Angiotensin II enhances GABA_B receptor-mediated responses and expression in nucleus tractus solitarii of rats

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Angiotensin II enhances GABA_B receptor-mediated responses and expression in nucleus tractus solitarii of rats. Am J Physiol Heart Circ Physiol 297: H1837–H1844, 2009. First published September 11, 2009; doi:10.1152/ajpheart.00354.2009.—Angiotensin II (ANG II) increases GABA_B receptor expression in neuronal cultures from the nucleus tractus solitarii (NTS). In the present study, the chronic effects of ANG II on GABA_B receptor expression and activity were examined in the NTS of Sprague-Dawley rats. Intracerebroventricular infusion of ANG II caused a significant elevation in blood pressure (BP) and an increase in GABA_B receptor expression in the NTS. Conversely, chronic N^3-nitro-L-arginine methyl ester (L-NAME) treatment also increased BP, but had no effect on GABA_B receptor expression in the NTS. Next, we examined the BP response to the GABA_B receptor agonist baclofen microinjected into the NTS of ANG II- or artificial cerebrospinal fluid (aCSF)-infused rats. NTS microinjection of baclofen increased BP in both groups of rats. However, the pressor response to baclofen was enhanced in ANG II-infused rats compared with aCSF-infused rats. In addition, bilateral microinjection of the GABA_B receptor antagonist CGP-35348 into the NTS evoked a decrease in BP in both group of rats, and the depressor responses to CGP-35348 were enhanced in the ANG II-infused rats. In contrast, the pressor responses to the GABA_A receptor agonist muscimol and the depressor responses to the GABA_A receptor antagonist bicuculline were comparable between aCSF- and ANG II-infused rats. These results indicate that chronic ANG II infusion stimulates GABA_B receptor expression and augments GABA_B receptor-mediated responses in the NTS. This effect could contribute to the central nervous system actions of ANG II that result in dampening of baroreflexes and elevation in arterial BP.

Hypertension; γ-aminobutyric acid; blood pressure; gene expression

Angiotensin II (ANG II) participates in cardiovascular regulation, not only by its direct effect on vascular smooth muscle, but also via its actions in the central nervous system (2, 8, 10, 18, 21). ANG II receptors are present at many sites in the rat brain, such as paraventricular nucleus, rostral ventrolateral medulla, and nucleus tractus solitarii (NTS) (2, 10, 18). The NTS, where baroreceptor and chemoreceptor afferents terminate, plays an important role in central cardiovascular regulation and in the pathogenesis of hypertension (7, 11). The effects of ANG II in the NTS on baroreflex and cardiovascular control have been well studied. Microinjection of ANG II into the medial portion of the NTS produces either a decrease or an increase in blood pressure (BP), depending on the dose injected (6, 23, 30). Furthermore, microinjection of ANG II into the NTS attenuates baroreceptor reflex sensitivity (10, 17, 29). These previous reports indicate that ANG II may modulate the central integration of the baroreceptor inputs in the NTS (30). The cardiovascular regulation of ANG II is mediated not only by acute modulation of neuronal transmission and neuronal activity, but also via changes in gene expression to influence long-term BP regulation. However, the identity of the particular genes that are altered by and mediate the chronic actions of ANG II is not established. To this end, our previous study demonstrated that chronic ANG II treatment increases γ-aminobutyric acid B (GABA_B) receptor expression and enhances the inhibitory response to the GABA_B receptor agonist, baclofen, in neuronal cultures (36).

The inhibitory amino acid GABA is a potent modulator of neurons within the NTS (5, 24, 27). Moreover, the NTS contains a high density of both GABA_A and GABA_B receptors (19, 38). GABA_B receptors are metabotropic, G protein-coupled receptors that mediate presynaptic and postsynaptic inhibition by reductions in calcium conductance or increases in potassium conductance (38). GABA_B receptors play an important role in the integration of baroreceptor afferent inputs and baroreflex function (8, 19, 31, 34, 35, 38). Microinjection of GABA_B agonists or antagonists into the NTS produce a marked pressor or depressor response, respectively (14, 31, 37). More interestingly, the GABA_B agonist (baclofen)-induced pressure response is enhanced in chronically hypertensive rats, such as spontaneously hypertensive rats (SHR), deoxycorticosterone-salt-induced hypertensive rats, and one-kidney renal wrap hypertensive rats (9, 19, 31, 34, 38). However, the exact cellular mechanisms underlying the enhanced activation of GABA_B receptors under hypertensive conditions are still obscure.

That the potentiated pressor response to GABA_B receptor agonists in the NTS of these hypertensive animal models is similar suggests that a common mechanism may be involved in the enhanced GABA_B receptor-mediated response in the NTS. The activity of ANG II is increased in central cardiovascular regulatory regions, including NTS, in hypertensive animal models (3, 13). Thus the present study was performed to examine the interaction between ANG II and the GABA system in the NTS. Specifically, we determined the effect of chronic central administration of ANG II on GABA_B receptor-mediated responses in the rat NTS.

METHODS

Animals. Adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA), weighing 290–350 g, were used in this study. All animals were housed under controlled conditions with a 12:12-h light-dark cycle. Food and water were available to the animals ad libitum. All protocols were approved by the North Dakota State University Institutional Animal Care and Use Committee (protocol A0741).

Recording of arterial pressure. Chronic BP recording was carried out with a radiotelemetry system. Rats were anesthetized with a
mixture of oxygen (1 l/min) and isoflurane (3%; Halocarbon, River Edge, NJ), which was delivered through a nose cone. A telemetry BP probe (model TA11PA-C40, Data Sciences International, St. Paul, MN) was positioned intra-abdominally and secured to the ventral abdominal muscle with the catheter inserted into the lower abdominal aorta. During the surgery, rats received an intraperitoneally delivered warm sterile 0.9% saline (−3% of body weight) to ensure proper fluid balance. The telemetry signals were processed and digitized as radio frequency data, which were recorded and stored in a computer using the Dataquest IV system (Data Sciences International). In the conscious state, mean values of BP and heart rate (HR) were recorded continuously for 1 h every day between 10:00 AM and 11:00 AM. Continuous recordings were started 4 days after probe implantation.

Acute BP recording was carried out using PE-10 catheters fused to PE-50 catheters under isoflurane anesthesia. The vascular catheters were prefilled with heparinized saline (100 IU/ml), placed in the right femoral artery, and connected to a BP transducer and a bridge amplifier (AD Instrument, Colorado Springs, CO). The BP and HR data were collected and analyzed with PowerLab software (AD Instrument, Colorado Springs, CO).

Intracerebroventricular ANG II infusion. After basal BP recording for a 3-day control period using radiotelemetry, rats were placed in a stereotaxic apparatus under isoflurane anesthesia. A 28-gauge, stainless steel cannula (Alzet Brain Infusion Kit 2, Durect, Cupertino, CA) was implanted into the right lateral cerebral ventricle and fixed on the skull with dental cement. The cannula was placed 1.0 mm posterior to the primary site, 1.4 mm lateral to the bregma. The lower end of the cannula was drilled into the skull with dental cement. The cannula was placed 1.0 mm posterior to the primary site, 1.4 mm lateral to the bregma. The lower end of the cannula was drilled into the skull with dental cement. The cannula was placed 1.0 mm posterior to the primary site, 1.4 mm lateral to the bregma. The lower end of the cannula was drilled into the skull with dental cement. The cannula was placed 1.0 mm posterior to the primary site, 1.4 mm lateral to the bregma. The lower end of the cannula was drilled into the skull with dental cement. The cannula was placed 1.0 mm posterior to the primary site, 1.4 mm lateral to the bregma. The lower end of the cannula was drilled into the skull with dental cement.

Microinjection experiments. After 7-day ICV ANG II infusion, rats were anesthetized with isoflurane. PE50 catheters filled with heparinized saline (100 IU/ml) were inserted into the femoral artery for the measurement of BP and HR, as described above. The GABA<sub>B</sub> receptor agonist or antagonist was microinjected bilaterally into the NTS, according to procedures described previously (12, 31). In brief, the anesthetized animals were placed in a stereotaxic frame. After the ICV infusion minipump and cannula were taken off, a multiple-barrel glass injection pipette (tip size 20–40 μm) was positioned in the NTS. The coordinates for the NTS were determined from the Swanson rat atlas (28), which were 0.5 mm rostral to the caudal tip of the area postrema, 0.5 mm lateral to the midline, and 0.5 mm below the dorsal surface of the brain stem. Proper placement was confirmed by checking for a l-glutamate-induced (200 pmol, in 50 nl) depressor response. This would induce a characteristically abrupt decrease in BP (ΔBP > 35 mmHg) and HR (ΔHR > 50 beats/min), if the needle tip was located precisely in the NTS. After a responsive site was identified by L-glutamate, the probe remained in this site throughout the remainder of the experiment. GABA<sub>B</sub> receptor antagonist (CGP-35348, 100 pmol, in 50 nl, Sigma-Aldrich), GABA<sub>B</sub> receptor agonist (baclofen, 50 pmol, in 50 nl, Sigma-Aldrich), GABA<sub>A</sub> receptor antagonist (bicuculline, 10 pmol, in 50 nl, Sigma-Aldrich), and GABA<sub>A</sub> receptor agonist (muscimol, 100 pmol, in 50 nl, Sigma-Aldrich) were dissolved in saline and microinfused bilaterally into the NTS. The volume of microinjection was determined by the displacement of fluid meniscus in the micropipette barrel under a microscope. The duration of each injection was 10–20 s. For bilateral microinjections of a given drug, the time interval between the two microinjections was <1 min. Throughout the experiment, rat body temperature was maintained in the range of 36.5–37.5°C with a heating pad (Gaymar Industries, Orchard Park, NY). After the protocol, the injection position was also confirmed by microinjection of methylene blue dye (50 nl).

Western blot analysis of GABA<sub>B</sub> receptor protein. The animals were euthanized with an excessive dose of pentobarbital sodium. The brains were removed and immediately put on ice, blocked in the coronal plane, frozen on dry ice, and sectioned at 100-μm thickness in a cryostat. The NTS was punched using Harris Micro-Punch tools. The size of the punches varied from 0.5 to 1.0 mm, depending on the position of the brain section, which was determined by the brain atlas (28). The NTS tissues were collected from all brain sections containing the NTS, and both sides of the NTS from two rats were pooled together to collect enough tissue for Western Blots. The tissue was then homogenized in a lysing buffer containing 20 mM Tris· HCl (pH 6.8), 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, and 8 μM inhibitor cocktail (125 mM PMSF, 2.5 mg/ml aprotinin, 2.5 mg/ml leupeptin, 2.5 mg/ml antipain, and 2.5 mg/ml chymostatin). The tissue lysate was centrifuged, and the supernatant was collected. The protein concentration was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA). The samples were boiled for 5 min, followed by loading on 8% SDS-PAGE gel (10 μg of protein, 20 μl per well) for electrophoresis using a Bio-Rad mini-gel apparatus at 100 V for 60 min. The fractionized protein on the gel was electrophoretically transferred onto nitrocellulose membranes at 350 mA for 90 min. The membrane was probed with primary antibody (GABA<sub>B</sub> receptor rabbit polyclonal antibody, Chemicon, 1:1,000 or GABA<sub>B</sub> receptor rabbit polyclonal antibody, Santa Cruz, 1:500) and secondary antibody (goat anti-rabbit IgG horseradish peroxidase, Bio-Rad, 1:3,000) and then treated with chemiluminescence substrate (ECL Western blotting detection kit, Amersham Pharmacia Biotechnology) for 2 min at room temperature. The bands in the film were visualized and analyzed using Quantity One Software (Bio-Rad).

Statistical analyses. All data are expressed as means ± SE. Comparisons between experimental groups were performed with ANOVA followed by a Newman-Keuls test. Differences were considered significant at P < 0.05.

RESULTS

BP responses to chronic infusion of ANG II. ANG II was infused into the right lateral cerebral ventricle of rats at 120 μg·kg<sup>−1</sup>·day<sup>−1</sup> via osmotic minipumps. Mean arterial BP (MAP) and HR were measured via radiotelemetry before and during a 6-day infusion period. The baseline MAP and HR values were similar between the ANG II infusion group and aCSF group. As shown in Fig. 1A, chronic ICV infusion of ANG II significantly increased MAP from 87 ± 1 to 109 ± 2 mmHg. The time-dependent pressor effect of ICV ANG II infusion started at 24 h and lasted at least 6 days during infusion (Fig. 1A). The aCSF control produced no significant changes in MAP (Fig. 1A). In addition, no significant alteration in HR was observed during ICV infusion with either ANG II or aCSF (Fig. 1B). The results demonstrate that 1 wk of ICV ANG II infusion produces a significant increase in MAP without altering HR.

GABA<sub>B</sub> and GABA<sub>A</sub> receptor expression in the NTS of ANG II-infused and L-NAME-treated rats. To investigate the effects of ANG II infusion on GABA<sub>B</sub> receptor expression in the NTS, GABA<sub>B</sub> receptor protein levels were determined by Western blot analysis in the micropunched NTS of ANG II- or aCSF-infused rats. Data in Fig. 1, C and D, demonstrate that GABA<sub>B</sub> receptor expression levels were increased by twofold in ANG II-infused rats, compared with aCSF-infused control rats. In contrast, the expression of GABA<sub>A</sub> receptor γ2 subunit was comparable between ANG II- and aCSF-infused rats (Fig. 1, E
These results demonstrate that chronic, central administration of ANG II selectively increases GABA\(_B\) receptor expression and had no effect on GABA\(_A\) receptor expression in the NTS.

To rule out the possibility that the ANG II-induced increase in GABA\(_B\) receptor expression in the NTS is not caused by the elevated BP, we examined GABA\(_B\) receptor expression in the NTS of rats made hypertensive by chronic administration of NOS inhibitor N\(^{\text{G}}\)-nitro-L-arginine methyl ester (L-NAME). L-NAME (40 mg·kg\(^{-1}\)·day\(^{-1}\)) was added in the drinking water for 2 wk to evoke chronic inhibition of NOS and elevation of BP in vivo (1). Basal MAP (Fig. 1G) was significantly higher (\(P < 0.01\)) in the L-NAME treatment group (115 ± 2 mmHg; \(n = 7\)) compared with the control group (88 ± 1 mmHg; \(n = 6\)). The protein levels of GABA\(_B\) receptor and GABA\(_A\) receptor within the NTS were assessed by Western analysis in L-NAME-infused, t-NAME-treated, or control (Con) rats shown in A and G. Values are normalized using \(\beta\)-actin. F: bar graphs summarizing the quantitation of GABA\(_B\) protein levels in the NTS of rats shown in A and G. Data are normalized using \(\beta\)-actin. G: bar graphs showing the MAP of rats treated with t-NAME (40 mg·kg\(^{-1}\)·day\(^{-1}\)) added in drinking water or regular water for 2 wk. Values are means ± SE (\(n = 8\) per group). *\(P < 0.01\) vs. the MAP of control rats.

**Effect of microinjection of CGP-35348 into the NTS.** To determine whether chronic ANG II infusion alters GABA\(_B\) receptor function in the NTS, we microinjected the GABA\(_B\) receptor antagonist, CGP-35348, into the NTS of ANG II- or aCSF-infused rats. BP and HR were recorded before and after CGP-35348 microinjection, as described in METHODS. Figure 2 shows the time course of MAP and HR before and after CGP-35348 microinjection.
after microinjection of CGP-35348 into the NTS of rats infused intracerebroventricularly with either ANG II or aCSF. Bilateral microinjection of CGP-35348 (100 pmol, 50 nl) into the NTS induced a significant decrease in MAP in rats infused with either ANG II or aCSF (Fig. 2, A and B). Time course data showing the MAP (A) and HR (B) changes evoked by CGP-35348 (100 pmol in 50 nl) microinjected into the NTS of chronic ANG II infusion rats. Values are means ± SE (n = 7 or 6 in each group) *P < 0.05, **P < 0.01 vs. MAP or HR of saline control at corresponding time points. C and D: time course data showing MAP (C) and HR (D) changes evoked by NTS microinjection of CGP-35348 (100 pmol in 50 nl) in aCSF infusion rats. Values are means ± SE (n = 7 or 6 in each group). *P < 0.05 vs. MAP of saline control rats at corresponding time points. E and F: Identification of the microinjection sites in the NTS. E: a representative photomicrograph showing the NTS microinjection site. F: the location of this microinjection site shown in E, based on the rat brain atlas of Swanson (28). Arrow indicates the injection site. AP, area postrema; C, central canal; NTSm, medial NTS; NTSl, lateral NTS; ts, tractus solitarii; GR, gracile nucleus; SPVC, spinal nucleus of the trigeminal caudal part; PY, pyramid; XII, hypoglossal nucleus.

Effect of microinjection of baclofen into the NTS. In another group of rats, we examined the effect of microinjection of a selective GABA<sub>B</sub> agonist, baclofen, into the NTS of ANG II-
or aCSF-infused rats. Microinjection of baclofen into the NTS induced an elevation in BP in both ANG II-infused rats (Fig. 4A) and aCSF-infused rats (Fig. 4B). As shown in Fig. 4A, NTS microinjection of baclofen (50 pmol, 50 nl) caused a time-dependent increase in MAP that started at 1 min, peaked at 3–4 min, and lasted ~8 min in the ANG II group. MAP was increased from 106 ± 3 to 140 ± 4 mmHg by microinjection of baclofen into the NTS of ANG II-infused rats (Fig. 4A). In aCSF-infused rats, baclofen also induced an elevation in MAP (from 86 ± 4 to 106 ± 3 mmHg, as shown in Fig. 4B). However, the pressor effect of baclofen in these rats lasted only ~5–6 min (Fig. 4B). These data demonstrate that injection of baclofen into the NTS produced a greater increase in MAP in the ANG II-infused hypertensive rats than that in aCSF-infused control rats (Fig. 4C). However, NTS microinjection of baclofen did not alter HR in either ANG II-infused or aCSF-infused rats (Fig. 4D). In addition, the BP and HR responses to baclofen microinjected into the NTS were not significantly different between L-NAME-treated rats and control rats (Fig. 4, C and D). In summary, these data indicate that chronic ICV ANG II infusion increases the pressor effect of baclofen in the NTS of rats. In contrast, L-NAME treatment has no effect on the pressor response evoked by microinjection of baclofen into the NTS.
Effect of microinjection of bicuculline and muscimol into the NTS. To investigate whether chronic ANG II infusion alters GABA<sub>A</sub> receptor function in the NTS, we microinjected the GABA<sub>A</sub> receptor antagonist, bicuculline, into the NTS of ANG II- or aCSF-infused rats. BP and HR were recorded before and after bicuculline microinjection (10 pmol in 50 nl), as described in METHODS. Microinjection of bicuculline into the NTS resulted in a decrease in MAP from 111 ± 2 to 90 ± 4 mmHg (n = 6, P < 0.05) in ANG II-infused rats and from 89 ± 2 to 70 ± 6 mmHg (n = 6, P < 0.05) in aCSF-infused rats. However, the depressor responses to bicuculline microinjected into the NTS were not different between ANG II- and aCSF-infused rats (Fig. 5A). The HR in ANG II-infused rats was 359 ± 11 and 350 ± 9 beats/min (n = 6, P < 0.05) before and after NTS microinjection of bicuculline, and the HR in aCSF-infused rats was 363 ± 12 and 350 ± 13 beats/min (n = 6, P < 0.05) before and after NTS administration of bicuculline, respectively. The HR responses evoked by microinjection of bicuculline into the NTS are shown in Fig. 5B. In addition, microinjection of the same volume of saline control into the NTS altered neither MAP nor HR in both group of rats. These results demonstrate that chronic ICV infusion of ANG II has no effect on the depressor response to the GABA<sub>A</sub> receptor antagonist, bicuculline, microinjected into the NTS.

In another group of rats, we examined the effect of the GABA<sub>A</sub> receptor agonist muscimol microinjected into the NTS on the BP and HR in ANG II- and aCSF-infused rats. Bilateral microinjection of muscimol (100 pmol in 50 nl) into the NTS significantly increased BP from 110 ± 4 to 132 ± 5 mmHg (n = 7, P < 0.05) in ANG II-infused rats and from 90 ± 3 to 112 ± 4 mmHg (n = 6, P < 0.05) in aCSF-infused rats. The pressor responses evoked by NTS microinjection of muscimol were not different between ANG II- and aCSF-infused rats (Fig. 5C). The HR in ANG II-infused rats was significantly increased from 340 ± 11 to 368 ± 12 beats/min (n = 7, P < 0.05), and the HR in aCSF-infused rats was also significantly increased from 347 ± 6 to 373 ± 10 beats/min (n = 6, P < 0.05). However, the increases in HR evoked by microinjection of muscimol into the NTS were comparable between ANG II- and aCSF-infused rats (Fig. 5D). In addition, microinjection of the same volume of saline into the same area of NTS did not alter MAP and HR in both groups of rats. These data indicate that chronic ICV administration of ANG II did not alter the pressor effect of the GABA<sub>A</sub> receptor agonist, muscimol, microinjected into the NTS on BP.

DISCUSSION

The present study provides the first evidence that 1) elevated BP induced by chronic ICV infusion of ANG II is associated with increased GABA<sub>B</sub> receptor expression in the rat NTS; 2) the depressor response to a GABA<sub>B</sub> receptor antagonist injected into the NTS is enhanced by chronic ANG II infusion; 3) chronic ANG II infusion also exaggerated the pressor effect of a GABA<sub>B</sub> receptor agonist injected into the NTS; and 4) the depressor response to a GABA<sub>A</sub> receptor antagonist and the pressor response to a GABA<sub>B</sub> receptor agonist are not altered by chronic ANG II infusion. These results suggest that ANG II selectively enhances functional GABA<sub>B</sub> receptor expression and inhibitory GABAergic neurotransmission in the NTS. This ANG II-induced enhancement of GABA<sub>B</sub> receptor activity could contribute to the development of ANG II-related hypertension.

Brain ANG II plays an important role in central BP control and in the pathogenesis of hypertension, as evidenced by the effective attenuation of hypertension with ANG II type 1 receptor antagonists or genetic downregulation of ANG II type 1 receptors (12, 22, 29). The actions of ANG II are mediated by stimulating sympathetic nerve activity and dampening the baroreflex. In chronic hypertension, the baroreceptor reflex remains functional; however, baroreflex regulation of sympathetic outflow is reset to a higher BP level in chronic hypertension via neurohormonal mechanisms (33, 37). The mecha-
nisms involved in ANG II-mediated dampening of the baroreflex are still not clear.

The present observations demonstrate that chronic ICV infusion of ANG II induces a long-term elevation of BP that is associated with enhanced expression of GABA\(_B\) receptors in the NTS, a crucial area for regulating arterial pressure and baroreceptor reflex function (18, 32). These results suggest that ANG II in the NTS increases inhibitory GABAergic neurotransmission, leading to inhibition of baroreceptor afferent input signals triggered by increased BP (20, 37, 38), thus causing dampening of the baroreflex. The inhibited baroreflex could cause central desensitization to peripheral BP elevation and reset the BP regulatory set points to a higher BP level. One concern raised in the present study is whether the ANG II-induced GABA\(_B\) receptor expression in the NTS is mediated by the direct stimulatory effect of ANG II or by a secondary response to the elevation in BP, since elevated peripheral BP may increase the central input signal, leading to altered gene expression in brain cardiovascular regulatory areas. However, our findings demonstrate that elevated BP induced by chronic administration of the NOS inhibitor, L-NAME, did not alter our findings demonstrate that elevated BP induced by chronic ICV administration of ANG II increases BP, which triggers baroreflex-mediated cardiovascular responses, thus buffering the ANG II-induced tachycardia. However, this speculation must be confirmed in future studies.

In summary, chronic infusion of ANG II selectively increases GABA\(_B\) receptor expression and enhances responses to GABA\(_B\) receptor agonists and antagonists in the NTS of rats. The ANG II-induced increase in GABA\(_B\) receptor expression and GABA\(_B\) receptor-mediated response in the NTS could inhibit baroreceptor input signals and dampen baroreflexes, thus leading to an increase in sympathetic outflow and elevated BP. Interaction between the ANG II and GABA systems may contribute to the central resetting of long-term BP control and the development of chronic hypertension.

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