Role of Brain Corticosterone and Aldosterone in Central Angiotensin II–Induced Hypertension

Bing S. Huang, Roselyn A. White, Monir Ahmad, Frans H.H. Leenen

Abstract—Circulating angiotensin II (Ang II) activates a central aldosterone–mineralocorticoid receptor neumodulatory pathway, which mediates most of the Ang II–induced hypertension. This study examined whether specific central infusion of Ang II also activates this central aldosterone–mineralocorticoid receptor pathway. Intracerebroventricular infusion of Ang II at 1.0, 2.5, and 12.5 ng/min for 2 weeks caused dose-related increases in water intake, Ang II concentration in the cerebrospinal fluid, and blood pressure. Intracerebroventricular Ang II, at 2.5 and 12.5 ng/min, increased hypothalamic aldosterone and corticosterone, as well as plasma aldosterone and corticosterone without affecting plasma Ang II levels. Intracerebroventricular infusion of the aldosterone synthase inhibitor FAD286—but not the mineralocorticoid receptor blocker eplerenone—inhibited by ≥60% the Ang II–induced increase in hypothalamic aldosterone. Both blockers attenuated by ≥50% the increase in plasma aldosterone and corticosterone with only minimal effects on hypothalamic corticosterone. By telemetry, intracerebroventricular infusion of Ang II maximally increased blood pressure within the first day with no further increase over the next 2 weeks. Intracerebroventricular infusion of FAD286 or eplerenone did not affect the initial pressor responses but similarly prevented 60% to 70% of the chronic pressor responses to intracerebroventricular infusion of Ang II. These results indicate distinctly different patterns of blood pressure increase by circulating versus central Ang II and support the involvement of a brain aldosterone–mineralocorticoid receptor–activated neuromodulatory pathway in the chronic hypertension caused by both circulating and central Ang II. (Hypertension. 2013;62:00-00.) ● Online Data Supplement

Key Words: aldosterone synthase ■ cerebrospinal fluid ■ eplerenone ■ infusions, central or intracerebroventricular.

A chronic increase in circulating angiotensin II (Ang II) causes a gradually developing neurogenic pressor response,1,2 associated with a progressive increase in neuronal activity in the paraventricular nuclei (PVN) and supraoptic nuclei (SON) of the hypothalamus.3,4 Recent studies have provided new insights into the brain mechanisms mediating these responses to Ang II. We showed that in Wistar rats, chronic subcutaneous infusion of Ang II increases both plasma and hypothalamic aldosterone levels.4 Intracerebroventricular infusion of an aldosterone synthase inhibitor attenuates the Ang II–induced increase in hypothalamic aldosterone but not in plasma aldosterone.4 Intracerebroventricular infusion of an aldosterone synthase inhibitor or mineralocorticoid receptor (MR) blocker attenuates the Ang II–induced neuronal activation in the magnocellular and parvocellular parts of the PVN but not in the SON.4 Intracerebroventricular infusion of an aldosterone synthase inhibitor,4 an MR blocker,5,6 or Digibind to bind endogenous ouabain (EO)4 also largely prevent the Ang II–induced hypertension. We proposed7 that circulating Ang II may stimulate nuclei outside the blood–brain barrier, such as the subfornical organ (SFO) and the organum vasculosum of lamina terminalis leading acutely to direct activation of the PVN causing the initial pressor response, but chronically also to activation of an aldosterone–MR–EO neuromodulatory pathway. Aldosterone synthesis may possibly occur in the SON, and aldosterone via MR stimulates EO synthesis/release possibly in magnocellular neurons in the SON and PVN.6 Activation of this aldosterone–MR–EO–amplifying mechanism results in upregulation of Ang II type I (AT1) receptors and nicotinamide adenine dinucleotide phosphate oxidase subunits and a decrease in nitric oxide synthase expression in the PVN,7 and thereby further activation of pressor mechanisms, such as sympathetic tone,8 plasma vasopressin,9 or plasma EO10 leading to progressive hypertension. We proposed that the central pressor response to circulating Ang II depends on MR in the central nervous system (CNS), apparently as a result of functionally active aldosterone synthesis and release in the CNS. Supporting this concept, Xue et al11 recently reported a 1.8-fold increase in CYP11B2 mRNA in the lamina terminalis and a 2.6-fold increase in the PVN after subcutaneous infusion of Ang II. Circulating Ang II stimulates production and release of adrenal aldosterone, and an increase in hypothalamic aldosterone may also reflect uptake from the circulation,12 despite its poor penetration.13 Direct central infusion of Ang II also causes
sympathetic hyperactivity and hypertension, and may be a more specific stimulus for CNS aldosterone synthesis. In this study, we evaluated whether central infusion of Ang II also increases blood pressure (BP) via activation of the aldosterone–MR–EO pathway. We, therefore, evaluated the effects of (1) subcutaneous versus intracerebroventricular infusion of Ang II at various doses on BP, water intake, and Ang II concentration in the cerebrospinal fluid (CSF); (2) intracerebroventricular infusion of Ang II on hypothalamic and plasma aldosterone and corticosterone; and (3) intracerebroventricular infusion of an aldosterone synthase inhibitor or MR blocker on the chronic effects of central Ang II on hypothalamic and plasma aldosterone and corticosterone and on BP.

Methods
Male Wistar rats, weighing 200 to 250 g (Charles River, Montreal, Canada), were housed in a climatized room on a 12-h light/dark cycle and given standard laboratory chow (120 μmol Na/g) and tap water ad libitum. All surgeries and experiments were approved by the University of Ottawa Animal Care Committee, and conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (8th Edition, 2011).

For all surgeries, rats were anesthetized with 2% isoflurane in oxygen. For actual number of rats per group, intracerebroventricular or intra-arterial cannulation, BP measurement, and CSF withdrawal, see the online-only Data Supplement.

Protocol I: Dose-Related Responses for BP, Water Intake, and CSF Ang II Concentration in Rats With Chronic Subcutaneous Versus Intracerebroventricular Infusion of Ang II
Rats were divided into 6 groups (n=5–8 per group) for a 12- to 13-day subcutaneous or intracerebroventricular infusion by osmotic minipumps (Model 2002) of (1) subcutaneous Ang II at 150 ng/kg per minute; (2) subcutaneous Ang II at 500 ng/kg per minute; (3) intracerebroventricular vehicle (artificial Cerebrospinal fluid [aCSF]); (4) intracerebroventricular Ang II at 1.0 ng/min; (5) intracerebroventricular Ang II at 2.5 ng/min; and (6) intracerebroventricular Ang II at 12.5 ng/min. Subcutaneous infusion of Ang II at 2.5 or 12.5 ng/min had no effects on resting BP and plasma Ang II levels. An additional control group of 6 rats underwent sham intracerebroventricular or subcutaneous surgery without minipumps. Water intake was assessed on days 11 and 12 of the infusion. In the afternoon of day 12, a catheter was placed into the right femoral artery; BP and heart rate (HR) were measured in conscious rats in the next morning. The rat was then anesthetized and trunk blood and brain tissue were collected for aldosterone and corticosterone. Responses to intracerebroventricular infusion of FAD286 at 25 and 50 μg/d, or to intracerebroventricular Ang II alone and Ang II and eplerenone vehicle were similar, and data from each set of 2 treatments were pooled together.

To increase the sample size for the steroid measurements, in another 4 groups of Wistar rats (n=7–8 per group), intracerebroventricular cannula and osmotic minipumps (Model 2002) were implanted for a 2-week intracerebroventricular infusion of (1) aCSF; (2) Ang II at 2.5 ng/min alone; (3) Ang II (2.5 ng/min) and FAD286 at 25 μg/d; and (4) Ang II (2.5 ng/min) and eplerenone at 5 μg/d. Resting rats were decapitated and blood and brain tissue were collected. The results from the 2 sets of animals were similar and combined for analysis.

For details of telemetry probe implantation and accuracy of the position of the intracerebroventricular cannula, see the online-only Data Supplement.

Biochemical Assays for Aldosterone, Corticosterone, and Ang II
See the online-only Data Supplement for details.

Data Analysis
Values are expressed as mean±SE. For BP and HR sequential responses over time, area under each curve was calculated with SigmaPlot. For dose-related responses and biochemical assays in the central blockade study, and areas under the curve, 1-way ANOVA was performed. For BP and HR changes from baselines in the central blockade study, 1-way ANOVA with repeated measures was performed. For these tests, when the F values were significant for main effect, Bonferroni t test was used for multiple comparisons. Statistical significance was defined as P<0.05 (see the online-only Data Supplement for details).

Results
BP, Water Intake, and CSF Ang II Concentration After Subcutaneous Versus Intracerebroventricular Infusion of Ang II
Subcutaneous or intracerebroventricular infusion of Ang II for 2 weeks did not affect gain of body weight significantly. Subcutaneous infusion of Ang II at both doses did not significantly increase daily water intake (Figure 1A). In contrast, intracerebroventricular Ang II at 1.0, 2.5, and 12.5 ng/min caused marked, dose-related increases in daily water intake up to 6- to 9-fold.

Compared with sham rats, subcutaneous infusion of Ang II at 150 ng/kg per minute did not change the CSF [Ang II], and at 500 ng/kg per minute significantly increased CSF [Ang II].
II) from ≈10 to 90 pg/mL (Figure 1B). CSF [Ang II] was significantly higher in rats with intracerebroventricular infusion of aCSF versus sham rats without intracerebroventricular infusion of aCSF. Intracerebroventricular infusion of Ang II at the 3 rates caused marked dose-related increases in CSF [Ang II] from ≈90 pg/mL in rats with intracerebroventricular aCSF up to ≈1000 pg/mL at the highest rate of intracerebroventricular Ang II.

Subcutaneous infusion of Ang II at 150 and 500 ng/kg per minute for 2 weeks significantly increased mean arterial pressure (MAP) in a dose-related manner (Figure 1C). Intracerebroventricular infusion of aCSF alone did not change resting BP. In contrast to increases in CSF [Ang II] and water intake, intracerebroventricular Ang II at 1 ng/min did not significantly increase BP, whereas intracerebroventricular infusion of Ang II at 2.5 and 12.5 ng/min significantly increased MAP in a dose-related manner (Figure 1C).

**Chronic Intracerebroventricular Ang II and Hypothalamic and Plasma Aldosterone and Corticosterone**

In Protocol II, intracerebroventricular infusion of Ang II at 2.5 and 12.5 ng/min for 2 weeks also increased MAP in a dose-related manner (122±4 and 150±7 versus 103±4 mm Hg of intracerebroventricular vehicle; *P*<0.05 for both comparisons).

Measured in samples collected the day after surgery, intracerebroventricular Ang II increased hypothalamic aldosterone and corticosterone levels by 4- to 7-fold. The extent of the increases induced by Ang II at the 2 rates was similar (Figure 2A). Effects of intracerebroventricular Ang II on aldosterone and corticosterone levels in the hippocampus were similar to those in the hypothalamus (Figure 3A).

Intracerebroventricular Ang II at both rates had no effects on plasma Ang II levels (Figure 3B), but intracerebroventricular Ang II significantly increased plasma aldosterone and corticosterone levels in a dose-related manner (Figure 4A).

**Central Blockade of Aldosterone Synthase and MR**

Intracerebroventricular infusion of Ang II at 2.5 ng/min increased MAP by 20 mm Hg during day 1, and BP remained at this level throughout the 2 weeks (Figure 5). HR was not affected (Figure 5). Intracerebroventricular infusion of FAD286 at 25 to 50 μg/d did not affect the Ang II–induced increase in BP on days 1 and 2, but from day 3 onward, attenuated the pressor effects of Ang II by 60% to 70%. Intracerebroventricular infusion of eplerenone similarly influenced the Ang II–induced pressor responses, but was somewhat more effective, decreasing the chronic BP response by 70% to 80%. Subcutaneous infusion of FAD286 did not affect the intracerebroventricular Ang II–induced increase in MAP. Intracerebroventricular infusion of FAD286 or eplerenone did not affect the intracerebroventricular Ang II–induced increase in water intake (277±23 or 265±33 versus 259±25 mL/d for intracerebroventricular Ang II alone, not significant).

Tissue and plasma aldosterone and corticosterone levels in stressed (Protocol II) versus nonstressed (Protocol III) rats are shown in Figures 2 and 4. In nonstressed control rats, hypothalamic aldosterone and corticosterone levels were significantly lower than in stressed rats with recent surgery in Protocol II (139±24 versus 327±67 pg/g; and 13±2 versus 33±5 ng/g; *P*<0.05 for both comparisons). Hypothalamic aldosterone and corticosterone levels of nonstressed rats also increased after intracerebroventricular infusion of Ang II at 2.5 ng/min for 14 days, but the extent of increases is clearly smaller compared with the increases observed in those in Protocol I (Figure 2). Intracerebroventricular infusion of FAD286 significantly inhibited the increase in aldosterone, but did not affect the increase in hypothalamic corticosterone. Eplerenone caused no significant changes.

In nonstressed control rats, plasma aldosterone and corticosterone levels were also lower compared with stressed rats in Protocol II (82±10 versus 226±88 pg/mL; 30±7 versus 96±31 ng/mL; *P*<0.05 for both; Figure 4). Intracerebroventricular infusion of Ang II at 2.5 ng/min significantly increased plasma aldosterone and corticosterone, but less in nonstressed compared with stressed animals (Figure 4). The increase in
plasma aldosterone was significantly attenuated by intracerebroventricular infusion of FAD286 and eplerenone. Intracerebroventricular eplerenone attenuated ($P=0.04$) and intracerebroventricular FAD286 tended ($P=0.15$) to attenuate the Ang II–induced increase in plasma corticosterone.

**Discussion**

The main new findings of this study are (1) intracerebroventricular infusion of Ang II at 2.5 and 12.5 ng/min rapidly increases BP associated with increases in hypothalamic and plasma aldosterone and corticosterone without affecting plasma Ang II levels; (2) intracerebroventricular infusion of an aldosterone synthase inhibitor prevents most of the increases in hypothalamic and plasma aldosterone by intracerebroventricular infusion of Ang II; and (3) intracerebroventricular infusion of an aldosterone synthase inhibitor or MR blocker does not affect the initial pressor responses but prevents most of the chronic pressor responses to intracerebroventricular infusion of Ang II.

Intracerebroventricular-administered Ang II increases BP, HR, and renal sympathetic nerve activity via stimulation of...
Central Ang II, CNS Aldosterone, and BP

Intracerebroventricular infusion of Ang II for 90 minutes stimulates neurons in the SFO, organum vasculosum of lamina terminalis, median preoptic nucleus (MnPO), and PVN, as assessed by Fos immunoreactivity. Lesions of the ventral anteroventral third ventricle attenuate the pressor response induced by acute intracerebroventricular injection of Ang II. These findings together would suggest that short-term intracerebroventricular infusion of Ang II directly stimulates neurons in the SFO/organum vasculosum of lamina terminalis and via the MnPO activates neurons in the PVN and the rostral ventrolateral medulla leading to sympatho-excitation and rapid increase in BP. Chronic intracerebroventricular infusion of Ang II causes a persistent increase in BP associated with clear increases in both hypothalamic and plasma aldosterone and corticosterone. Although intracerebroventricular infusion of the aldosterone synthase inhibitor FAD286 or the MR blocker eplerenone does not affect the initial BP increase by intracerebroventricular infusion of Ang II, the chronic pressor responses to intracerebroventricular infusion of Ang II are inhibited by 70% to 80% by intracerebroventricular infusion of FAD286 or eplerenone. Intracerebroventricular infusion of FAD286 largely prevents the Ang II–induced increase in hypothalamic aldosterone but not the increase in hypothalamic corticosterone, and eplerenone does not affect either. The MR blocker is only modestly (not significant) more effective than the aldosterone synthase inhibitor in inhibiting the Ang II–induced hypertension, suggesting that aldosterone is responsible for most of the MR activation. However, an additional role for corticosterone cannot be excluded (see Limitation section of this article). Altogether, it seems that similar to the responses to an increase in circulating Ang II, an increase in CSF Ang II chronically also increases local production of aldosterone, leading to activation of the MR–EO neuromodulatory pathway, and thereby, most of the chronic hypertension induced by central Ang II. Further studies are needed to assess whether responses to MR activation involve nongenomic and genomic actions in the CNS, and where the relevant MR populations are located. Both aldosterone synthase and MR are expressed in forebrain, hypothalamic, and brain stem nuclei involved in cardiovascular regulation. Knockdown of MR in specific nuclei will be more informative in this regard than intracerebroventricular infusions.

Subcutaneous and intracerebroventricular infusions of Ang II cause distinctly different changes in plasma and CSF Ang II concentration, water intake, as well as the pattern of BP increases. Subcutaneous infusion of Ang II at 500 ng/kg per minute (but not 150 ng/kg per minute) increases the Ang II concentration in the plasma from ≈6 to 22 pg/mL and in CSF from ≈10 to 90 pg/mL. Circulating Ang II seems not to cross the blood–brain barrier, and the increase in CSF Ang II after subcutaneous infusion of Ang II may, therefore, reflect an increase in endogenous brain Ang II. Intracerebroventricular infusion of Ang II at 2.5 or 12.5 ng/min did not change plasma Ang II levels. This would be expected because subcutaneous infusion of Ang II at these rates also does not affect plasma Ang II concentration. Intracerebroventricular...
acSF itself increased CSF [Ang II] by 4-fold, and intracerebroventricular infusion of Ang II at the 3 rates markedly increased CSF [Ang II] to ≈500 to 1000 pg/mL versus ≈90 pg/mL in rats with intracerebroventricular acSF. Neurons in circumventricular organs, such as the SFO, respond to an increase in [Ang II] in both CSF and circulation.\textsuperscript{19,27} AT\textsubscript{1} receptors in different neuronal populations in the SFO may contribute to circulating versus central Ang II–induced responses\textsuperscript{19} (ie, AT\textsubscript{1} receptors sensitive to circulating Ang II may detect modest increase in Ang II, and those sensitive to CSF Ang II may only be stimulated by a large increase in Ang II). The pathophysiological relevance of this marked increase in CSF [Ang II] by intracerebroventricular infusion of Ang II is unclear at present. In dogs with pacing-induced heart failure, CSF [Ang II] significantly increased to ≈600 versus ≈200 pg/mL in sham dogs.\textsuperscript{28} No studies have reported CSF [Ang II] in other models of heart failure or hypertension models. Further studies are needed to clarify whether, for example, in rats, postmyocardial infarction CSF [Ang II] increases and contributes to persistent activation of central aldosterone-activated neuro-modulatory pathways.

Consistent with a previous study,\textsuperscript{29} intracerebroventricular Ang II at 1 ng/min did not increase BP, but increased daily water intake. Intracerebroventricular FAD286 or eplerenone attenuated the pressor responses to intracerebroventricular Ang II at 2.5 and 12.5 ng/min but not the Ang II–induced dipsogenic response. The pressor response to central Ang II is predominantly mediated by AT\textsubscript{1} receptor stimulation, and drinking response and release of vasopressin are mediated by both AT\textsubscript{1} and AT\textsubscript{2} receptors stimulation.\textsuperscript{30} Peterson et al\textsuperscript{10} showed that both nicotinamide adenine dinucleotide phosphate oxidases, NOX2 and NOX4, in the SFO contribute to central Ang II–induced pressor response, but only NOX2 is required for the dipsogenic response to central Ang II. It seems that different central pathways/mechanisms are involved in various responses to central Ang II, and the aldosterone–MR pathway only contributes to the chronic pressor responses to central Ang II.

Intracerebroventricular infusion of Ang II at 2.5 ng/min increases BP maximally by 20 mm Hg within the first 1 to 2 days, and BP remains at this level during the next 2 weeks. Intracerebroventricular infusion of FAD286 or eplerenone does not affect the initial increase in BP, but from day 3, reverses the elevated BP toward control values. In contrast, in rats on regular salt intake subcutaneous infusion of Ang II at 150 and 500 ng/kg per minute increases MAP by ≈5 and 20 mm Hg for the first 3 days, and then further increases BP with peaks of ≈20 and 60 mm Hg after 2 weeks.\textsuperscript{4} Intracerebroventricular infusion of FAD286 or eplerenone prevents most of this progressive increase in BP after the initial 2 to 3 days.\textsuperscript{4} The similar effects of intracerebroventricular infusion of FAD286 or eplerenone on the chronic pressor responses elicited by central and circulating Ang II indicate that activation of the central aldosterone–MR pathway contributes to chronic hypertension induced by both central and circulating Ang II. The central actions of Ang II per se seem to cause a rapid and maximal increase in BP within 1 to 2 days with no progression over time. In contrast, a combination of peripheral and central actions may contribute to the progressive pressor responses to circulating Ang II. Our findings suggest that the central actions are essential, but the progressive increase in BP may depend on amplifying effects of circulating Ang II on arteries and kidneys.

Intracerebroventricular infusion of Ang II did not increase plasma Ang II levels, but significantly increased plasma aldosterone and corticosterone levels. Also considering that subcutaneous infusion of Ang II only increases plasma aldosterone,\textsuperscript{4} and intracerebroventricular infusion of FAD286 or eplerenone both inhibit these increases by 60% to 70%, CNS effects of intracerebroventricular Ang II mediate these responses rather than leakage of the intracerebroventricular Ang II into the circulation. Several mechanisms may mediate these effects of central Ang II on plasma aldosterone and corticosterone levels. Intracerebroventricular-infused Ang II may enhance EO release from the pituitary and adrenal,\textsuperscript{31} and plasma EO may increase aldosterone production and secretion.\textsuperscript{32} Because plasma corticosterone also clearly increases, Ang II–induced adrenocorticotropic hormone (ACTH) release may also contribute. Enhanced ACTH release may also mediate the much larger increase in the plasma levels of both steroids by central Ang II in stressed animals as compared with resting rats (Figure 5). Unexpectedly, this difference was also apparent for levels of both steroids in the hypothalamus (Figure 4). Whether these levels are stress-sensitive because of higher local production or higher uptake from the circulation still needs to be assessed.

**Limitation**

FAD286 is a relatively selective aldosterone synthase inhibitor, which also inhibits corticosterone synthesis. In rats with ACTH infusion, FAD286 is 50-fold selective for reducing plasma aldosterone versus corticosterone.\textsuperscript{33} In this study, intracerebroventricular FAD286 prevented the Ang II–induced...
increase in hypothalamic aldosterone and caused only a minor (not significant) attenuation of the increase in hypothalamic corticosterone. However, we cannot exclude that FAD286 decreases corticosterone in specific nuclei, such as the SON, and this decrease contributes to less MR activation.14

Perspectives
We demonstrate that both circulating and central Ang II increase hypothalamic levels of corticosterone and aldosterone, whereas circulating Ang II causes a gradual increase in BP and central Ang II increases BP maximally within 1 to 2 days. Intracerebroventricular infusion of an aldosterone synthase inhibitor largely prevents the increase in hypothalamic aldosterone but not corticosterone, and after the initial 2 to 3 days, prevents most of the BP increase as does an MR blocker in response to either circulating or central Ang II. These findings provide further evidence to support a novel CNS mechanism for Ang II–induced hypertension (ie, a chronic increase in Ang II seems to activate a brain MR–dependent neuromodulatory pathway, which plays a major role in the chronic phase of Ang II–induced hypertension). The rapid versus slow increase in BP may reflect pure CNS responses to central Ang II versus CNS and peripheral interactions for circulating Ang II.

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**Novelty and Significance**

**What Is New?**
- Chronic intracerebroventricular infusion of angiotensin (Ang) II increases
  hypothalamic aldosterone and corticosterone and causes a rapid, sus-
  tained increase in blood pressure.
- Intracerebroventricular infusion of an aldosterone synthase inhibitor
  prevents most of the increase in hypothalamic aldosterone, and intra-
  cerebroventricular infusion of an aldosterone synthase inhibitor or min-
  eralocorticoid receptor blocker prevents most of the chronic pressor
  responses to intracerebroventricular Ang II.

**What Is Relevant?**
- This study provides evidence to support the involvement of aldosterone,
  and perhaps, corticosterone produced locally in the brain and a min-
  eralocorticoid receptor–activated neuromodulatory pathway in the hyper-
  tension caused by both circulating and central Ang II.

**Summary**
Central infusion of Ang II rapidly causes a sustained increase in
blood pressure associated with increases in hypothalamic corti-
costerone and aldosterone. Central infusion of an aldosterone
synthase inhibitor prevents most of the increase in hypothalamic
aldosterone induced by central infusion of Ang II. Central infusion
of an aldosterone synthase inhibitor or mineralocorticoid receptor
blocker does not affect the initial pressor responses but prevents
≈70% of the chronic pressor responses to central infusion of Ang II.
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Role of brain corticosterone and aldosterone in central angiotensin II induced hypertension
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Methods
For all surgeries, rats were anesthetized with 2% isoflurane in oxygen. Effective levels of anesthesia were maintained by observing reactions to physical stimulation such as toe-pinch, as well as monitoring the pattern of respiration. For pain relief, buprenorphine (0.04mg/kg) was injected sc ½ hour before and twice daily for 3 days following major surgeries, and only one dose was injected pre-surgery for arterial cannulation.

Protocol I
Rats were divided into 6 groups for sc or icv infusion by osmotic minipumps (Model 2002) of: 1) sc Ang II at 150 ng/kg/min (n=5); 2) sc Ang II at 500 ng/kg/min (n=6); 3) icv vehicle (artificial cerebrospinal fluid, aCSF, n=6); 4) icv Ang II at 1.0 ng/min (n=8); 5) icv Ang II at 2.5 ng/min (n=6); and 6) icv Ang II at 12.5 ng/min (n=6). An additional control group of 6 rats underwent sham icv or sc surgery without minipumps. For icv infusions, a 23-gauge right-angled icv cannula was implanted into the left lateral cerebral ventricle (1) and connected to the osmotic minipump via a polyethylene (PE) tubing.

In the afternoon of day 12, a catheter (PE10 fused to PE50) was placed into the right femoral artery and exteriorized and sealed. Measurements of BP and HR were performed in conscious rats in the next morning (about 18 hour after the surgery). The arterial line was connected to a pressure transducer for recordings of BP and HR via a personal computer equipped with AcqKnowledge software (ACQ 3.2). Animals were allowed to settle for at least 30 min prior to recording of resting BP and HR levels for 5 minutes. The rat was then anesthetized and a hole was drilled in the skull at the sagittal midline immediately rostral to the interparietal-occipital bone suture. A 25-gauge, Pencan® pencil point spinal needle (B. Braun Medical Inc.) was inserted through the hole at 70-75° angle to the skull surface, and advanced about 7.5 mm. With a 1 ml syringe, about 150-200 µl of CSF was collected from the cisterna magna at < 10 µl/sec.

Protocol III
A telemetry probe (DSI model TA11PA-C40) was implanted into the abdominal cavity and secured to the ventral abdominal wall with the catheter inserted into the abdominal aorta (1). Icv cannulas and sc minipumps (Model 2002) were implanted in 6 groups of rats, for a 2-week icv infusion of: 1) aCSF (n=6); 2) Ang II alone at 2.5 ng/min (n=5); 3) Ang II (2.5 ng/min) plus the aldosterone synthase inhibitor FAD286 icv at 25 µg/day (n=4); 4) Ang II (2.5 ng/min) plus the aldosterone synthase inhibitor FAD286 icv at 50 µg/day (n=6); 5) Ang II (2.5 ng/min) plus the MR blocker eplerenone icv at 5 µg/day (n=8); 6) Ang II (2.5 ng/min) plus the vehicle for eplerenone (4% acetonitrile, n=5). FAD286 hydrogen tartrate (Novartis Institutes for BioMedical Research, NJ) was used because it is soluble in aCSF. Each 1.67 mg of FAD286 hydrogen tartrate provides 1 mg of FAD286 free base, and the amount of the drug in the pumps was adjusted accordingly. Responses to icv infusion of FAD286 at 25 and 50 µg/day (for BP changes after the icv infusion of Ang II: +10±3 versus +8±1 mmHg, N.S.), or to icv Ang II alone and
Ang II plus eplerenone vehicle (for BP changes after the icv infusion of Ang II: +23±3 versus +20±2 mmHg, N.S.) were similar, and data from each set of 2 treatments were pooled together. For all protocols, the accuracy of the position of the icv cannula and integrity of its connection to osmotic pump were verified by visual examination during tissue collection. No problems related to icv cannula or pumps were found in Protocol I and II. In protocol III, 8 out of 62 rats were excluded because of unsuccessful icv cannulation, or broken or disconnected pump catheters.

**Biochemical assays for aldosterone, corticosterone, and angiotensin II**

Plasma and brain aldosterone were measured by RIA after extraction on Sep-Pak C18 cartridges as previously described (2). Brain tissue was first homogenized, on ice, in 10 volumes 100% methanol using a polytron. After centrifugation (3000 rpm for 30 min at 4°C using a Sorvall Legend RT), the supernatants were dried in a vacuum concentrator. The residues were re-dissolved in 3 ml 0.1% TFA, centrifuged to pellet any insoluble material, and the supernatants applied to preconditioned Sep-Pak C18 cartridges. Aldosterone was eluted with 4 ml 80% methanol after pre-washing with 12% methanol. Dried eluates were re-dissolved in 1.2 ml RIA buffer (0.1M PBS containing 0.5% BSA.) Duplicate 0.2 ml aliquots (plus 0.3 ml RIA buffer) were incubated with 100µl each aldosterone antibody (ICN, #07-108216, final dilution 1:90,000) and [125-I]aldosterone (ICN, #07-108226) for 16-24 hr at 4°C. After separation with dextran-coated charcoal, supernatants were counted using a Canberra Packard (CP) AutoGamma counter. The detection limit is calculated based on the statistical difference between the zero standard and the first non-zero standard (using the AutoGamma’s curve fitting software). For aldosterone, this value was 1.0 pg/tube. Since the average weight of hypothalamic tissue extracted was 80-90 mg and the amount put into each RIA tube was ~14 mg, the sensitivity for hypothalamic aldosterone was 1.0/14 = 0.071 pg/mg or 71 pg/g. The lowest aldosterone tissue values were 50% higher than the assay detection limit. For the hippocampus, the average tissue weight was 175 mg, the amount in each RIA tube was ~29 mg, and the sensitivity was 0.034 pg/mg or 34 pg/g. The lowest aldosterone values in the hippocampus were ~2 times higher than the assay detection limit. The intra-assay variation was 7% and all samples from an experiment were done in one assay. The recovery i.e. spiking with known concentrations of aldosterone was ≥ 88%. Cross re-activities were 0.03 and 0.14 % for corticosterone and DOC, respectively. Plasma aldosterone was measured similarly, except 0.5 ml was applied directly to the preconditioned cartridges, the dried eluates were re-dissolved with 2.5 ml RIA buffer, and duplicate 0.5 ml aliquots were used for the RIA. The sensitivity for plasma aldosterone was equivalent to 10pg/ml.

For tissue corticosterone measurements, the brain tissues were prepared as described above for the aldosterone assay, then the re-dissolved Sep-Pak eluates were further diluted 1:5 with steroid diluent (MP Biomedicals product #07-166197) before assay using an [125I]- corticosterone RIA kit (MP Biomedicals, NY, product #07-120103) according to the manufacturer’s instructions. Briefly, 200µl [125I] labeled corticosterone was added to 100µl standard or diluted sample, followed by the addition of 200µl anti-corticosterone. Tubes were mixed well, and incubated for 2 hours at room temperature. The second antibody was added to precipitate the bound fraction, and after centrifugation, and removal of the supernatant, the pellet was counted using the CP AutoGamma. Corticosterone standards in the range of 6.25 – 500 pg/tube were run in parallel. In the present study, the detection limit calculated from the corticosterone standard curve was 2.0 pg/tube. Based on the average hypothalamic weight of 80-90 mg and the amount of tissue put into each RIA tube was ~1.4 mg, the sensitivity for hypothalamic corticosterone was 2.0 /1.4 =
1.4 pg/mg or 1.4 ng/g. For the hippocampus, the equivalent of ~3.0 mg was put into each RIA tube, giving a sensitivity of 0.67 pg/mg or ng/g. The lowest tissue values were 5 times higher than the assay detection limit. The intra-assay variation was 7% and all samples from the experiment were done in one assay. Recovery was >80%. The corticosterone antibody had 100% cross reactivity with corticosterone, 0.34% with desoxycorticosterone, and 0.1% with testosterone. The same kit was used to measure plasma corticosterone according to the manufacturer’s instructions.

Plasma and CSF Ang II were measured by RIA after extraction on C18 Sep-Pak cartridges and separation by HPLC, as described previously (3, 4). The angiotensin II antibody used in the RIA was a generous gift from Drs. Schalekamp and Danser (Erasmus University Rotterdam, The Netherlands) and its specificity and sensitivity have been extensively validated (2-5). Intra- and inter-assay variabilities and recovery were 5 and 13%, and >80%, respectively. The cross-reactivity of the Ang II antibody with other Ang peptides is 55% with Ang III, 73% with Ang-(3-8), 100% with Ang-(4-8), <3.0% with Ang-(1-7) and <0.1% with Ang I. However, the HPLC peaks for other peptides are well separated from Ang II, and were not included in the calculations.

**Statistical analysis for the data in Figures 1-5 in the manuscript**

Fig 1. By one-way ANOVA.
   - For CSF [Ang II], F=8.53, p=0.001.
   - For water intake, F=34.33, p=0.0002.
   - For MAP, F=19.8, p=0.0001.

Fig 2. By one-way ANOVA.
   - (A) For aldosterone, F=13.6, p=0.0002; for corticosterone, F=14.3, p=0.00005.
   - (B) For aldosterone, F=7.1, p=0.004; for corticosterone, F=5.3, p=0.007.

Fig 3. By one-way ANOVA.
   - (A) For aldosterone, F=14.9, p=0.0001; for corticosterone, F=13.2, p=0.0001.
   - (B) F=2.1; p=0.82.

Fig 4. By one-way ANOVA.
   - (A) For aldosterone, F=16.4, p=0.00002; corticosterone, F=14.3, p=0.00005.
   - (B) For aldosterone, F=8.4, p=0.001; for corticosterone, F=10.6, p=0.0006.

Fig 5. By one-way ANOVA for areas under the curve, and one-way ANOVA with repeated measures for BP and HR changes from baseline.
   - For areas under curve, F=59.5, p<0.0001.
   - For BP changes from baseline, F=24.8, 32.4, 19.8 and 18.7; p=0.0001, 0.0001, 0.001, 0.003, for Ang II, Ang II+sc FAD, Ang II+icv Eple and Ang II+icv FAD, respectively.
   - For HR changes from baseline, F=3.4-4.2; p>0.05 for all groups.
References


