinking in response to systemic dehydration (*3*). Furthermore, the opioid agonist morphine causes a threefold increase in consumption of a 0.5 percent saccharine solution (*4*) while exerting little effect on water intake after fluid deprivation (*2*). Thus activation of the opioid system enhances the intake of sweet solutions. It has not been known whether the reverse relation also holds, that is, whether increased ingestion of a saccharine solution results in a greater release and utilization of endogenous opioids. If such a relation exists, one would expect animals consuming large volumes of saccharine solution over long periods to be more tolerant to morphine than their controls. We now report that repeated consumption of a 3 m*M* sodium saccharin solution induced cross-tolerance to morphine and to stress-induced analgesia.

The availability of LC2 lines of rats made this experiment feasible (*5*). The LC2-Hi line (*6*), especially the females, are distinguished by elevated rates of

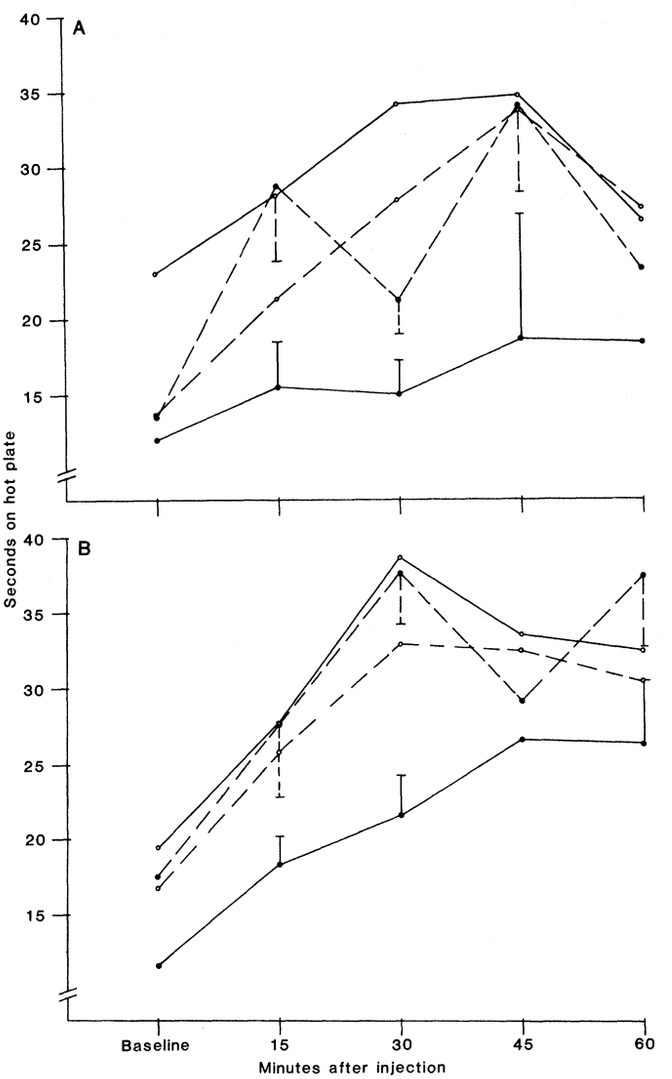

Fig. 1. Mean latencies of the pain response after the injection of morphine hydrochloride at 2.5 mg/kg (A) and 5.0 mg/kg (B). Open and closed circles represent LC2-Lo and LC2-Hi lines, respectively. Dashed and continuous lines represent water and saccharine exposure, respectively. Vertical lines represent standard errors of the means of LC2-Hi groups consuming water or saccharine.