Cerebrospinal Fluid Angiotensin II Immunoreactivity Is Not Derived from the Plasma

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SUMMARY To elucidate whether the presence of angiotensin II immunoreactivity (ANG II-ir) in the cerebrospinal fluid (CSF) of the dog is in part due to passage of the peptide across the CSF-blood-brain barrier, [111I]angiotensin II (ANG II) was infused intravenously for 7 days in conscious, trained dogs at a rate of 10 µg/kg/day. Mean arterial pressure (MAP) and heart rate were monitored each day, and samples of arterial blood and CSF (with a catheter secured into the cisterna magna) were drawn at regular intervals for determination of catecholamine levels, ANG II-ir, and electrolyte levels. Within 2 days after ANG II infusion, MAP stabilized at 35 ± 1 mm Hg (mean ± se, p < 0.001) above control values. The hypertension was associated with bradycardia, suppressed plasma renin activity, and a fall in both plasma and CSF Na⁺ concentrations. These changes coincided with a considerable and sustained decrease in the levels of plasma and CSF norepinephrine. On the other hand, levels of epinephrine and K⁺ in the two compartments remained unchanged. Although concentration of ANG II-ir in plasma was augmented markedly (368% above control values, p < 0.001), ANG II-ir in the CSF remained within the low values measured in the control period. We conclude that ANG II-ir in the CSF of the cisterna magna of the dog does not originate from and is not related to the concentration of the peptide in the plasma, even after considering a possible pressure-dependent increase in the permeability of the blood-brain barrier. These data provide further evidence that CSF ANG II-ir may be synthesized in brain tissue and may either be released or diffuse into the extracellular fluid contained in the brain ventricles. (Hypertension 7: 65–71, 1985)

Key Words - angiotensin II • arterial hypertension • renal hypertension • blood-brain barrier • blood pressure • brain peptides • cerebrospinal fluid • plasma renin activity

An increasing body of evidence suggests that angiotensin II (ANG II) and/or structurally related peptides synthesized by neural elements, may influence cardiovascular function. Although immunohistochemical, biochemical, and physiological data support the concept of a separate protein system forming ANG II in the brain, the information is by no means conclusive. Because other studies have shown that the presence of hormones in the cerebrospinal fluid (CSF) may reflect their activity in the cerebral interstitial fluid, functional evidence about the role of ANG II in the central nervous system (CNS) may be ascertained by repeatedly monitoring the levels of the peptide in the CSF. As it is possible that a part of the ANG II present in the CSF may be derived from the plasma, we evaluated this mechanism by measuring the concentration of ANG II in both blood and CSF before, during, and after a 7-day continuous intravenous infusion of the peptide. The experiments were performed in trained, conscious dogs, and the dose of ANG II infused was estimated to produce hypertension as well as plasma ANG II levels similar to those obtained in conscious dogs with chronic renovascular hypertension.

Methods

Animal Surgery

The experiments were performed in five trained mongrel dogs (average body weight, 21 ± 1 kg) conditioned for 2 weeks. After this time the dogs were anesthetized with sodium pentobarbital (15 mg/kg) intravenously 30 minutes after premedication with morphine sulfate (2 mg/kg, intramuscular [i.m.]). Arterial and venous catheters (Tygon, U.S. Stoneware, Akron, OH) were inserted into an iliac artery and vein; the free

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end of each catheter was tunneled through the subcutaneous tissue to exit at the base of the dorsal neck. A specially constructed catheter was positioned into the cisterna magna as described by Suzuki and Ferrario. To reach this region of the brain, the dog's head was positioned in a stereotaxic frame and the atlanto-occipital membrane was reached through a midline incision in the dorsal neck; the flanged end of the catheter was anchored to the atlanto-occipital membrane, and the free end of the tubing was tunneled to the scapular region. Dexamethasone sodium phosphate (Decadron) was given (4 mg, i.m.) after completion of the surgical procedure; antibiotics (800,000 units of procaine penicillin G plus 1.0 g of dihydrostreptomycin sulfate, i.m.) were administered for 3 days after surgery.

**Experimental Protocol**

Dogs were brought to the laboratory for daily training and conditioning 1 week to 10 days after the surgical procedure. During daily 90-minute recording sessions the animals were housed in a pen situated in a dimly lit laboratory, shielded from ambient visual or auditory stimuli. Although measurements were performed while the dogs were confined to the pen, they were free to change posture, turn about, or rest quietly.

To facilitate housing of the pump used to infuse ANG II continuously, dogs wore a jacket (Alice King Chatham, Medical Arts, Los Angeles, CA) throughout the experiment. The system used for the continuous infusion of the peptide consisted of a battery-driven ambulatory infusion pump (Model ML-6, Cormed Inc., Middleport, NY) coupled to a 150-ml reservoir (Viaflex, Travenol Laboratories, Inc., Deerfield, IL). Both the pump and the plastic reservoir were housed in the pockets of the jacket. During the initial 4 days, isotonic NaCl was infused continuously at a rate of 5.0 ml/hour. After this time, \( \text{[He}^5\) ANG II (synthesized by M.C. Khosla, Cleveland Clinic Foundation) was added to the saline vehicle in amounts calculated to provide an infusion rate equivalent to 7 ng/kg/minute.

Fresh solutions were prepared each day from aliquots of \( \text{[He}^5\) ANG II stored (−20 °C) at a concentration of 100 μg/ml in 0.9% NaCl with 0.1% bacitracin added by weight. The plastic bag containing the solution of ANG II and the batteries for the pump were changed daily by interrupting the infusion for no more than approximately 4 minutes. Samples of the ANG II infused remaining in the reservoir at the end of each day were tested for pressor activity in a rat bioassay system to verify that the peptide remained active at 37 °C for longer than 24 hours. At the termination of the infusion dogs continued to receive 0.9% NaCl intravenously for an additional 4 days.

Twice before and on each day of the ANG II infusion, a 10-ml sample of arterial blood was taken from each dog for the assay of plasma renin activity (PRA), ANG II immunoreactivity (ANG II-ir), norepinephrine (NE) and epinephrine (EPI) concentrations, hematocrit, and serum electrolyte levels. In addition, 3 ml of CSF also was collected from the cisterna magna twice during both the control and postinfusion periods and four times during the 7-day period of ANG II infusion. The CSF samples were demonstrated to contain less than 0.01% contamination of hemoglobin as judged by the Hemastix (Ames Co., Elkhart, IN) method. Both blood and CSF samples were drawn at similar times each day (0900–1000 hrs) to minimize variability due to circadian rhythms.

**Chemical Analysis**

All of the biochemical determinations performed in the plasma and CSF of these dogs were carried out as detailed previously. The evidence included documentation of the range and mean values of these variables in a large population of conscious, unrestrained dogs.

To briefly summarize, PRA was measured by radioimmunoassay and serum and CSF Na⁺ and K⁺ concentrations were determined by flame photometry. For measurements of ANG II-ir, samples were collected in a prechilled syringe (0 °C) containing 200 μL of a solution of 15% ammonium EDTA and 9.25 mmol orthophenanthroline. After centrifugation at 4 °C, plasma and CSF samples were stored at −20 °C until assayed. Measurements of ANG II-ir were performed using a radioimmunoassay with an antibody that has less than 1% crossreactivity with ANG I. We have shown before that the lowest detectable level of ANG II-ir averages 0.5 pg/tube, this value is equivalent to a concentration of 2.5 pg of ANG II-ir per milliliter of either plasma or CSF.

For measurements of catecholamine levels, samples of blood and CSF were collected in prechilled tubes containing 20 μL of EGTA (90 mg/ml) and glutathione (60 mg/ml) centrifuged at 1,000 g (4 °C) and frozen until assayed at −70 °C. The radioenzymatic assay has an average sensitivity of 33 ± 13 pg/ml for EPI and 45 ± 15 pg/ml for NE (mean ± 1 SD respectively). The coefficient of variation is 6.7% for EPI and 9.0% for NE.

**Data Analysis**

Beat-by-beat recordings of arterial pressure were stored on an FM analog tape recorder (Model 5600, Honeywell Instruments Div., Denver, CO) for later processing by a digital computer (Eclipse S/140, Data General Corp., Waltham, MA) with algorithms described elsewhere. Values in each cardiac cycle were stored on digital tape and used to compute histograms and calculate means and standard deviations. The mean and the standard deviation for each variable were computed for each recording session and from the grouping of recording sessions at designated time intervals (i.e., control, ANG II infusion, etc). The mean of all session standard deviations for systolic, diastolic, and mean pressures was computed for each dog to estimate variability. We refer to this value as an index of inrasession variability. Unless denoted otherwise, all values are expressed as means ± SE. One-way analysis of variance was performed to assess the effects of ANG II infusion over time on the measured variables. The Dunnett's test was used to assess significance of differences. The Student's t test for paired
Linear regression analyses were determined by the least-squares method. A $p$ value of less than 0.05 was considered significant.

**Results**

Figure 1 shows the effects of a continuous infusion of ANG II in five trained, conscious dogs maintained on a normal intake of sodium (65 mEq Na+/day). Beginning with the first day after initiation of the infusion, hypertension developed with regularity in all animals, with increases in mean arterial pressure (MAP) reaching a plateau by the second day (Figure 1). For the group as a whole, the increases in MAP between the second and seventh days of infusion amounted to $35 \pm 1$ mm Hg ($p < 0.01$) above a baseline value of $102 \pm 2$ mm Hg. Hypertension was associated with mild bradycardia that reached statistical significance after the second day of infusion. The hemodynamic changes were accompanied by a marked and sustained suppression of PRA. Interruption of the ANG II infusion after 7 days caused MAP to fall below, and heart rate to increase above, control values (Figure 1). One day after stopping the ANG II infusion, MAP fell from $138 \pm 4$ mm Hg to $97 \pm 2$ mm Hg ($p < 0.01$). At the same time heart rate increased from $71 \pm 5$ beats/minute (seventh day) to $101 \pm 8$ beats/minute ($p < 0.01$). Although PRA was still significantly depressed 24 hours after stopping the infusion ($p < 0.05$), it increased toward baseline values by the third day postinfusion.

Since infusion of subpressor doses of ANG II are reported to cause blood pressure lability, this was evaluated as described in Methods. During the control period, average values of the intrasession variability of MAP and heart rate were $9.3 \pm 0.7$ mm Hg and $23 \pm 1$ beats/minute respectively; these data are in agreement with those reported previously. Between the first and seventh day of the ANG II infusion there was a significant increase ($p < 0.05$) in the intrasession variability of systolic pressure without any change in the standard deviation of MAP and heart rate. Between the second and seventh day of infusion the intrasession variability of systolic pressure was $15 \pm 0.2$ mm Hg versus $9.7 \pm 0.3$ mm Hg ($p < 0.01$) before the infusion.

The hypertension produced by the infusion of ANG II caused a transient fall in the hematocrit (first day) and a gradual decrease in plasma Na+ concentrations that persisted for 24 hours after the infusion ended. On the other hand, plasma K+ concentrations remained unchanged (Figure 2). Although there was a tendency for the animals to weigh more during the ANG II infusion, the change did not attain statistical significance ($p > 0.05$). Figure 2 also shows the corresponding changes in CSF Na+ and K+ concentrations. As shown previously in normal dogs, values of CSF Na+ are higher than those in the plasma, whereas the converse is true for CSF K+. In the present experiments baseline values of CSF Na+ and CSF K+ averaged $153 \pm 0.3$ mEq/L and $2.9 \pm 0.1$ mEq/L, compared with $146 \pm 0.6$ mEq/L and $4.3 \pm 0.1$ mEq/L in plasma respectively. The hyponatremia associated with the ANG II infusion was paralleled by a similar fall in CSF Na+ levels ($149 \pm 0.5$ mEq/L on the seventh day, $p < 0.01$) that persisted for 24 hours after stopping the infusion. The CSF K+ concentrations did not change.

Figure 3 shows that there was a significant increase ($p < 0.05$) in the ANG II-ir of the plasma without any parallel changes in CSF ANG II-ir. Before the infusion of ANG II, plasma and CSF ANG II-ir averaged 16 ± 4 pg/ml and 4.9 ± 2.4 pg/ml respectively. These values are in agreement with those reported by us elsewhere. Between the first and seventh day of ANG II infusion, plasma ANG II-ir rose to 75 ± 15 pg/ml ($p < 0.01$); after stopping the infusion, plasma ANG II-ir fell below baseline levels (7 ± 3 pg/ml) but the difference did not attain statistical significance ($p > 0.05$). The CSF ANG II-ir averaged 4.5 ± 3.0 pg/ml 24 hours after initiation of the infusion; it did not change during the following 6 days and remained at these levels during the postinfusion period (Figure 3).
FIGURE 2. Development of hypertension is associated with a transient fall in the hematocrit, no significant changes in body weight, and a delayed, but statistically significant, fall in plasma and CSF Na+ concentrations. For graphs depicting the changes in the concentration of Na+ and K+ in plasma and CSF, solid lines and filled circles are values derived from plasma, solid lines and filled squares are CSF values. * = p < 0.05.

The ANG II-dependent hypertension produced significant changes (p < 0.05) in the concentration of NE in both compartments (Figure 3). During the control period, plasma and CSF levels of NE averaged 140 ± 11 pg/ml and 263 ± 20 pg/ml respectively. On the first day of the ANG II infusion plasma NE levels fell to 62 ± 15 pg/ml (p < 0.01) while CSF NE levels averaged 197 ± 41 pg/ml (p < 0.05). By the seventh day plasma and CSF NE levels averaged 59 ± 7 pg/ml and 164 ± 20 pg/ml respectively. These changes were statistically significant at p < 0.01 (Figure 3). Both plasma and CSF NE returned to baseline values after stopping the infusion of ANG II. Before the infusion of ANG II, baseline values of EPI in plasma and CSF averaged 74 ± 25 pg/ml and 29 ± 9 pg/ml respectively. Twenty-four hours and seven days into the infusion period, plasma EPI levels averaged 33 ± 6 pg/ml and 43 ± 17 pg/ml (p > 0.05) respectively. Corresponding values for CSF EPI were 35 ± 4 pg/ml and 21 ± 3 pg/ml (p > 0.05) respectively.

Comparison of the changes in MAP with other recorded variables revealed the existence of a positive correlation (Figure 4) with plasma ANG II-ir (r = 0.77, p < 0.0005) and a negative correlation (Figure 4) with plasma NE (r = -0.69, p < 0.0005), CSF NE (r = -0.51, p < 0.001), and CSF Na+ (r = -0.56, p < 0.001) concentrations. Plasma NE levels correlated with CSF NE levels (r = 0.49, p < 0.005), and plasma Na+ levels correlated with CSF Na+ levels (r = 0.51, p < 0.001). Other correlations were not statistically significant.

FIGURE 3. Corresponding values of plasma (top panel) and CSF ANG II-ir (middle panel) and CSF and plasma NE levels (solid squares and circles of bottom panel respectively) in the five dogs infused with ANG II. * = p < 0.05.
FIGURE 4 In these experiments there was a direct correlation between MAP and plasma ANG II-ir \((r = 0.77, p < 0.0005)\), while a negative correlation was found when MAP was related to the levels of NE in the plasma \((r = -0.69, p < 0.0005)\). Open circles denote the inclusion of both baseline and postinfusion data points in the computation of the regression values. Parameters for the regression lines \((y = a + bX)\) are. Top panel \(- a = 104 \text{ mm Hg, } b = 0.36 \text{ mm Hg (pg/ml), } X = \text{ plasma ANG II-ir (pg/ml); bottom panel } - a = 150 \text{ mm Hg, } b = -0.31 \text{ mm Hg (pg/ml), } X = \text{ plasma NE levels (pg/ml).}"

Discussion

A relatively prolonged infusion of ANG II into the systemic circulation of conscious dogs, in amounts sufficient to produce hypertension and a marked rise in plasma ANG II-ir, did not cause any changes in the ANG II-ir present in the CSF. The action of increasing the circulating levels of ANG II on blood and CSF NE and EPI was also characterized. The importance of these new findings is underscored by the use of baseline and postinfusion data points in the computation of the regression values.

One major reason for undertaking this study is that previous attempts to evaluate the mechanism accounting for the presence of ANG II-ir in the CSF of humans and animals were not conclusive, because either the entry of ANG II into cerebral ventricles was estimated by the detection of radioactivity in the CSF after systemic injection of labeled ANG II or the direct effect of a systemic infusion of the peptide on CSF ANG II-ir was estimated from short-term infusions in anesthetized animals. A better understanding of the anatomical structures and functional characteristics of the brain barriers regulating transport of blood peptides into the cerebral interstitial fluid and CSF casts some doubt about conclusions obtained by previous studies. Accordingly, it was necessary to carry out experiments that more closely resembled those situations in which changes in CSF ANG II-ir have been used to validate the possible importance of the brain renin-angiotensin system in the regulation of blood pressure.

It is now known that transport of blood peptides into the fluid matrix of the brain may occur by one of two mechanisms. One is the passage of the peptide across the blood brain barrier by either diffusion or specific enzymelike carriers located in the luminal and antiluminal sides of the endothelial membrane. The other mechanism entails penetration of the peptide into circumventricular organs as their ependyma have low-resistance tight junctions. Although the hydrophilic nature of the ANG II molecule excludes the passive diffusion of the peptide across the blood-brain barrier, a pressure-dependent change in the permeability of the blood-brain barrier, as in the presence of hypertension, may disrupt the tight junctions between brain capillary endothelial cells. This effect would permit the peptide to gain access to the interstitial fluid of the brain and, hence, the CSF. Since the levels of CSF ANG II-ir were not elevated by producing a sustained increase in arterial pressure of a magnitude similar to that reported in chronically hypertensive animals, our data suggest that a hypertension-dependent disruption of the blood-brain barrier may not facilitate the passage of ANG II into the CSF. In addition, the data do not support the existence of a specific endothelial transport system for ANG II. This interpretation is in agreement with other studies showing the absence of a specific transport system for other peptides in the endothelial lining of brain capillaries.

On the other hand, the absence of the blood-brain barrier in brain circumventricular organs may constitute a route by which ANG II migrates into the CSF. Recent studies indicate that circumventricular organs have two major functions with regard to peptide entry into the brain. First, the existence of low-resistance tight junctions in the circumventricular organs allows blood-borne peptides to gain rapid entrance into the brain interstitium. Second, after distribution into the interstitial fluid space of the circumventricular organ, peptides may slowly be transported into adjacent areas including the CSF. Although substances circulating in blood may slowly and nonspecifically enter the brain parenchyma at the circumventricular organs and therefore gain access to the CSF, our data also exclude this mechanism as a possible source for the presence of ANG II-ir in the dogs' CSF. This finding is of importance because, as indicated by Partridge, evaluation of this particular mechanism required attaining sustained increases in the plasma concentration of the peptide. In all dogs studied the ANG...
II infusion produced an increase in plasma ANG II-ir that lasted for the 7 days of infusion. If ANG II can gain access to the CSF via a circumventricular organ pathway, it should have been possible for us to detect an increase in the peptide levels in the CSF. It is possible that ANG II may be effectively degraded by angiotensinases at the perivascular spaces of the circumventricular organs, the cerebral interstitial fluid, or the CSF. Although brain tissue has high angiotensinase activity, the opposite is found in the CSF. While the present findings do not belittle the importance of an action of ANG II at or within the near vicinity of circumventricular organs, the maintenance of normal levels of CSF ANG II despite large increases in the plasma compartment suggest that circumventricular organs are not a pathway by which ANG II can gain access to the CSF. By inference, the data provide further support for the view that CSF ANG II-ir originates within the CNS. Until now, a brain source for the existence of the peptide in the CSF has been assumed from indirect evidence such as (1) a CSF-plasma ratio of ANG II of less than 1.0,13; (2) the observation by us15 and others16 that the concentrations of ANG II-ir in blood do not correlate with the values of the peptide in the CSF; and (3) radioactively labeled ANG II injected intravenously could not be detected in brain tissue or the CSF.17-20 The results of our more direct approach indicate that it is unlikely that either pressure-dependent changes in the permeability of the blood-brain barrier or passage of the peptide from the interstitial space of brain circumventricular organs to the CSF contributes appreciably to the detection of ANG II-ir in the fluid contained within the cerebral ventricles.

We have also examined the effect of the ANG II infusion on circulatory variables and the sympathetic nervous system. The data are in agreement with previous studies,14-20 which indicate that peripheral vasconstriction rather than volume expansion is the predominant factor accounting for the elevation in MAP. Although the transient changes in hematocrit and body weight observed in the dogs suggest the existence of an early volume-dependent component on this kind of hypertension, the later and more sustained falls in plasma and CSF Na+ levels, without corresponding changes in K+ concentration, do not support this possibility. This interpretation is compatible with studies showing that in both dogs26 and rats29 the slow pressor response produced by long-term infusion of ANG II did not produce Na+ and water retention or a persistent stimulation of aldosterone.

Angiotensin II stimulates the sympathetic nervous system at both peripheral and central sites.5,11,15 Although previous studies13 have emphasized that these small amounts of ANG II can potenti ate sympathetically mediated vasomotor activity, this was not confirmed in the present experiments. This conclusion is based on (1) the demonstration of a weak increase in the lability of systolic pressure only and (2) the presence of bradycardia in association with marked decreases in the concentration of NE in both plasma and the CSF. Failure to demonstrate an appreciable increase in the lability of MAP may be related to experimental conditions, as in previous studies hypertension was the result of an increased responsiveness to environmental stimuli that had no discernible effect on pressure before the infusion. Because our dogs were monitored while they were recumbent and were isolated from either environmental disturbances or startling behaviors, only minor changes in the responsiveness of MAP would be documented. Although measurements of circulating catecholamine levels as a gauge of sympathetic function have recognized limitations, the suppressed level of NE in both the plasma and CSF of these conscious hypertensive dogs raises an important question regarding a sympathetically mediated increase in vascular resistance. Since we do not know of any other study in which catecholamine concentrations were measured during the continuous infusion of ANG II, it is not possible to make comparisons. In the current experiments baseline values of both NE and EPI were within the range reported for normal dogs.4,10 In none of the dogs did we observe high values during either the infusion or the postinfusion periods. Therefore, we suggest that the reduction in plasma and CSF NE levels produced by the continuous infusion of ANG II reflects a blunting of sympathetic nerve activity. These data do not belittle an important role for ANG II in the modulation of adrenergic mechanisms. Failure to document this function may be due to one of several reasons: (1) the relatively short duration of the experiment, (2) a potent stimulation of the baroreceptor reflex by the relatively rapid increase in blood pressure, and (3) the possibility that the neural actions of ANG II are influenced by the state of sodium balance.5

Conclusion

The results of 7 days of continuous intravenous infusion of ANG II in conscious dogs indicate that bloodborne ANG II is not a factor accounting for the presence of the peptide in the CSF. These data also suggest that increased activity of the peripheral renin-angiotensin system does not necessarily result in the activation of the protein components that may be responsible for the formation of the active peptide in the brain parenchyma.1

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