Aldosterone Acting Through the Central Nervous System Sensitizes Angiotensin II-Induced Hypertension


Abstract—Previous studies have shown that preconditioning rats with a nonpressor dose of angiotensin II (Ang II) sensitizes the pressor response produced by later treatment with a higher dose of Ang II and that Ang II and aldosterone (Aldo) can modulate each other’s pressor effects through actions involving the central nervous system. The current studies tested whether Aldo can cross-sensitize the pressor actions of Ang II to enhance hypertension by employing an induction–delay–expression experimental design. Male rats were implanted for telemetered blood pressure recording. During induction, subpressor doses of either subcutaneous or intracerebroventricular Aldo were delivered for 1 week. Rats were then rested for 1 week (delay) to assure that any exogenous Aldo was metabolized. After this, Ang II was given subcutaneously for 2 weeks (expression). During induction and delay, Aldo had no sustained effect on blood pressure. However, during expression, Ang II-induced hypertension was greater in the groups receiving subcutaneous or intracerebroventricular Aldo during induction in comparison with those groups receiving vehicle. Central administration of mineralocorticoid receptor antagonist blocked sensitization. Brain tissue collected at the end of delay and expression showed increased mRNA expression of several renin–angiotensin–aldosterone system components in cardiovascular-related forebrain regions of cross-sensitized rats. Cultured subfornical organ neurons preincubated with Aldo displayed greater increases in [Ca2+]i after Ang II treatment, and there was a greater Fra-like immunoreactivity present at the end of expression in cardiovascular-related forebrain structures. Taken together, these results indicate that Aldo pretreatment cross-sensitizes the development of Ang II-induced hypertension probably by mechanisms that involve the central nervous system. (Hypertension. 2012;60:1023-1030.)

Key Words: aldosterone ■ angiotensin II ■ sensitization ■ blood pressure ■ brain renin–angiotensin–aldosterone system expression

Systemic and brain renin–angiotensin–aldosterone systems (RAASs) play critical roles in the regulation of blood pressure (BP) and body fluid homeostasis. In excess, the primary effectors of RAAS, angiotensin II (Ang II) and aldosterone (Aldo), exert similar direct and indirect adverse effects on various body tissues and organs. The interactions and synergism between Aldo and Ang II have been studied in both the periphery and central nervous system (CNS).1,2 We and others have demonstrated that central infusion of a mineralocorticoid receptor (MR) blocker or Aldo synthase (AS) inhibitor attenuates hypertension induced by systemic Ang II administration. Conversely, central blockade of angiotensin type 1 receptors (AT1-R) attenuates Aldo/salt-induced hypertension.3,4 Moreover, intracerebroventricular infusions of low doses of Ang II increase hypothalamic Aldo 4-fold, whereas subcutaneous infusion of the same dose has no effect on plasma or hypothalamic Aldo levels in rats.5 In rats, intracerebroventricular infusions of Aldo cause sympathetic hyperactivity, hypertension, and increased angiotensin-converting enzyme (ACE) and AT1-R mRNA expression in the hypothalamus.5 Blockade of MRs in the CNS by intracerebroventricular infusion of an MR antagonist prevents sympathetic hyperactivity in Dahl salt-sensitive rats during high salt intake6 and attenuates the upregulation of AT1-R and ACE in the hypothalamus in rats with heart failure.7,8

The sensitization of sodium appetite (ie, the ingestion of salty substances) and thirst (water drinking) has been associated with the central interactions of Aldo and Ang II.8,9 Epstein et al10 demonstrated that a low dose of deoxycorticosterone acetate pretreatment regimen, which by itself does not produce a salt appetite, enhances the actions of Ang II so that a subsequent, subthreshold intracerebroventricular dose of Ang II triggers a robust sodium appetite. Electrophysiological studies also indicate that forebrain regions, such as median preoptic nucleus, contain neurons that rapidly sensitize in response to Ang II when animals are preconditioned with Aldo.11

Our previous work demonstrated that a subpressor preconditioning dose of Ang II can act on the brain and sensitize the hypertensive response to a later Ang II treatment and that increased expressions of AS and MR in forebrain cardiovascular control regions are likely to mediate this enhanced pressor effect.12 Such results prompted us to hypothesize that, in addition to Ang II, Aldo may also have

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the capacity to cross-sensitize the actions of Ang II to enhance the hypertensive response to the octapeptide. To test this hypothesis, we conducted BP-recording studies using a 2-infusion protocol. This involved a period of induction (I) when a subpressor dose of Aldo was first infused either peripherally or centrally. Then, after a period of delay (D), a second Ang II infusion was given to test the expression (E) of hypertension. To gain further insight into the mechanisms underlying Aldo-induced cross-sensitization, additional cellular and molecular studies were also conducted.

### Methods

What follows is a brief summary of the experimental protocols. A detailed description of key methods can be found in an expanded Methods section in the online-only Data Supplement.

#### Animals

Sixty-one male Sprague–Dawley rats (10–12 weeks old, Harlan) were used. They were housed in temperature- and light-controlled animal quarters and were provided with rat chow (7013 National Institutes of Health-31 modified rat diet, 0.25% NaCl) ad libitum. All experiments were conducted in accordance with the National Research Council Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and were approved by the University of Iowa Animal Care and Use Committee.

#### Experimental Protocol

The present study followed an I-D-E experimental design, as described previously. During I, a subpressor dose of Aldo or vehicle was delivered subcutaneously (750 ng/h) or intracerebroventricularly (10 ng/h) by osmotic minipump (model 2001, ALZET) for 1 week. To assure that any exogenous Aldo was metabolized, the rats were then rested for 1 week (D). After this time, a second pump (model 2002, ALZET) was implanted to deliver Ang II (120 ng/kg per minute) for 2 weeks (E). Rats were randomly assigned to 1 of 6 groups: (1) I with Aldo plus E with saline (I-Aldo+E-S), (2) I with saline plus E with Ang II (I-Aldo+E-Ang II), (3) I with Aldo plus E with Ang II (I-Aldo+Ang II), (4) I with Aldo and intracerebroventricular MR antagonist (RU28318) plus E with Ang II (I-Aldo+intracerebroventricular MR antagonist (RU28318) +E-Ang II), (5) I with intracerebroventricular saline plus E with Ang II (I-intracerebroventricular saline +E-Ang II), and (6) I with intracerebroventricular Aldo plus E with Ang II (I-intracerebroventricular Aldo +E-Ang II). Brains were harvested at the end of E for analyses of tissue mRNA expression. The microdissected tissue samples for mRNA expression contained the paraventricular hypothalamic nuclei (PVN) or a set of structures lying along the lamina terminals (LT), including the subfornical organ (SFO), median preoptic nucleus, and organum vasculosum. Two additional control (saline, n=5) and experimental (subpressor dose of Aldo, n=5) groups received identical I and D procedures but had their brains collected at the end of D for analysis of mRNA expression. Likewise, 4 additional groups, including 1 control (saline) and 3 experimental groups (same treatments as groups 1–3 without physiological studies), were performed for assessments of Fos-related antigen immunoreactivity (Fra-IR, n=4 per group).

#### Physiological Studies

Under ketamine–xylazine anesthesia, rats were chronically instrumented with telemetry probes (TA11PA-C40; DSI) placed in the femoral artery for continuous monitoring of mean arterial pressure (MAP) and heart rate (HR), as described previously. Beginning 7 days after recovery from surgery, MAP and HR data collection was initiated. To study the cross-sensitizing effect of Aldo on Ang II-induced hypertension, the first osmotic pump containing saline or Aldo and the second pump containing saline or Ang II were implanted on the back of rats under isoflurane anesthesia.

### Measurement of mRNA Expression in the LT and PVN

Total RNA was isolated from LT and PVN tissue samples using the Trizol method (Invitrogen). Total RNA was reverse transcribed using random hexamers following the manufacturer’s instructions (Applied Biosystems). cDNA was amplified and analyzed using a C1000 thermocycler system (Bio-Rad). Changes in mRNA expression levels were normalized to GAPDH levels and calculated using the ΔΔCt method. Results are expressed as relative fold change, mean of fold change±SE.

#### Fluorescent Immunohistochemistry

Immunohistochemical studies were performed to assess neuronal activation in the SFO and PVN. Expression of Fra-like activity was used as an indicator of chronic neuronal activation. Brain sections were incubated with a rabbit polyclonal anti-Fos antibody (K-25, 1:1000, Santa Cruz) for 72 hours at 4°C. After being thoroughly washed with PBS, sections were incubated with Cy2-conjugated AffiniPure donkey antirabbit IgG (1:100, Jackson Laboratory) for 2 hours at room temperature. Fluorescence was then identified using confocal microscopy.

#### Measurement of Intracellular Calcium ([Ca²⁺]) in Cultured SFO Neurons

Experiments were performed in SFO cells after 3 days in culture. Cells were loaded with Fluo-4, an indicator of [Ca²⁺]. After incubation with Fluo-4 fluorescence was imaged using confocal microscopy and then was quantified using Fluoview 5 (Olympus, Japan) analysis software. To explore the effect of Aldo on Ang II-induced [Ca²⁺]i, cells were incubated overnight with Aldo (10 nmol/L) before an acute application of Ang II (100 nmol/L). The percentage changes of Fluo-4 fluorescence intensity after Ang II application were expressed relative to fluorescence of vehicle-treated neurons.

### Data Analysis

Baseline MAP and HR data were collected for 5 days and then for 28 consecutive days throughout I, D, and E. MAP and HR are presented as mean daily values averaged from daytime and night time measurements. Difference scores for MAP and HR were calculated for each animal based on the mean of the 5-day baseline subtracted from the mean of the final 5 days of treatment. One-way ANOVAs for the experimental groups were then conducted on the means of calculated difference scores. After establishing a significant ANOVA, post hoc analyses were performed with Tukey multiple comparison tests between pairs of mean change scores. The atlas of Paxinos and Watson was used to define regions of interest to evaluate Fra-IR in the SFO and PVN. Statistical evaluation of Fra-IR counts was performed by 1-way ANOVA and Tukey tests. The same statistical methods were used to analyze the differences in mRNA expression of brain RAAS components in the groups that had previously received Aldo during I versus the vehicle-treated group and in Fluo-4 fluorescence intensity response to Ang II in the neurons with or without overnight incubation with Aldo.

### Results

#### Effect of Aldo-Induced Sensitization During I on MAP and HR Induced by Ang II During E

During I and D, the subcutaneous subpressor dose of Aldo had no effect on MAP. However, over the course of E, Ang II induced a greater increase in MAP in the rats that received Aldo (Δ41.6±4.9 mm Hg, P<0.05, Figure 1A and 1C) during I as compared with the rats treated with saline (Δ19.8±4.1 mm Hg) during I. This augmentation of the pressor effect induced by Ang II was attenuated by concurrent intracerebroventricular infusions of the MR antagonist RU28318 along with the subcutaneous Aldo during I (Δ21.7±4.4 mm Hg, P<0.05).
To confirm that the effect of Aldo-induced sensitization was through its actions on the CNS, a low dose of Aldo, which had no effect on BP, was infused intracerebroventricularly for 1 week during I. This low-dose intracerebroventricular infusion of Aldo also enhanced the pressor effects induced by a subsequent Ang II (Δ35±1.4 mm Hg, P<0.05) infusion during E as compared with that of intracerebroventricular saline-treated rats (Δ21.5±3.8 mm Hg, Figure 1B and 1C).

Ang II infusion during E produced a significant decrease in HR in all groups (P<0.05). However, the fall in HR during Ang II treatment was similar in all groups (Figure 1D).

**Effect of Ang II Infusions on the mRNA Expression of RAAS Components in the LT**

In LT tissue collected at the end of D, a subpressor dose of Aldo during I induced a significant increase in the mRNA expression of angiotensinogen (AGT), AT\(_1\)-R, AT\(_2\)-R, ACE1, and MR in the LT when compared with controls (P<0.05). The expression of renin, ACE2, and AS in the LT was not higher after D (P>0.05, Figure 2).

At the end of E, Ang II induced a similar increase in the mRNA expression of RAAS components in the rats treated with saline during I, with an additional significant increase in AS (P<0.05). However, the Ang II infusion during E resulted in greater increases in mRNA expression of renin, AGT, AT\(_1\)-R, and ACE1 in the LT of the group that received either subcutaneous or intracerebroventricular Aldo during I (P<0.05). In contrast, ACE2 mRNA expression was significantly reduced (P<0.05). Central infusion of the MR antagonist during I blocked these augmented effects on mRNA expression (Figure 2).

**Effect of Ang II Infusions on mRNA Expression of RAAS Components in the PVN**

The subpressor dose of Aldo administered during I had no effect on mRNA expression of any of the RAAS components studied in the PVN at the end of D. At the end of E, Ang II induced only a slight, but significant increase in the mRNA expression of renin, AT\(_1\)-R, AS, and MR in the PVN of the animals that received saline during I (P<0.05, Figure 3). However, the subpressor dose of Aldo delivered peripherally or centrally during I enhanced Ang II-induced mRNA expression in AGT, AT\(_1\)-R, ACE1, and ACE2 during E (P<0.05), whereas the mRNA expression of renin and MR remained elevated above the control condition, but not significantly higher than the animals that received saline during I followed by Ang II during E. Central infusion of the MR antagonist during I enhanced ACE2 mRNA expression, but blocked the increased expression in ACE1 and MR and had no effect on increased expression of renin, AGT, and AT\(_1\)-R produced by the infusion of Ang II (Figure 3).

**Effect of Pretreatment With Aldo on Ang II-Induced [Ca\(^{2+}\)]\(_i\) in Cultured SFO Neurons**

Figure 4A shows representative bright field and Fluo-4 fluorescent images of cultured SFO neurons with or without Ang II and Aldo treatments. Acute application of Ang II increased Fluo-4 intensity in SFO neurons by 49.9±45.0% (n=55, P<0.05, Figure 4B). Overnight preincubation of Aldo augmented the Ang II-induced increase in Fluo-4 intensity in SFO neurons (70.6±5.3%, n=85, P<0.05, Figure 4B). Moreover,
pretreatment with Aldo also significantly increased the number of SFO neurons sensitive to acute application of Ang II when compared with SFO neurons pretreated with saline (57.4% versus 48.7%).

**Effect of Ang II Infusion on Neuronal Activation in the SFO and PVN of Rats Treated With Saline or a Subpressor Dose of Aldo During I**

A subpressor dose of Aldo during I induced significant (P<0.05) increases in Fra-IR in both the SFO (45.8±6.8 cells) and PVN (109.8±9.8 cells) compared with sparse labeling seen in these structures in the animals receiving saline during I and E (SFO 15.0±2.0 cells, PVN 45.8±7.4 cells). At the end of E, Ang II induced a similar increase in Fra-IR in both the SFO (47.3±4.4 cells) and PVN (99.4±7.9 cells) of the rats that received saline during I. A subpressor dose of Aldo given during I augmented Fra-IR in the SFO (95.7±10.4 cells, P<0.05) and PVN (241.3±13.0 cells, P<0.05) after Ang II infusion during E (Figure 5).

**Discussion**

Previous studies from our laboratory have shown that Ang II and Aldo interact in the CNS in a mutually cooperative manner to induce hypertension and that low doses of Ang II acting in the brain sensitize the hypertensive response to a subsequently administered slow pressor dose of Ang II. The present study investigated whether a conditioning treatment with Aldo acts to enhance the hypertensinogenic effects of Ang II and whether this is mediated through the CNS. The major results of these studies provide evidence indicating that previous systemic treatment with a subpressor dose of Aldo cross-sensitizes the pressor actions of Ang II. Aldo–Ang II cross-sensitization can also be produced when Aldo is administered intracerebroventricularly. This observation, plus the demonstration that MR antagonist administered intracerebroventricularly during I blocked the cross-sensitizing action of systemic Aldo, indicates that the activation of MR is necessary for Aldo–Ang II cross-sensitization and that the sensitization process is likely to involve the CNS. Furthermore, the cross-sensitizing action of Aldo is associated...
with increased neuronal activation as demonstrated by elevated levels of immunoreactivity for immediate early gene protein (ie, Fra-like fluorescence) and elevated mRNA expression of components of the brain RAAS in the LT and PVN. Also, studies of SFO neurons demonstrated that conditioning cultured cells with Aldo enhanced the neuronal responses to Ang II. Taken together, these and previous results indicate that subpressor levels of either circulating Aldo or Ang II can

Figure 3. Angiotensin II (Ang II) infusion induced greater increases in mRNA expression of angiotensinogenin (AGT) (A), angiotensin type 2 receptors (AT2-R) (B), and angiotensin-converting enzyme 1 (ACE1) and ACE2 (C) in the paraventricular hypothalamic nuclei (PVN) of rats receiving a subpressor dose of aldosterone (Aldo) vs those receiving saline during induction (I). E indicates expression; icv, intracerebroventricular. I-Aldo, peripheral treatment with Aldo during I; I-S, peripheral treatment with saline during I; E-Ang II, peripheral treatment with a pressor dose of Ang II during E; S, saline; RU, RU28318. *P<0.05 vs control; #P<0.05 vs I-Aldo alone or I-S+E-Ang II, †P<0.05 vs I-(icv)Aldo+E-Ang II.

Figure 4. Overnight preincubation of aldosterone (Aldo) augmented angiotensin II (Ang II)-induced increase in [Ca2+]i in subfornical organ (SFO) neurons. A, Representative bright field and Fluo-4 fluorescent images of cultured SFO neurons with or without Ang II and Aldo treatment. B, Summary of the changes in fluorescence intensity when treated with Ang II in SFO neurons with or without Aldo preincubation. *P<0.05 vs pretreatment with saline.
deoxycorticosterone acetate pretreatment elicited a long-term enhancement of median preoptic nucleus neuronal activity and increased the sensitivity to Ang II.\textsuperscript{11} In in vitro studies investigating the interactions of Aldo and Ang II on vascular smooth muscle cells, pretreatment with Aldo decreased the threshold for increases in $[\text{Ca}^{2+}]_i$ elicited by the octapeptide.\textsuperscript{16} Using a similar in vitro strategy in the present study, we found that cultures of SFO neurons conditioned with Aldo displayed an increased number of neurons responsive to Ang II and that these neurons showed a greater increase in $[\text{Ca}^{2+}]_i$ in response to Ang II. This finding is consistent with the observations of others\textsuperscript{11} and the idea that Aldo and Ang II can act on the same forebrain neurons to synergize and cross-sensitize each other’s actions.

All components of the RAAS involving both Aldo and Ang II synthesis and receptors are expressed within the CNS.\textsuperscript{17,18} In the periphery, renin production is generally considered to be rate limiting for the RAAS. However, the regulation of AGT, ACE, and the number and affinity of AT-R are also important for regulating activity of this system.\textsuperscript{18,19} Increased renin and AGT expression in the brain results in an increase in Ang II production, which is associated with elevated thirst and salt intake.\textsuperscript{20} Aldo has also been shown to upregulate AGT through an Ang II-dependent pathway to enhance ischemia-induced neovascularization.\textsuperscript{21} In our current investigations where Aldo was delivered during I, LT tissue collected at the end of D showed elevated expression of ACE1, AT$_1$-R, MR and AGT mRNAs. Interestingly, at the end of D, there were no changes in expression of mRNA for components of the RAAS in the PVN with Aldo given during I. Taken together, these observations suggest that there are LT RAAS components that are common mediators of sensitization/cross-sensitization processes. The capacity of Ang II or Aldo to mutually upregulate their receptors may be indicative of actions within central positive feed-forward systems, which are responsible for sensitization/cross-sensitization and which can accelerate the onset and rate of development of hypertension.

Pretreatment with Aldo not only increased expression of several RAAS pressor-related mRNAs in the LT after D, but also enhanced renin, AGT, AT$_1$-R, and ACE1 expression in the LT and AGT and ACE1 expression in the PVN in I-Aldo+E-Ang II rats. Because BP was higher in I-Aldo+E-Ang II rats as compared with I+S+E-Ang II animals, it also cannot be ruled out that the observed increase in mRNA expression was not attributable to the increased elevation of BP. However, it seems reasonable to consider that there was an accelerated increase in the activity of the brain RAAS as a result of Aldo cross-sensitization. One explanation for the apparent enhancement of the hypertensive response is the possibility that the act of terminating Aldo administration itself might actually be the cause of sensitization. However, this does not seem to be as parsimonious as the interpretation that it was the initial exposure to Aldo during I that induced persistent CNS molecular and structural changes. This view is one that is most consistent with sensitization studies in other fields of neuroscience (drug addiction, learning and memory, long-term potentiation, pain, enhancement of salt appetite, etc). The simple reason we chose the experimental design of removing Aldo at the end of the I

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Figure 5. Treatment with a subpressor dose of aldosterone (Aldo) during I enhanced subsequent angiotensin II (Ang II)-induced Fra expression (indicating neuronal excitation) in the subfornical organ (SFO) and paraventricular hypothalamic nuclei (PVN) vs those treated with saline during induction (I). A, Representative fluorescence microscopic images of sections from all groups of rats. B, Quantification of Fra-positive neurons in the SFO and PVN of each group. E indicates expression; E-Ang II, peripheral treatment with a pressor dose of Ang II during E; E-S, peripheral treatment with saline during E; I+Aldo, peripheral treatment with Aldo during I; I-S, peripheral treatment with saline during I; S, saline. *P<0.05 vs I-S+E-S; #P<0.05 vs I-Aldo+Ang II or I-S+E-Ang II.
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induce long-term changes in CNS function that increase the vulnerability to hypertension.

Forebrain structures located along the LT and hypothalamic nuclei, particularly the PVN, play important roles in the long-term regulation of BP and body fluid homeostasis.\textsuperscript{13} Many forebrain regions have been shown to contain both MR and AT$_1$-R,\textsuperscript{14,15} and such a close proximity of these receptors raises the possibility that there are common cellular sites of interaction between mineralocorticoids and Ang II. Using iontophoretic and electrophysiological techniques, Thornton et al\textsuperscript{11} investigated in vivo synergistic actions produced by peripheral priming doses of deoxycorticosterone acetate followed at a later time by centrally administered Ang II. They found that
period was to assure that we had eliminated the possibility that exogenous Aldo was still available at the time E began.

The complete set of enzymes for the synthetic cascade of Aldo has been documented to be present in the brain by demonstrations of mRNA expression and of AS activity. Peripheral or intracerebroventricular administration of either MR antagonists or specific AS inhibitors significantly attenuates Ang II-induced kidney injury and hypertension, suggesting that an increase in the local synthesis of Aldo acting on brain MR can generate increased AT1-R.3,4,22 Although such functional studies indicate that increased endogenous brain Aldo and MR might enhance the pressor effects of Ang II, previous studies did not report evidence of increased AS expression or elevated Aldo.4,21 These previous studies attempted to find increased AS expression by isolating mRNA from the entire hypothalamus. This procedure may have resulted in diluting AS mRNA, because within the entire hypothalamus there may be only a small amount of AS localized to specific nuclei. In the present study, we microdissected the PVN from the entire hypothalamus and, in addition, collected the LT region in order to obtain greater concentrations of critical mRNA from key tissues, thereby permitting better quantification of mRNA expression. We found that Aldo given during I induced a small increase in MR expression in the LT when measured at the end of D and that there were marked increases in AS and MR mRNA expression in both the LT and PVN in I-S+E-Ang II-treated rats. Interestingly, I-Aldo+E-Ang II animals did not evidence increased AS or MR expression beyond that seen in I-S+E-Ang II-treated rats.

Recently identified components of the RAAS, ACE2 and Ang(1–7)/Mas-R, have been shown to have important roles in the control of BP and sympathetic activity associated with their pressor-attenuating actions.3,4,13 Xia et al25 reported that AT1-R in the brain of chronically hypertensive mice overexpressing human renin and human AGT has reduced ACE2 activity, but not ACE2 expression. Aldo has also been shown to downregulate ACE2 expression in rat cardiocytes and in the kidney.3,27 MR blockade increases ACE2 activity and expression in congestive heart failure patients and in experimental animals.28 In the present study, we found that Aldo alone or systemic Ang II infusion by itself did not affect ACE2 expression in the LT or PVN. However, in I-Aldo+E-Ang II-treated rats, there was a decrease in ACE2 expression in the LT, but an increase in the PVN. This dissociation of ACE2 mRNA expression in the LT versus PVN indicates that there is site-specific regulation of ACE2 in cardiovascular-related forebrain regions and that this may represent a shift in homeostatic equilibrium when transitioning from a normotensive to a prohypertensive state. The increased ACE2 expression in the PVN may reflect the response of counterregulatory mechanisms attempting to correct the enhanced Ang II-induced hypertension. Previous studies have shown that blockade of endogenous Ang(1–7) in the PVN reduced renal sympathetic tone29 and that overexpression of ACE2 in brain reduced BP and counteracted most of the other typical actions of Ang II.30

Similar to the effects on ACE2 expression, there was enhanced AT1-R expression in the PVN in response to systemic Ang II infusion after sensitization with Aldo. These results are consistent with our recent studies on Ang II sensitization.12 AT1-Rs have been proposed to act as functional antagonists to counterregulate the effects of Ang II on AT1-R.11 Either overexpression or selective activation of central AT1-R reduced BP and plasma norepinephrine levels probably by reducing sympathetic outflow.3,31 The enhanced central AT1-R expression seen in the present study might reflect activation of inhibitory mechanisms that buffer the actions of Ang II acting on AT1-R. However, recent studies have shown that AT1-R is involved in Ang II-stimulated Aldo release. In rat adrenal glomerulosa or in human adrenals with an Aldo-producing adenoma, AT1-R agonists increased Aldo secretion, and this response was completely inhibited by the AT1-R antagonist, PD123319.34,35 If there is a similar relationship in the brain between AT1-R and Aldo, enhanced AT1-R expression might be expected to increase brain Aldo production to facilitate increased BP. Further studies will be necessary to clarify the roles of Ang II/AT1-R and ACE2/Ang(1–7)/Mas-R in Aldo and Ang II sensitization/cross-sensitization processes.

**Perspectives**

Both earlier studies and the present experiments demonstrate that preconditioning with nonpressor infusions of either Ang II or Aldo can sensitize/cross-sensitize the hypertensinogenic actions of Ang II. These findings indicate that the Ang II-sensitizing and Aldo-cross-sensitizing effects are sustained and are associated with maintained changes in the expression of pressor components of the brain RAAS. In particular, expression of ACE1, AT1-R, and AS is increased in structures of the LT. Such enduring preconditioning effects must be mediated by some form of neuroplasticity, which involves a cascade of cellular and molecular events in the coupling between I and E. Further experiments will be performed to determine the role of some particular factors (e.g., brain-derived neurotrophic factors) or pathways in the neuroplasticity, which mediates the sensitizing effects induced by Aldo or Ang II.

Because circulating Aldo can access many brain regions, and evidence indicates that Aldo may be synthesized de novo in the CNS, increased mineralocorticoid levels in the brain may lead to generation of a hypertensinogenic viscous cycle by accelerating the activation of pressor components of the central RAAS. Regardless of the source of Aldo, the application of MR blockers with systemic and central actions in conjunction with Ang II antagonists provides an important rationale for a strategy to reduce the Ang II and Aldo CNS sensitizing/cross-sensitizing effects that may contribute to and accelerate the onset and progression of hypertension.

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**Disclosures**

None.

**References**

Novelty and Significance

What Is New?
- These studies demonstrate that exposure to systemic or central nonpressor doses of aldosterone is capable of sensitizing the angiotensin II-induced hypertensive response.

What Is Relevant?
- The demonstration of cross-sensitization between the central actions of aldosterone and angiotensin II indicates that central nervous system neuroplasticity is likely to play an important role in the pathogenesis and progression of some forms of hypertension.

Summary
The study indicates that aldosterone acts on the brain to cross-sensitize the hypertensive response to angiotensin II and that cross-sensitization is associated with maintained altered expression of renin–angiotensin–aldosterone systems within components of a forebrain cardiovascular control network.
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Aldosterone Acting through the CNS Sensitizes Angiotensin II-Induced Hypertension

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Methods
Measurement of mRNA Expression in the LT and PVN

Total RNA was isolated from LT and PVN using Trizol method (Invitrogen) and treated with DNase I (Invitrogen). RNA integrity was checked by gel electrophoresis. Total RNA was reverse transcribed using random hexamers following the manufacturer’s instructions (Applied Biosystems). Real time PCR was conducted using 200-300 ng of cDNA and 500 nM of each primer in a 20 μl reaction with iQ SYBR Green Supermix (Bio-Rad). Amplification cycles were conducted at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and annealing/extension at 60°C for 30 s. Reactions were performed in duplicate and analyzed using a C1000 thermocycler system (Bio-Rad). Samples that did not yield homogeneous melt curves were excluded. Changes in mRNA expression levels were normalized to GAPDH levels and calculated using the ΔΔCt method. Results are expressed as relative fold change, mean of fold change ± SE. Primers were purchased from Integrated DNA Technologies (Coralville, IA). The sequences of the primers are shown in Table S1.

SFO cells culture

Five to six rat pups (18-20 days old) were decapitated, and the head was placed in ice-cold 70% ethanol. The brain was removed immediately and placed in a petri dish with ice-cold cutting solution (220 mM sucrose, 3 mM KCl, 0.2 mM CaCl2, 10 mM dextrose, 6 mM MgSO4, 1.25 mM NaH2PO4, 26 NaHCO3). A thick coronal slice was made to include tissue from the rostral level of the optic chiasm caudal to the collicular level. Previous dissection studies carried out in animals that had been systematically injected with Evans blue (1% solution), which labels brain areas outside the blood-brain barrier (i.e., circumventricular organs, such as the SFO), have shown that the SFO can be isolated and removed from these coronal slices with minimal non-SFO tissue attached (non-SFO tissue is mainly from the hippocampal commissure). Isolated SFOs from five to six pups were transferred to a tube containing Earle’s balanced salt solution (Sigma) and dispase I (4 U/2 ml; Roche) and incubated for 1 h at 37°C. After three washes in culture medium, a cell suspension was prepared by trituration of the fragments through a fire-polished Pasteur pipette until the tissue fragments were visibly dissociated, and then several drops of the medium containing the cells were plated onto previously precoated (0.1 mg poly-L-lysine/ml; Sigma) round 22-mm coverslips and incubated in a humidified atmosphere (plus 5% CO2) at 37°C. The culture medium was Dulbecco’s modified Eagle’s medium (Sigma) with 10% fetal bovine serum (heat inactivated at 56°C for 30 min; Sigma) and 1% L-glutamine-penicillin-streptomycin solution (Sigma) added. After 2-4 h postplating to allow the cells to adhere to the coverslips, additional medium was added.

Intracellular calcium measurement

Intracellular calcium concentration was measured with Fluo-4AM. Cells were loaded with Fluo-4 by incubating cells grown on 22-mm coverslips in artificial cerebrospinal fluid (aCSF; 126 mM NaCl, 3 mM KCl, 2 mM CaCl2, 10 mM dextrose, 1 mM MgSO4, 1.25 mM NaH2PO4, 26 NaHCO3) containing 0.02 mM Fluo-4 for 60 min at 37°C. During the incubation, fluo-4-AM is absorbed by the cell and hydrolyzed only within living cells to impermeant Fluo-4. For cytosolic calcium measurements and pharmacological manipulations, the coverslip was mounted in a bath chamber that was attached to the microscope stage and artificial CSF was used as the recording
solution. Fluorescence intensity was measured using a video microscopic digital image analysis system (FluoView 500 Confocal Laser Scanning Microscope; Olympus) with a 488 nm Argon laser as an excitation source. An increase in cytosolic calcium concentration produces an increase in fluorescence intensity with only a little shift in wavelength. At the end of the experiment data was analyzed and the fluorescence intensity converted to numerical values. A response to stimulation was discerned as a peak increase in fluorescence intensity and represented as a percentage change from baseline recording. Cell viability was checked at the end of the experiment by applying artificial CSF containing 50 mM KCl (substituting an equimolar amount of NaCl). All procedures were performed at room temperature (24 ± 2°C) in a dark room. The manipulation of removing and applying bath solutions can induce a small change in fluorescence intensity (2.99 ± 0.67%). Therefore, only cells that presented a change in fluorescence intensity greater than 8.7% (average plus two standard deviations) were considered as being responsive to the stimulus applied.

Immunohistochemistry

The day after the conclusion of the treatments, the rats were anesthetized and perfused with 0.1 M PBS, followed by 4% paraformaldehyde (PF). Free-floating sections (40 μm) were incubated in 5% normal donkey serum for 1 h, followed by a primary antibody, rabbit polyclonal anti-Fra (K-25, 1:1000, Santa Cruz) in 5% donkey normal serum with 0.2% Triton X-100 for 72 h at 4°C. After being thoroughly washed with PBS, sections were incubated with Cy™-conjugated AffiniPure donkey anti-rabbit IgG (1:100, Jackson) in PBS for 2 h at room temperature. Fluorescence was then identified using confocal microscopy. In the PVN, level 1 (-1.60 mm) is the most rostral and included the dorsal parvocellular, medial parvocellular, and ventrally located posterior magnocellular subnuclei. Level 2 (-1.88 mm) contains a prominent posterior magnocellular region and both dorsal and ventrolateral parvocellular divisions. Level 3 (-2.12 mm) is the most caudal and consisted of the medial and lateral parvocellular divisions. The number of retrogradely labeled cells from the spinal cord and RVLM is significantly greater at levels 2 and 3 vs level 1 (Stocker et al, Am J Physiol Regul Integr Comp Physiol 287:R1172-R1183, 2004). We used levels 2 and 3 for summarizing the Fra results.

Table S1: Primer Sequences for Real Time PCR

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<th>Gene</th>
<th>Forward primer</th>
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<th>Accession number</th>
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AGT, angiotensinogen; MR, mineralocorticoid receptor; AT-R, angiotensin receptor; ACE, angiotensin converting enzyme; AS, aldosterone synthase