Augmented Upregulation by c-fos of Angiotensin Subtype 1 Receptor in Nucleus Tractus Solitarii of Spontaneously Hypertensive Rats


Abstract—Our laboratory demonstrated previously that spontaneously hypertensive rats (SHR) exhibited an elevated basal Fos expression in the nucleus tractus solitarii (NTS), the terminal site for primary baroreceptor afferents, and that Fos protein is required for the re-expression of angiotensin subtype 1 receptor (AT1R) mRNA in the NTS after baroreceptor activation. The present study evaluated the hypothesis that this re-expression of AT1R is augmented in SHR and is promoted by the heightened Fos expression. Reverse transcription–polymerase chain reaction analysis revealed that baroreceptor activation via sustained increase in systemic arterial pressure resulted in a discernible reduction in the expression of AT1R mRNA at the dorsomedial medulla of SHR and normotensive Wistar-Kyoto rats. However, SHR manifested an appreciably larger magnitude of decline, followed by a faster time course of re-expression in AT1R mRNA. Parallel findings were obtained from the pressor response induced by microinjection unilaterally of angiotensin II (40 pmol) into the NTS. Whereas the re-expression of AT1R at both transcriptional and functional expression levels after baroreceptor activation was discernibly blunted by prior bilateral application into the NTS of an antisense c-fos oligonucleotide (50 pmol), the suppression in SHR was again significantly more intense. Control pretreatment with the corresponding sense or scrambled c-fos oligonucleotide was ineffective. We conclude that the heightened Fos expression in SHR is causatively related to the augmented re-expression of AT1R in the NTS at both transcriptional and functional levels. (Hypertension. 2002;40:335-341.)

Key Words: receptors, angiotensin Â· Fos protein Â· nucleus tractus solitarii Â· transcription Â· hypertension, genetic Â· rats

Fos protein, the translational product of immediate early gene c-fos, exerts influence on cellular functions by regulating the induction of its downstream target genes as a transcription factor.1 After translation, Fos protein couples with Jun protein to form a heterodimer nucleoprotein complex that binds with high affinity to a DNA-specific sequence identified as activating protein-1 (AP-1) site.1,2 A number of candidate genes with AP-1 binding site sequence were proposed as potential target genes for Fos protein.1 Among these candidates is the angiotensin receptor gene.3,4 The cloning and sequencing of angiotensin receptor genes,4,5 as well as the discovery of specific nonpeptide angiotensin receptor antagonists devoid of agonist activity,6 have led to the definition of at least angiotensin subtype 1 (AT1R) and subtype 2 (AT2R) receptors. Interestingly, the AP-1 binding site, which contains the octomer consensus sequence AT-CACTCA, is found in the regulatory domain of both AT1R and AT2R genes.3,4

Arterial baroreceptor reflex (BRR) is one of the fundamental operating mechanisms via which the central nervous system maintains stable blood pressure.7 Consequently, hypertensive patients8,9 and animals with genetic10,11 or experimentally induced12,13 hypertension manifest retarded BRR sensitivity. In spontaneously hypertensive rats (SHR), hyper-activity of the brain angiotensin system, including an increase in the number of AT1R in brain areas that are relevant to cardiovascular control,14–16 is suggested to play an important role in this form of genetic hypertension. One of the modes of action in this regard is the reduction in BRR sensitivity exerted by angiotensin (Ang) II via an action on the AT1R, but not AT2R,17–19 in the nucleus tractus solitarii (NTS), the principal terminal site of the primary baroreceptor afferents.20

Our laboratory demonstrated recently19 that Fos expression induced via activation of AT1R underlies the inhibitory modulation of BRR by Ang II at the caudal NTS. Fos protein is also required for the re-expression of the downregulated AT1R mRNA21 in the NTS after baroreceptor activation. In addition, an elevated basal Fos expression in the NTS is associated with the reduced BRR sensitivity during hypertension.22 It follows that to ensure that Ang II may continuously
inhibit BRR despite the enhanced baroreceptor afferent traffic during hypertension, the heightened Fos expression in the SHR is causatively related to an augmented re-expression of AT1R at the caudal NTS. By comparing the molecular synthesis and functional expression of AT1R and/or AT2R at the NTS elicited by baroreceptor activation and their regulation by Fos protein in SHR and normotensive Wistar-Kyoto rats (WKY), the present study supports this hypothesis.

**Methods**

**Animals**

The experimental procedures used in this study were approved by our institutional animal care committee. Male adult SHR and WKY (age, 8 to 10 weeks; weight, 200 to 230 g; n = 338 for each strain) purchased from the Experimental Animal Center, National Science Council, Taiwan, were used.

**Microinjection of Test Agents**

Three 15-mer phosphorothioated c-fos oligonucleotides (Quality System, Taipei, Taiwan) were used,22–24 including a key antisense oligonucleotide (ASON1) that targets against a region of the c-fos mRNA that flanks the initiation codon (5'-129 to 143-3') and a scrambled (ASON2) c-fos oligonucleotide. Each oligonucleotide was microinjected bilaterally, at a volume of 50 nl, into the NTS (n = 25 to 30 animals per group) under pentobarbital anesthesia (50 mg/kg IP) 24 hours before the experimental sessions. The stereotoxic coordinates were 0.35 to 0.7 mm below the surface of the fourth ventricle and 0.5 to −0.5 mm from and 0.35 to 0.5 mm lateral to the obex. Ang II (40 pmol, n = 6 to 7 animals per group) was microinjected unilaterally into the NTS before and at 15-minute intervals for 120 minutes after baroreceptor activation. Microinjection of artificial cerebrospinal fluid (aCSF; pH 7.4) served as the volume and vehicle control.

**General Preparation**

During the experimental session, rats were anesthetized initially with pentobarbital sodium (50 mg/kg IP) to perform preparatory surgery. Animals received thereafter continuous intravenous infusion of pentobarbital sodium (10 to 15 mg·kg⁻¹·h⁻¹), which provided satisfactory anesthetic maintenance while preserving the capacity of central cardiovascular regulation, including the BRR response.25 Pulsatile and mean systemic arterial pressure (MSAP), as well as heart rate, were recorded on a polygraph (Gould RS3400). Animals were mechanically ventilated to maintain end-tidal CO₂ within 4% to 5%, as monitored by a capnograph (Datex Normocap). All data were collected from animals with a maintained rectal temperature of 37±0.5°C.

**Baroreceptor Activation**

The arterial baroreceptors were activated by a sustained increase in MSAP evoked by intravenous infusion of phenylephrine (100 μg/mL; Sigma) for 30 minutes (n = 25 to 30 animals per group). The infusion rate was adjusted (5 to 7 μL·kg⁻¹·h⁻¹) to maintain an average elevation in MSAP of 45 to 50 mm Hg.22 Intravenous infusion of saline (n = 25 animals per group) served as our volume and vehicle control, and animals that received preparatory surgery and maintained under pentobarbital anesthesia served as our sham control (n = 26 animals per group). In some experiments, the sensitivity of BRR control of the heart rate was evaluated by determining the slope of the linear regression line that relates reflex bradycardia to transient increase in MSAP evoked by intravenous bolus administration of phenylephrine (2.5, 5, or 10 μg/kg; n = 6 to 7 animals per group).21,26

**Isolation of Total RNA and Reverse Transcription–Polymerase Chain Reaction**

At the conclusion of some physiological experiments, the brain was rapidly removed on dry ice. The dorsomedial medulla (1 mm rostral to 1 mm caudal to the obex) that contains the NTS, area postrema, and dorsal motor nucleus of the vagus nerve were removed by micropunches made with a stainless steel bore (1.5 mm ID), and subject to total RNA isolation.21 Quantification of AT1R, AT2R, or GAPDH mRNA was performed by reverse transcription–polymerase chain reaction (RT-PCR)21–27 using primers21 obtained from Gene-emed Synthesis. PCR was performed at 94°C (1 minute), 58°C (1 minute), and 72°C (1 minute) for 30 cycles. This condition was determined quantitatively in initial experiments by cyclic lineage analysis21 to ascertain that amplifications of probes were within their linear ranges. Each PCR product was size-fractionated by 2% agarose gel electrophoresis, using a 100-bp DNA marker (New England BioLabs). The gel was stained with ethidium bromide (1 μg/mL), visualized by an ultraviolet transilluminator, and photographed. The density of each PCR band was measured and analyzed by ImageMaster VDS analysis software (Pharmacia Biotech), and the amount of mRNA products for AT1R or AT2R was expressed as the ratio to GAPDH mRNA product, which served as the internal control.

**Immunohistochemical Staining**

At the conclusion of other physiological experiments, free-floating sections (20 μm) of the caudal medulla oblongata were processed for immunohistochemical staining of Fos protein.21–24 A rabbit polyclonal anti-Fos antiserum (sc-52, 1:4000; Santa Cruz) was used, and clonal anti-Fos antiserum (sc-52, 1:4000; Santa Cruz) was used, and clonal anti-Fos antiserum (sc-52, 1:4000; Santa Cruz) was used, and a scrambled cDNA oligonucleotide probe (SON1) that targets against a region of the c-fos mRNA that flanks the initiation codon (5'-129 to 143-3') and a corresponding sense (SON) and a scrambled (ASON2) c-fos oligonucleotide. Each oligonucleotide was microinjected bilaterally, at a volume of 50 nl, into the NTS (n = 25 to 30 animals per group) under pentobarbital anesthesia (50 mg/kg IP) 24 hours before the experimental sessions. The stereotoxic coordinates were 0.35 to 0.7 mm below the surface of the fourth ventricle and 0.5 to −0.5 mm from and 0.35 to 0.5 mm lateral to the obex. Ang II (40 pmol, n = 6 to 7 animals per group) was microinjected unilaterally into the NTS before and at 15-minute intervals for 120 minutes after baroreceptor activation. Microinjection of artificial cerebrospinal fluid (aCSF; pH 7.4) served as the volume and vehicle control.

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The criterion for identification of Fos-LI was a distinctly stained nucleus, and the number of NTS neurons that displayed Fos-LI was quantified as reported previously.21–24 Double immunohistochemical staining for Fos-LI and AT1R or AT2R was processed in some experiments as described.29 In brief, sections were similarly processed for Fos-LI, using a polyclonal goat anti-Fos antiserum (sc-52G, 1:4000; Santa Cruz) and visualized by a Vectastain ABC peroxidase kit with nickel intensification. The same sections were subsequently incubated with a polyclonal rabbit anti-AT1R (sc-1173, 1:1000; Santa Cruz) or anti-AT2R (sc-9040, 1:1000; Santa Cruz) antiserum. Immunoreactive product was visualized by a Vectastain ABC alkaline phosphatase kit. The final immunohistochemical product of Fos-LI was stained in black; that of AT1R or AT2R, in red.

Statistical Analysis
All values are expressed as mean±SEM. One-way or 2-way ANOVA with repeated measures was used, as appropriate, followed by the Scheffé multiple-range test. P<0.05 was considered statistically significant.

Results
Differential Effect of Baroreceptor Activation on AT1R mRNA Level in the Dorsomedial Medulla of SHR and WKY
RT-PCR analysis revealed that compared with WKY, SHR exhibited a significantly augmented basal level of AT1R mRNA in the dorsomedial medulla that includes the NTS (Figure 1). Baroreceptor activation resulted in a discernible reduction in the expression of AT1R mRNA in both strains of rats (Figure 1), with 2 major differences. First, the maximal decline in SHR, which occurred 30 minutes after sustained elevation in MSAP and reached a level comparable with that of AT1R or AT2R, in red.

Differential Distribution of Fos-LI and Immunoreactivity for AT1R or AT2R in NTS
Baroreceptor activation resulted in Fos expression in the commissural, medial and dorsomedial subnuclei of NTS21–24,26,29 of SHR and WKY (Figure 2). Whereas a majority of NTS neurons that showed Fos-LI was double labeled for AT1R (Figures 2A and 2B), fewer Fos-positive neurons in the NTS were immunoreactive to AT2R (Figures 2C and 2D).

Effect of Antisense c-fos Oligonucleotide Pretreatment on Changes in AT1R mRNA Level in the Dorsomedial Medulla After Baroreceptor Activation
Microinjection bilaterally of c-fos ASON1 (50 pmol) into the NTS 24 hours before the experiments did not discernibly affect the basal level of AT1R mRNA in the dorsomedial medulla of SHR and WKY (Figure 3). The reduction in AT1R mRNA 30 minutes after baroreceptor activation was again comparable to aCSF control in both strains of rats. Intriguingly, c-fos ASON1 pretreatment significantly blunted the re-expression of AT1R mRNA detected 60 minutes in SHR or 90 minutes in WKY after sustained elevation in MSAP (Figure 3). There was also a trend of progressive decline in AT1R mRNA in the SHR, which approached the level in WKY.

As we observed previously,22 immunohistochemical analysis showed an elevated basal Fos-LI in the NTS of SHR (Table). Furthermore, sustained increase in MSAP appreciably increased the number of Fos-positive cells to comparable levels in the NTS of both strains of rats when measured 120
minutes after stimulation. Such an increase was significantly blunted by pretreatment with c-fos ASON1. The effectiveness of c-fos ASON1 to block Fos expression was also confirmed when the magnitude of BRR potentiation in SHR
(60.3±5.2%, n=7 animals per group) was discernibly greater than that in WKY (47.8±3.7%, n=6 animals per group).

Effect of Antisense c-fos Oligonucleotide Pretreatment on Ang II–Induced Pressor Response After Baroreceptor Activation

Microinjection unilaterally of Ang II (40 pmol) into the NTS resulted in an appreciable increase in MSAP (Figure 4). Interestingly, the magnitude of such a functional expression of AT1R served as a target for its long-term control. Hypertensive rats exhibit exaggerated Ang II–mediated synaptic transmission, and functional overexpression of AT1R contributes to the heightened pressor response to brain Ang II in SHR. Baroreceptor activation in normotensive rats downregulates AT1R mRNA in the dorsomedial medulla, and Fos protein in the NTS plays a permissive role in its re-expression.

The present study extended these observations to reveal that this upregulation of AT1R at the NTS by Fos protein after sustained elevation in MSAP is further augmented in SHR at both transcriptional and functional levels.

At the transcriptional level, we demonstrated that the elevated basal level of AT1R mRNA in the dorsomedial medulla of SHR plays a role during hypertension by 2 observations. First, the magnitude of maximal decline in AT1R mRNA induced after baroreceptor activation, which reached a level comparable with that in WKY, was significantly greater in SHR. Second, the re-expression of AT1R mRNA in the dorsomedial medulla after sustained hypertension, which was blunted by prior microinjection of c-fos ASON1 into the NTS, exhibited a faster time course in SHR. Fos expression evoked by baroreceptor activation is mediated via both N-methyl-D-aspartate and non–N-methyl-D-aspartate receptors in the NTS. Glutamatergic neurotransmission at the NTS is also involved in the downregulation of AT1R mRNA after baroreceptor activation. Angiotensin and glutamate receptors exhibit augmented interaction in the brain of SHR, and an elevated basal Fos expression is present in the NTS during hypertension.

Pretreatment with microinjection bilaterally of c-fos ASON1 into the NTS prevented the gradual return of Ang II–induced pressor response that began 60 minutes after sustained elevation in MSAP in both strains of rats (Figure 4). Instead, there was a progressive reduction in the magnitude of this functional expression of AT1R, which was more intense in SHR.

Lack of Effect of Control c-fos Oligonucleotides

Microinjection bilaterally into the NTS of the SON or ASON2 c-fos oligonucleotide did not discernibly affect the temporal alterations in the level of AT1R mRNA in the dorsomedial medulla (Figure 3), the Ang II–induced pressor response (Figure 4), or the increase in number of Fos-positive NTS neurons (Table) induced by baroreceptor activation in both SHR and WKY rats. In addition, pretreatment with aCSF or ASON1, SON, or ASON2 c-fos oligonucleotide did not appreciably affect the level of AT1R mRNA in the dorsomedial medulla in our sham-control animals and in SHR or WKY that received intravenous infusion of saline.

Microinjection Sites

Histological verification in randomly selected animals demonstrated that microinjection of test agents was delivered to sites distributed mainly within the anatomic confines of the NTS (Figure 5).

Discussion

Upregulation of brain AT1R gene was suggested to play an important role in the pathogenesis of hypertension and serves as a target for its long-term control. Hypertensive rats exhibit exaggerated Ang II–mediated synaptic transmission, and functional overexpression of AT1R contributes to the heightened pressor response to brain Ang II in SHR. Baroreceptor activation in normotensive rats downregulates AT1R mRNA in the dorsomedial medulla, and Fos protein in the NTS plays a permissive role in its re-expression. The present study extended these observations to reveal that this upregulation of AT1R at the NTS by Fos protein after sustained elevation in MSAP is further augmented in SHR at both transcriptional and functional levels.

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interplay in the NTS among glutamatergic neurotransmission, Fos protein, and AT1R during hypertension is our demonstration that transcriptional regulation of AT1R mRNA re-expression in the dorsomedial medulla by c-fos after baroreceptor activation, which is already present in WKY, is enhanced in SHR. The exact mechanism that underlies this enhanced interplay awaits further investigation.

Based on the pressor response induced by microinjection of Ang II into the NTS, we ascertained that the functional expression of AT1R23 exhibited changes, with a time delay, that paralleled alterations in AT1R mRNA in the dorsomedial medulla after sustained elevation in MSAP. Thus, the Ang II–induced pressor response was augmented in SHR, along with greater degree of suppression after baroreceptor activation and faster rate in returning to baseline level. Of note was that the progressive reduction in the magnitude of our functional expression of AT1R was more intense in SHR on pretreatment with c-fos ASON1. These observations reinforced our contention that upregulation of AT1R mRNA by c-fos in the dorsomedial medulla after baroreceptor activation may be augmented in SHR. It should be mentioned that we have established previously21 that the suppression of pressor responses to repeated administration of Ang II into the NTS after sustained increase in MSAP is not related to tachyphylaxis.

It is interesting to note that Fos protein exerted selectively a regulation on AT1R, but not AT2R, mRNA in the dorsomedial medulla of both SHR and WKY. Activation of AT1R contributes mainly to the brain Ang II–induced cardiovascular responses,26 including the inhibitory modulation of BRR response via Fos expression in the caudal NTS. This subpopulation of neurons in the NTS located ventromedial to the solitary tract. This subpopulation of NTS neurons is where AT1R, but not AT2R, mRNA is distributed.44 Our immunohistochemical findings further revealed that the majority of Fos-positive NTS neurons also exhibited AT1R, but not AT2R, immunoreactivity.

We are confident that the blunting effects of c-fos ASON1 on our experimental indices were related to its complementarity with c-fos mRNA because pretreatment with SON or ASON2 oligonucleotide was ineffective. In addition, micro-injection bilaterally into the NTS of aCSF, ASON1, SON, or ASON2 c-fos oligonucleotide did not elicit discernible changes in the level of AT1R mRNA in SHR or WKY that received intravenous infusion of saline. This confirmed that our results obtained after sustained increase in MSAP were not caused by fluid loading in the circulatory system. The concern for the confounding influence of pentobarbital sodium is also minimized because we previously demonstrated that our scheme of anesthetic management provided stable anesthesia and preserved the capacity of central cardiovascular regulation. The lack of discernible changes in AT1R mRNA in sham-control animals further attested that anesthesia was not a confounding factor.

In conclusion, the present study showed that the upregulation of AT1R re-expression at the NTS by Fos protein after sustained hypertension is further augmented, at both transcriptional and functional levels, in SHR.

**Perspectives**

A hallmark of hypertension is depressed BRR response,8–13 which may result from the inhibitory action on this reflex by the exaggerated angiotensinergic neurotransmission at the NTS.31 At the same time, Fos expression induced via activation of AT1R underlies the inhibitory modulation of BRR by Ang II at the caudal NTS.19 As a prelude to hypertension, we demonstrated that Fos expression, which is responsible for the augmented re-expression of AT1R at the caudal NTS, is heightened in the SHR to ensure that Ang II may continu-
tion of basal Fos expression in NTS contributes to the restoration of BRR sensitivity in SHR that received antihypertensive treatment.

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