Sensitization of Slow Pressor Angiotensin II (Ang II)–Initiated Hypertension

Induction of Sensitization by Prior Ang II Treatment

Baojian Xue, Zhongming Zhang, Ralph F. Johnson, Alan Kim Johnson

Abstract—Sensitization involving the central nervous system has been studied in many conditions but has received little attention in investigation of the pathogenesis of hypertension. Our experiments were initiated to determine whether angiotensin II (Ang II)–induced hypertension can be sensitized by prior Ang II treatment and the role of the brain renin-angiotensin-aldosterone system (RAAS) in this process. To demonstrate Ang II–induced sensitization, we used an experimental design of induction-delay-expression. Male rats were implanted for telemetered blood pressure (BP) recording. During induction (I), low doses of subcutaneous or intracerebroventricular Ang II were delivered for 1 week, and then the rats were rested for 1 week (delay [D]) to ensure that any exogenous Ang II was metabolized. After this, a second higher dose of Ang II was given subcutaneously for 2 weeks (expression [E]). During I and D, the low doses of Ang II had no sustained effects on BP. However, during E, the Ang II–induced BP increase was greater in the groups that had received low doses of Ang II during I in comparison to the group receiving saline during I. Central angiotensin type 1 receptor antagonist delivery blocked this sensitization. Brain tissue collected at the end of D and E showed increased mRNA expression of several RAAS components in key forebrain regions of sensitized rats. Fos-related antigen–like immunoreactivity was also increased at the end of E in the sensitized forebrain. These results indicate that subpressor doses of Ang II act on the brain to sensitize the hypertensive response to subsequent Ang II and that sensitization is associated with altered expression of RAAS components in forebrain cardiovascular control structures.

Key Words: angiotensin II ▪ blood pressure ▪ sensitization ▪ neuronal activation ▪ RAAS component expression

It has been nearly 50 years since Dickinson and colleagues first described the effect of infusing nonpressor doses of angiotensin II (Ang II) into rabbits to gradually produce hypertension over the course of several days. This slow Ang II–induced pressor phenomenon, defined as autopotentiation, has been demonstrated in several species, including rats and humans. Recently, it has been shown that autopotentiation to Ang II in the induction of hypertension resembles several models of neuroplasticity and behavior that are investigated in the context of sensitization. For example, the sensitization of sodium appetite (ie, the ingestion of salty substances) and thirst (water drinking) has been associated with the central actions of Ang II and aldosterone (Aldo). Although such behavioral studies indicate that increased activity of the brain renin-angiotensin-Aldo system (RAAS) is involved in fluid-related behavioral and neurohormonal sensitization, the involvement of brain RAAS components in similar processes has received little attention in the context of the pathogenesis of hypertension.
kidney, and brain. In experimental animals or hypertensive patients, infusions of Ang II induced an increased expression of renin, AGT, ACE, and AT₁R in the kidney, brain, or T lymphocyte, which resulted in generating higher Ang II levels and hypertension. These results suggest that Ang II can act in a feed-forward manner to enhance the activity and expression of several RAAS components and that such increases in RAAS activity can potentially contribute to an augmented rise in BP induced by Ang II. The present experiments were initiated to determine whether Ang II has the capacity to sensitize the brain in the process of the generation of Ang II–induced hypertension. This study also investigated which components of the brain RAAS are likely to play a role in such an Ang II–initiated sensitization process.

**Methods**

What follows is a brief summary of the experimental protocols. A detailed description of some methods can be found in the expanded Methods section, available in the online Data Supplement at http://hyper.ahajournals.org.

**Animals**

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

**Experimental Protocol**

Operationally, the process of sensitization can be viewed as consisting of a period of induction (I) followed by a period of expression (E). During E, the consequences of I can be evaluated. Because it is possible to vary the amount of time between I and E—in other words, introduce a period of delay (D)—there is an opportunity to observe the response elicited at E in the absence of the treatment (eg, a low dose of Ang II) presented during I. Therefore, the present studies followed an I-D-E experimental design. During I, a low subpressor dose of Ang II or vehicle was delivered subcutaneously (10 ng/kg per minute) by osmotic minipump (model 2001, Alzet) for 1 week. The rats then rested for 1 week (D), after which time, a second pump (model 2002, Alzet) was implanted to deliver a higher subcutaneous dose of Ang II (120 ng/kg per minute) for 2 weeks (E). Rats were randomly assigned to 1 of 6 groups: (1) I with Ang II plus E with saline, (2) I with saline plus E with Ang II, (3) I with Ang II plus E with Ang II, (4) I with Ang II and icv AT₁R antagonist (irbesartan; 125 µg/day, Sigma-Aldrich) plus E with Ang II, (5) I with icv saline plus E with Ang II, and (6) I with icv Ang II plus E with Ang II. Brains were collected at the end of E for regional (the lamina terminalis [LT] and the paraventricular hypothalamic nucleus [PVN]) analysis for mRNA expression or Fos-related antigen immunoreactivity (Fra-IR, indicating neuronal excitation). Two additional [PVN] analysis for mRNA expression or Fos-related antigen immunoreactivity (Fra-IR, indicating neuronal excitation). Two additional

**Immunohistochemistry**

Free-floating sections (40 µm) were incubated in a primary antibody, rabbit polyclonal anti-Fra (K-25, 1:1000, Santa Cruz Biotechnology) at room temperature overnight, followed by incubation in biotinylated goat anti-rabbit immunoglobulins (1:200, Vector Laboratories) for 1 hour. Then, the immunoreaction was detected with an ABC kit (Vector Laboratories) and metal-enhanced diaminobenzidine (DAB) (Sigma-Aldrich).

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

Total RNA was isolated from LT and PVN 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).

**Data Analysis**

MAP and HR data were collected for 5 baseline 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 nighttime 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 analysis was performed with Fisher least significant difference multiple comparison tests between pairs of mean change scores. To test for differences in the means of the 5 baseline days versus the means of the final 5 days of treatments, t tests were performed.

A brain atlas was used to define regions of interest to evaluate Fra-IR in the subfornical organ (SFO) and PVN. An experimenter who was blind to the experimental group counted cells showing Fra-IR in 2 or 3 histological sections from each structure for each animal. Statistical evaluation of Fra-IR counts was performed by 1-way ANOVA and Student t tests. The same statistical methods were used to analyze the differences in mRNA expression of brain RAAS components in the groups that previously had received the low dose of Ang II during I versus the vehicle-treated group.

**Results**

**Effect of Ang II–Induced Sensitization During I on MAP and HR Induced by Subsequent Ang II During E**

During I and D, the low subcutaneous dose Ang II had no effect on MAP. However, over the course of E, the high dose of Ang II induced a greater increase in MAP in the rats that received the low dose of Ang II (Δ40.4±2.8 mm Hg, n=6, P<0.05, Figure 1A and 1C) during I as compared with the rats treated with saline (Δ21.2±5.3 mm Hg, n=6) during I. This augmentation of the pressor effect induced by the high dose of Ang II was attenuated by concurrent icv infusions of the AT₁R antagonist irbesartan along with the low subcutaneous dose of Ang II during I (Δ17.9±6.0 mm Hg, n=5, P<0.05).

To confirm that the effect of Ang II–induced sensitization occurred through its actions on the central nervous system (CNS), a low dose of Ang II was infused icv for 1 week during I. This resulted in a mild but significant increase in MAP during the first 3 days, which gradually returned to the basal level by the end of D. The icv infusion
Effect of Ang II Infusion on the mRNA Expression of RAAS Components in the LT

In LT tissue collected at the end of D, a low dose of Ang II during I induced a significant increase in the mRNA expression of AT,R (1.35±0.08-fold, P<0.05, Figure 2B), AT,R (1.46±0.09-fold, P<0.05, Figure 2B), ACE1 (1.34±0.11-fold, P<0.05, Figure 2C), ACE2 (1.48±0.09-fold, P<0.05, Figure 2C), Aldo synthase (AS) (1.84±0.11-fold, P<0.05, Figure 2D), and mineralocorticoid receptor (MR) (1.36±0.15-fold, P<0.05, Figure 2D) in the LT compared with controls (1.00±0.04). The expression of renin and AGT in the LT was not higher (P>0.05, Figure 2A) after D.

At the end of E, the higher dose of Ang II induced a similar increase in the mRNA expression of RAAS components in the rats treated with saline during I. However, the Ang II infusion during E resulted in greater increases in mRNA expression of renin (1.57±0.09-fold, P<0.05), AGT (1.86±0.10-fold, P<0.05), AT,R (3.34±0.27-fold, P<0.05), AT,R (3.76±0.22-fold, P<0.05), ACE1 (3.73±0.24-fold, P<0.05), and AS (2.62±0.05-fold, P<0.05) in the LT of the group that received a low dose of Ang II during I. Central infusion of the AT1R antagonist during I blocked these augmented effects on mRNA expression.

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

The low dose of Ang II 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, the higher dose of Ang II induced a mild but significant increase in mRNA expression of AS (1.22±0.06-fold, P<0.05) and MR (1.21±0.66-fold, P<0.05) in the PVN of the animals that received saline during I. The mRNA expression of other components of RAAS in the PVN of rats receiving saline during I was not increased. However, a low dose of Ang II delivered during I enhanced Ang II–induced mRNA expression only of AS during E (2.66±0.18-fold, P<0.05), whereas the mRNA expression of 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 (1.54±0.12, Figure 3). Central infusion of the AT1R antagonist during I blocked the effects produced by the infusion of the higher Ang II dose.
Effects of Ang II Infusion on Neuronal Activation in the SFO and PVN of Rats Treated With Saline or a Low Dose of Ang II During I

In the rats that received saline during I, the higher dose of Ang II infused during E produced significant increases in Fra-IR in both the SFO (55.3 ± 13.5 cells, P < 0.05) and PVN (88.3 ± 15.7 cells, P < 0.05) compared with sparse labeling seen in these structures in the animals receiving saline during I and E (SFO 15.7 ± 5.8, PVN 41.3 ± 16.7 cells). A low dose of Ang II given during I augmented Fra-IR in the SFO (107.3 ± 19.3 cells, P < 0.05) and PVN (153.3 ± 2.4 cells, P < 0.05, Figure 4A and 4B) after the higher dose of Ang II infusion during E.

Discussion

The present study investigated the capacity of Ang II to sensitize the brain and thereby enhance the onset of hypertension to a subsequent treatment with Ang II. The activation of forebrain areas and the expression of mRNA for components of the brain RAAS was also studied to gain insight into the role of the brain and neurohumoral mediators in this process. The most important finding of these studies demonstrated that a low dose of Ang II can sensitize the brain to produce an enhanced hypertensive response to Ang II. The amplified pressor effects produced by sensitizing the brain are associated with increased neuronal activation and elevated mRNA expression of RAAS components in 2 key forebrain cardiovascular control regions. Both the functional sensitization and the enhanced mRNA expression were blocked by central AT1R antagonist administration. These results suggest that subpressor levels of Ang II can induce sustained CNS changes that increase the vulnerability to hypertension.
The initiation and development of hypertension involves the interactions of multiple organs and systems. Targeting experimental ablations to specific organs (cf. ablation of the organum vasculosum of the LT [OVLT]) can interrupt the course of development or maintenance of high BP produced by the systemic infusion of Ang II. Current evidence indicates that there are at least 2 distinct phases of Ang II–induced hypertension. First, an initial elevation in pressure associated with an Ang II–mediated systemic vasoconstriction, which is then replaced by a second, long-term, neurogenic component. In the present study, the initial increase in BP in response to systemic Ang II administration (ie, within 4–7 day) was comparable for both groups with or without the low dose of Ang II as a pretreatment. After days 4 to 7, the increase in BP accelerated in the low dose of Ang II–pretreated group. This suggests that the sensitization occurs during the time when neurogenic mechanisms are contributing to the progression of increased BP.

Forebrain structures, along with the LT (ie, the SFO, median preoptic nucleus, and organ vasculosum of the LT) and the hypothalamus (particularly the PVN), play important roles in the long-term regulation of BP and body fluid homeostasis. LT structures are involved in both sensing and processing input derived from humoral factors (eg, Ang II and extracellular osmolality) and transmitting this information to the PVN, which in turn projects to hindbrain and spinal cord cardiovascular control structures. Recent studies demonstrate that chronic 14-day infusions of pressor doses of Ang II into rabbits and rats result in sustained SFO and PVN activation (ie, increased Fra-IR) and increased BP. The present results are consistent with these studies and extend them by showing that a low, sensitizing dose of Ang II during I augments both hypertension and neuronal SFO and PVN activation produced by 14 days of Ang II infusion.

Figure 3. Angiotensin II (Ang II) infusion induced significant increases in mRNA expression of aldosterone synthase (AS) and mineralocorticoid receptor (MR) but not mRNA expression of other components of the renin-angiotensin-aldosterone system in the paraventricular hypothalamic nucleus (PVN) of rats receiving either saline or a low dose of Ang II during induction (I). Intracerebroventricular (icv) infusion of the angiotensin type 1 receptor (AT1R) antagonist blocked these effects. I-S indicates peripheral treatment with saline during I; I-Ang II, peripheral treatment with a low dose of Ang II during induction (I); I-Ang II/icv Irbe, peripheral treatment with a low dose of Ang II plus central treatment with irbesartan (Irbe) during I; E-Ang II, peripheral treatment with a high dose of Ang II during expression. *P<0.05 vs saline, #P<0.05 vs I-S+E-Ang II, †P<0.05 vs I-Ang II+E-Ang II.

Figure 4. Treatment with a low dose of angiotensin II (Ang II) during induction (I) enhanced subsequent Ang II–induced Fos-related antigen (Fra) expression (indicating neuronal excitation) in the subfornical organ (SFO) and paraventricular hypothalamic nucleus (PVN) as compared with those treated with saline during I. A, Representative fluorescence microscopic images of section from all groups of rats. B, Quantification of Fra-positive neurons in the SFO and PVN of each group. I-S+ expression (E)-S indicates I with saline plus E with saline; I-S+E-Ang II, I with saline plus E with Ang II; I-Ang II+E-Ang II, I with low dose Ang II plus E with Ang II. *P<0.05 vs I-S+E-S, #P<0.05 vs I-S+E-Ang II.
In hypertensive patients and experimental animals, it has been shown that Ang II can induce increases in both local AT,R expression and Ang II levels, which are likely in turn to contribute to increased BP. In the present studies, the low dose of Ang II administered during I may have facilitated the generation of brain Ang II and increased the number of AT,R. The failure to find an enhancement in BP response during E in animals receiving low doses of Ang II in conjunction with central infusion of an AT,R antagonist during I indicates that activation of AT,R in the brain is necessary during the I phase to sensitize the hypertensive response.

Ang II upregulates AGT and renin expression in the kidney and increases intrarenal Ang II levels along with systolic BP, thereby suggesting a direct link between AGT or renin gene expression and BP. In the brain, AGT is widely distributed in astroglia but has a more restricted regional distribution in neurons. Renin has been identified in neurons, and renin and AGT are located in close proximity to AT,R-containing neurons, suggesting a model for the local production and action of Ang II. Double-transgenic mice, which overexpress human renin (hRen) and human AGT (hAGT) in the brain, exhibit a marked increase in Ang II–like immunoreactivity in the SFO and elevated water and salt intakes, which are significantly reduced after chronic icv delivery of an AT,R antagonist. This provides evidence that increased renin and AGT expression results in an increase in Ang II production in the brain. In the present study, although the low dose of Ang II alone did not increase renin and AGT expression in the LT after D, it did increase expression of message at the end of E. Although it cannot be ruled out that this increase in expression was a result of the elevated level of BP, it may be more parsimonious to propose that it was a progressive increase in brain Ang II through a positive feedback mechanism during systemic Ang II infusion that contributed to the elevated hypertension.

Of the AT-Rs, AT,R is known to mediate most actions of Ang II. Abnormal regulation and function of the AT,R have been proposed to contribute to development and maintenance of various forms of hypertension. In contrast, AT,R have been suggested to act as a functional antagonist of AT,R. Yu et al demonstrated AT,R protein expression in the brain. Either overexpression or selective activation of central AT,R decreases BP and norepinephrine levels, partially by reducing sympathetic outflow. The upregulation of AT,R and downregulation of AT,R expression creates a functional imbalance that may be associated with the pathogenesis of hypertension and heart failure. However, in the present study, Ang II induced a significant increase in expression of both AT,R and AT,R, suggesting that a low dose of Ang II sensitizes the expression of message for both receptors. This enhanced central AT,R expression may reflect the activation of inhibitory mechanisms that attempt to buffer against the actions of Ang II, which collectively act to increase BP. This is in agreement with a recent study conducted in obese Zucker rats.

High ACE has been associated with increased susceptibility to hypertension and end organ damage. Ang II can increase ACE expression and cause renal pathology in rats. A recent study showed that Ang II can interact with ACE through a calcium signaling mechanism to activate ACE and promote increased reactive oxygen species. This result suggests that not only is ACE an Ang II–generating enzyme but that it is also a signaling receptor for this peptide. Inhibition of ACE alone prevents increased ACE protein expression, enhanced formation of Ang II, and increased BP during systemic Ang II infusion.

ACE2 has a high substrate specificity for Ang II to convert it into Ang(1-7). Infusion of Ang(1-7) or overexpression of ACE2 has been shown to counteract most typical actions of Ang II and to reduce BP. In the present study, we found that infusion of a high dose of Ang II during E did not increase ACE2 expression beyond the significant increase produced by a low dose of Ang II given during I. This result is consistent with a recent study from Xia et al showing that AT,R in the brain of chronically hypertensive mice overexpressing hRen and hAGT have reduced ACE2 activity but not reduced ACE2 expression. In contrast, in the present study, a low dose of Ang II was found to sensitize ACE1 expression in the LT, with ACE1 showing higher expression in response to the Ang II administered during E in the rats that received the low dose of Ang II during I. This high ACE1 expression and unchanged ACE2 expression may reflect a shift of homeostatic equilibrium from a normotensive to a prohypertensive state.

In recent years, Aldo has come to be recognized as a key mediator of RAAS-related effects. Like Ang II, Aldo is not only a major regulator of extracellular fluid volume and electrolytes but is also linked to the pathogenesis of hypertension and heart failure. Synthesis of Aldo from 11-deoxycorticosterone in the adrenal cortex is catalyzed by AS, the product of the CYP11B2 gene. The brain has been identified as a major extra-adrenal site of CYP11B2 expression, and Ang II is a stimulus for CYP11B2-related Aldo synthesis. Recent studies highlight the interactions between Ang II and Aldo. Fiebeler et al reported that systemic administration of an AS inhibitor reduced circulating Aldo levels and ameliorated Ang II–induced organ damage in transgenic rats overexpressing both hRen and hAGT. Furthermore, we and others have demonstrated that central infusion of MR blocker or AS inhibitor attenuates hypertension induced by systemic Ang II administration. These data suggest that in the CNS, activation of a local Aldo system acts in a neuromodulatory mode to induce the pressor response to circulating Ang II and that a CNS Aldo-dependent neurogenic component contributes to hypertension induced by circulating Ang II.

In the present study, we performed microdissections of the PVN and LT to collect tissue to quantitate mRNA expression, and we found that the low dose of Ang II during I had no effect on mRNA expression of most RAAS components in the PVN but did produce a marked enhancement of AS mRNA in response to Ang II administered during E in the group that received Ang II during I. In the PVN, mRNA expression of MR was high both after D and E. In contrast to the PVN, the low dose of Ang II during I was associated with increased expression of all the RAAS components in the LT. It is also notable that the responses to Ang II in LT tissues...
were greater than in the PVN. These results suggest that there is likely to be differential regulation among different cardiovascular-related brain nuclei involved in the Ang II–induced sensitization process.

**Perspectives**

The present studies demonstrate that exposure to a nonpressor infusion of Ang II has a sensitizing effect that acts to facilitate an increase in BP produced by a subsequent slow pressor treatment with Ang II. The findings also indicate that this Ang II–induced sensitization involves the CNS and components of the brain RAAS. Through a brain-mediated mechanism, Ang II acts to increase the gain of its own action. One important implication of these observations is that in the course of producing hypertension, Ang II has the capacity for a feed-forward action so that low levels of circulating octapeptide progressively exert a greater and greater pressor action. This observation may account for why some hypertensive patients with plasma Ang II concentrations deemed to be within the normal range respond with lower BP after ACE inhibition or AT1R blockade.

The sensitization process is likely to involve a cascade of cellular and molecular events in the coupling between I and E in the CNS. The induction-delay-expression experimental paradigm we have developed and used in the present studies is likely to be a valuable model for future exploration of the cellular and molecular pathways involved in sensitization processes associated with Ang II–induced hypertension.

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**Disclosures**

None.

**References**


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Sensitization of Slow Pressor Angiotensin II (ANGII) Initiated Hypertension: Induction of Sensitization by Prior ANGII Treatment

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ANGII sensitization and ANGII-induced hypertension
Methods

Immunohistochemistry

The day after the conclusion of the physiologic experiments, 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 goat serum for 1 h, followed by a primary antibody, rabbit polyclonal anti-Fra (K-25, 1:1000, Santa Cruz) at room temperature overnight. Sections were washed in PBS before incubation in biotinylated goat anti-rabbit immunoglobins (1:200, Vector laboratories) for 1h. Then the immunoreaction was detected with a ABC kit (Vector Laboratories) and metal enhanced DAB (Sigma).

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 homogenous 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.

Table S1: Primer Sequences for Real Time PCR

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AGT, angiotensinogen; MR, mineralocorticoid receptor; AT-R, angiotensin receptor; ACE, angiotensin converting enzyme; AS, aldosterone synthase