Effects of Chronic Sodium Depletion on Canine Brain Renin and Cathepsin D Activities

K. BRIDGET BROSNIHAN, PH.D., ROBERT R. SMEBY, PH.D., AND CARLOS M. FERRARIO, M.D.

SUMMARY The activities of brain renin and cathepsin D were measured in brain regions of 10 dogs on a normal sodium intake (65 mEq Na+/day) and 10 other dogs placed on a low sodium diet (<4 mEq Na+/day) for 21 days and given a diuretic. The purpose of this study was twofold: to assess the effect of sodium depletion on brain renin activity; and to assess in the same regions alterations in brain renin and cathepsin D activities. Sodium depletion caused a ninefold increase in plasma renin activity, hemococoncentration, and hyponatremia. In the presence of marked hyperreninemia, the average cerebral renin activity was reduced significantly; the most pronounced changes occurred in the upper and lower brain-stem regions. Cerebrospinal fluid renin was increased by 30%, but this change was not significant in sodium-depleted dogs. There were no significant alterations in cathepsin D activity whether assessed in total or regional brain areas. These observations support the view that there is an inverse relationship between plasma and brain renin activity in chronically sodium-depleted dogs. Additionally, evidence is provided that brain renin activity is modified independently from cathepsin D activity.

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KEY WORDS cerebrospinal fluid • plasma renin activity • brain stem • sodium depletion • brain peptides and enzymes • isorenin • acid proteases

Although the brain renin angiotensin system has been the focus of extensive research in recent years, controversy surrounds what central function it normally serves. According to Ganten,1 this peptide system may function in the brain to regulate blood pressure via a presumed action upon central adrenergic neurons. Severs2 and Phillips3 suggest that brain renin affects the intake and excretion of water and sodium via activation of thirst, sodium appetite, and vasopressin release. A unifying hypothesis has been proposed by Ferrario et al.4 They suggest that the kidney and brain renin angiotensin systems function in a cooperative manner to exert total integration of central and peripheral autonomic nervous system function and sodium and water metabolism.

Before 1979, a welter of conflicting observations raised doubts about the existence of renin in the brain and handicapped efforts to establish its physiological significance. The ability of brain homogenates to generate angiotensin I was attributed to an acid protease (cathepsin D) normally present in lysosomes.5,6 Thus, in vivo this enzyme would be forced to exert its functions separated from renin substrate and at an unfavorable pH. Further, any angiotensin I formed by cathepsin D might be rendered inactive by this enzyme before it could have an action outside the cell. Using two different chromatographic procedures, Osman et al.,7 Smey et al.,8 and Hirose et al.9 independently showed that cathepsin D could be separated from brain renin and that the latter had the ability to form angiotensin I at a neutral pH. This discovery permitted us to evaluate the possible existence of an interplay between the brain and kidney renin angiotensin systems by inducing a stage of prolonged hyperreninemia due to sodium restriction. Both renin and cathepsin D activities were determined in subdivisions of the brain to ascertain if the protein that acted enzymatically to cleave angiotensin I from its substrate was modified independently of the acid protease.

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BRAIN RENIN DURING SODIUM DEPLETION/Brosnihan et al.

Methods

Control data were obtained in 10 dogs (23 ± 1 kg) fed a standard laboratory diet (Purina Lab Canine Diet No. 5000, Ralston Purina Company, St. Louis, Missouri) providing an intake of 65 mEq Na+/day. A sodium restriction protocol was implemented in another 10 dogs (20 ± 1 kg) by giving the animals a sodium-deficient diet (Prescription Diet, H/D, Hills Pet Products, Inc., Topeka, Kansas) containing no more than 4 mEq Na+/day for 3 weeks. To enhance the degree of sodium depletion, furosemide (40 mg, i.m., Lasix, Hoechst-Roussel Pharm. Inc., Somerville, New Jersey) was given on the last 3 days of the regime.

On the day of the experiment, 10 cc of venous blood was collected in conscious dogs for determinations of plasma renin activity, hematocrit, and electrolytes. The animals were then anesthetized with sodium pentobarbital (30 mg/kg i.v.). In a subgroup of four of the 10 normal and five of the other sodium-depleted dogs, 5 ml of cerebrospinal fluid (CSF) was taken by puncture of the cisterna magna 30 minutes after anesthesia, as described previously. All samples were immediately frozen at −20°C until assayed. The heads of the dogs were then rapidly perfused via a transthoracic approach with chilled, isotonic saline after the thoracic aorta had been clamped just below the subclavian artery. Following this perfusion, brain homogenates do not contain measurable amounts of plasma renin (unpublished observations). The cranium was opened with the aid of a Stryker autopsy saw and the brain washed quickly. Blocks of tissue from lower and upper brain stem, cerebellum, anterior and posterior cortex, and spinal cord were obtained and frozen immediately. The lower and upper brain stem regions were separated between the inferior and superior colliculi, and the cortex was divided along the temporal lobe.

Brain subdivisions were homogenized using a Polytron blender (Brinkman Instruments, Westbury, New York) in phosphate buffer 0.1 M at pH 6.5 (1 ml of buffer/g of wet tissue). The insoluble material was removed by centrifugation (3000 g for 30 minutes) and reextracted in the same manner. The entire extraction was conducted at 4°C to minimize enzymatic destruction of renin or other proteins. The two supernatant portions were combined and frozen until assayed. Dog renin substrate was purified as described elsewhere from plasma of dogs nephrectomized 48 hours before collection of blood. Brain renin activity was assayed as described by Smeby et al. Briefly, to 100 μl of substrate solution containing 20 mg/ml of purified dog renin substrate we added 100 μl of tissue extract and 100 μl of 0.1 M phosphate buffer at pH 6.5, the optimum pH for angiotensin I production by renin in the brain. It has also been shown previously that at this pH the contribution of cathepsin D to angiotensin I production is negligible. The buffer contained 10 mM disodium ethylenediaminetetraacetic acid (EDTA), 5 mM N-ethyl maleimide, and 0.5 mM p-methylphenylsulfonyl fluoride to inhibit activity from other neutral proteases. The solution was incubated at 37°C for 16 hours to detect the very low levels of renin activity present in brain tissue extracts, and the reaction was stopped by placing the tubes in a bath of boiling water for 10 minutes. The denatured protein was removed by centrifugation and the angiotensin I in the supernatant was measured by radioimmunoassay with a commercial kit (New England Nuclear Corporation, Boston, Massachusetts). Brain renin activity was expressed as nanograms (ng) of angiotensin I generated per hour per milligram (mg) of protein. Acid protease (cathepsin D) activity was measured at its optimum pH 3.5, in aliquots of the same brain tissue, by the method of Anson and expressed as mg of trichloroacetic acid soluble-tyrosine produced per hr per mg of protein (mg TYR/hr/mg protein). Protein determinations were performed according to the method of Lowry et al. Samples (5 cc) of CSF were lyophilized and reconstituted in 1 ml of phosphate buffer before being assayed for renin activity.

To assess the effect of diet on measured variables, analysis of variance was performed using the BMDP2V program with a repeated measures design. This BMDP2V program was run using PROPHET, a national computer resource supported in part by the Biotechnology Resources Program, Division of Resources, National Institutes of Health. Also the Student's t test for paired and unpaired data was used. Differences were considered statistically significant when p < 0.05.

Results

A deficit in sodium balance, persisting for 3 weeks, produced the following metabolic differences in the 10 normal (NS) and 10 sodium-depleted (LS) dogs. Plasma renin activity increased significantly to 17.9 ± 3.4 ng/ml/hr in LS dogs compared to 1.3 ± 0.5 ng/ml/hr in NS dogs. Sodium depletion caused hemoconcentration (45% ± 1% in NS vs 53% ± 1% in LS, p < 0.01), mild hypokalemia (4.6 ± 0.2 in NS vs 5.2 ± 0.1 mEq/liter in LS, p < 0.05), and mild hyperkalemia (4.6 ± 0.2 in NS vs 5.2 ± 0.1 mEq/liter in LS, p < 0.05). These findings agree with those reported previously.

In the CSF of NS anesthetized dogs, the renin activity was 0.099 ± 0.009 ng/ml/hr, a value 92% less than the activity of renin in plasma (p < 0.05), with a range between 0.073 and 0.116 ng/ml/hr (table 1). Following sodium depletion, the mean renin activity in the CSF averaged 0.129 ± 0.026 ng/ml/hr. This value was not significantly different from that measured in NS dogs, even though it averaged 30% above normal values. An estimate of the relationship between plasma (PI) and ventricular (CSF) renin activity was derived as the PI/CSF renin. This ratio averaged 15 ± 8 in normal animals compared to 106 ± 13 in the salt depleted ones. In other words, the sevenfold increase in the ratio of PI/CSF renin after sodium depletion was essentially due to the large increase in plasma renin activity. When both plasma and CSF renin activities were expressed as a function of the amount of protein present in the appropriate fluid, CSF levels of renin were significantly higher than the corresponding values of plas-
TABLE 1. Comparison of Plasma and Cerebrospinal Fluid Level of Renin Activity in Subgroups of Normal and Sodium-Depleted Dogs

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Sodium depleted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma renin activity</td>
<td>1.3 ± 0.5</td>
<td>12.6 ± 1.4†</td>
</tr>
<tr>
<td>(ng/ml/hr)</td>
<td></td>
<td></td>
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<tr>
<td>Plasma renin activity</td>
<td>0.019 ± 0.008</td>
<td>0.126 ± 0.024†</td>
</tr>
<tr>
<td>(ng/hr per mg protein)</td>
<td></td>
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<tr>
<td>CSF renin activity</td>
<td>0.099 ± 0.009†</td>
<td>0.129 ± 0.026‡</td>
</tr>
<tr>
<td>(ng/ml/hr)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSF protein (mg/dl)</td>
<td>23.8 ± 2.0</td>
<td>21.4 ± 2.0</td>
</tr>
<tr>
<td>CSF renin activity</td>
<td>0.542 ± 0.100†</td>
<td>0.588 ± 0.070‡</td>
</tr>
<tr>
<td>(ng/hr per mg protein)</td>
<td></td>
<td></td>
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<tr>
<td>Plasma/CSF</td>
<td>15 ± 8</td>
<td>106 ± 13*</td>
</tr>
<tr>
<td>Plasma/CSF (specific activity)</td>
<td>0.04 ± 0.02</td>
<td>0.23 ± 0.04*</td>
</tr>
</tbody>
</table>

Values are means ± SEM from four normal and five sodium-depleted dogs.

*p < 0.05, normal vs sodium-depleted.
†p < 0.01, normal vs sodium-depleted.
‡p < 0.05, plasma vs CSF renin activities, paired t test.

ma in both normal (p < 0.05) and sodium depleted dogs (p < 0.05) (table 1). The plasma to CSF ratio of the normalized renin activities was increased from 0.04 ± 0.02 in normal dogs to 0.23 ± 0.04 in sodium-depleted dogs, again reflecting alterations in plasma renin activity.

All subdivisions of the dog brain showed a capacity to form angiotensin I at a neutral pH. In normal animals, the overall cerebral activity calculated as the average of all values determined in brain and spinal cord was 0.25 ± 0.07 ng/hr/mg of protein; brain cathepsin D activity averaged 1.38 ± 0.18 mg TYR/hr/mg protein. In dogs subjected to a sodium restriction protocol, total brain renin activity averaged 0.09 ± 0.01 ng/hr/mg of protein (p < 0.05), a value 64% less than that determined in the normal group. Cathepsin D activity decreased to 1.18 ± 0.07 mg Tyr/hr/mg protein, a value which was not statistically different from that recorded in the normal group of dogs.

Figure 1 shows the pattern of renin activity within the brain subdivisions and the spinal cord in normal and sodium-depleted dogs. The corresponding values for the cathepsin D are shown in figure 2. In normal...
animals, renin activity ranged from 0.16 ± 0.04 ng/hr/mg protein in the spinal cord to 0.28 ± 0.08 ng/hr/mg protein in the upper brain stem. All subdivisions of the brain of sodium-depleted dogs and the spinal cord contributed to the significant fall in mean brain renin activity; within any individual region, however, the activity of brain renin was reduced significantly in the lower and upper brain stem only (fig. 1). On the other hand, none of the regions studied showed significant differences in the activity of cathepsin D (fig. 2).

**Discussion**

Osman et al. and Hirose et al. have now provided unequivocal evidence for the existence of a renin-like material in extracts of mammalian brains. They further showed that the angiotensin I generating activity of acid proteases such as cathepsin D did not contribute to the generation of angiotensin I when the assay was performed at a pH above 6.0. While the physicochemical characteristics of the protease forming angiotensin I at a neutral pH requires further investigation, compelling evidence exists regarding the presence of all necessary components of the renin angiotensin system in brain. The recent localization of renin to synaptosomes by Husain et al. and the presence of the converting enzyme in microsomal fractions of brain cells adds weight to the possibility that this protein system subserves an as yet undetermined, central function in the control of homeostasis.

The present findings indicate that in the dog the reactivity of renin in the brain is affected by sodium depletion lasting for 3 weeks. The well-characterized hyperreninemia that accompanies a restriction in sodium intake was accompanied by a mild increase in CSF renin activity and, conversely, a significant fall in the activity of renin in brain tissue, particularly pronounced in the upper and lower brain stem. These findings suggest that the regulatory processes affecting the reactivity of renin in the brain differ from those acting upon the peripheral system. In both normal and sodium-depleted dogs there was no obvious relationship between plasma and CSF renin activity. When expressed as ng/ml/hr, renin activity in the CSF was only 8% of the activity in plasma, and the large change in the ratio of plasma to CSF renin was essentially due to the increase in the activity of the plasma enzyme. The presence of renin in CSF in normal dogs and its nonsignificant increase after sodium depletion may reflect an increase in the local concentration of the enzyme in cerebral vessels or choroid plexus with further transport into the CSF compartment. Although the blood-brain barrier is impermeable to the renin molecule, sodium depletion may affect transport of proteins through this barrier. Alternatively, leakage could occur in the presence of pronounced hyperreninemia. It should also be considered that the slight increase in CSF renin reflects either a passive or active release of the neutral protease from the cerebral tissue into the CSF compartment. This possibility, however, does not conform to the parallel observation of a decrease in tissue brain renin activity. A chronic increase in the plasma concentration and/or activity of renin has been shown to be accompanied by a rise in the concentration of renin in the tissues, particularly the kidneys, whether the stimulus was due to sodium depletion, adrenalectomy, or clipping of a renal artery.

The distribution of renin activity in the various subdivisions of the dog's cerebrum conforms to that reported in other species although higher renin levels are reported for the choroid plexus and brain regions devoid of a blood-brain barrier (for example, pituitary and pineal glands). We are the first, however, to observe an apparently specific decrease in the renin activity of the brain stem, a finding which at first glance conforms with the possibility that the brain renin angiotensin system (RAS) may participate in the neuroendocrine abnormalities present in the sodium-depleted state.

The experiments of Ganten et al. in young and old dogs and Sen et al. in rats treated with an angiotensin antagonist provided evidence toward the possible existence of a negative feedback between the kidney and brain angiotensin systems. The experiments of Ganten were not entirely conclusive, however, because their methodology did not eliminate the participation of other proteases which could generate angiotensin I at a lower pH. Ferrario et al. have proposed that the reciprocal interplay between the activity of the kidney and the brain renin angiotensin system may be a function of sodium metabolism and/or fluid volume. While the present data support this hypothesis, further studies are required. Little is yet known about the factor or factors that both regulate and link the activity of the peripheral with the central renin angiotensin systems. That the problem is a complex one is illustrated by the observations of Schelling et al. in spontaneously hypertensive rats. They reported that spontaneously hypertensive rats have a similar profile of renin activities in most brain regions as found in Wistar Kyoto normotensive rats. In the neurohypophysis and in brain stem regions containing noradrenergic neurons, however, spontaneously hypertensive rats had an increase in renin activity with no change in cathepsin D activity.

The demonstration that sodium depletion reduces the activity of the brain renin in parallel with an increased activity of plasma renin is in accord with the idea that one function of the brain RAS may entail facilitation of central sympathetic vasomotor discharges. Ferrario et al. and Szilagyi et al. have shown that sodium depletion, as carried out in the present experiments, is associated with important changes in neuroendocrine function as reflected by reduced peripheral sympathetic nerve activity. Alterations in the noradrenaline concentration of both CSF and medulla oblongata constituent with increased central noradrenergic turnover and resetting of the interaction between high and low pressor baroreceptor reflexes. The present finding of reduced activity of the brain renin angiotensin system, at least as judged by the decreased activity of the brain iso-enzyme, is congruent with the possibility...
that the brain angiotensinergic system has a facilitative influence upon the sympathetic system.

The distribution of cathepsin D activity in brain regions of the dog expands upon the earlier observation of Day and Reid, by demonstrating that cathepsin D activity remains constant with sodium depletion, while renin activity does not. These latter findings are important for a number of reasons. First, they show that cathepsin D activity does not parallel renin activity; a dissociation between the two enzymes is consistent with the fact that antirenin antibodies do not cross-react with cathepsin D. Second, the change in renin activity following sodium depletion cannot be attributed to a nonspecific change in protein concentration since cathepsin D was not affected when expressed in the same units. Third, the contribution of acid protease activity to angiotensin I generation was avoided as much as possible. Confidence that brain renin activity can be measured as distinct from acid protease activity was gained by work from our laboratory and others. Recently demonstrated the separation of cathepsin D from brain renin by using CM-cellulose chromatography. Because the low sensitivity of these separation procedures prevented their application to the regional brain tissue samples studied here, a number of other procedures were used to ensure that the acid protease did not interfere with the brain renin assay. The incubation at pH 6.5 eliminated the contribution of angiotensin I generation by acid protease. When an amount of enzyme activity of bovine spleen cathepsin D approximately equal to the acid protease activity in these extracts at pH 4.5 was incubated at pH 6.5 with dog renin substrate, no angiotensin I formation could be detected. Additionally other neutral proteases, such as cathepsin L, cathepsin B, and elastase, were inhibited as described above. Similar procedures have recently been used by others. Furthermore, to eliminate neutral protease activity from plasma, the brains were perfused with saline extensively to remove any plasma renin contamination. In summary, our studies provide direct evidence for a possible involvement of brain renin in circumstances where sodium and water metabolism are affected. At a time when plasma renin and kidney renin activities were markedly elevated, there was a significant reduction in the level of brain stem renin activity. The demonstration of changes in the angiotensin I-forming activity of the neutral protease only without concomitant changes in cathepsin D activity in various brain regions points to the specific involvement of the brain renin angiotensin system in sodium depleted states.

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References

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