Inhibition of Baroreflex by Angiotensin II via Fos Expression in Nucleus Tractus Solitarii of the Rat

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Abstract—We evaluated the modulatory action of angiotensin II at the nucleus tractus solitarii on spontaneous baroreceptor reflex response, the angiotensin subtype receptors involved, and the role of Fos protein in this process, using Sprague-Dawley rats anesthetized with pentobarbital sodium. Microinjection bilaterally of angiotensin (Ang) II (5, 10, 20, or 40 pmol) into the nucleus tractus solitarii significantly suppressed the spontaneous baroreceptor reflex, as represented by the magnitude of transfer function between systemic arterial pressure and heart rate signals. There also was a concomitant increase in Fos-like immunoreactivity in the nucleus tractus solitarii. Both the suppression of spontaneous baroreceptor reflex and Fos expression in nucleus tractus solitarii neurons elicited by Ang II were discernibly attenuated by pretreatment with or coinjection into the bilateral nucleus tractus solitarii of a 15-mer antisense c-fos oligonucleotide that targets against the initiation codon of c-fos mRNA. In addition, those 2 actions of Ang II were reversed by the conadministration of the nonpeptide Ang type 1 (AT₁) receptor antagonist losartan (1.6 nmol) but not by the nonpeptide AT₂ receptor antagonist PD 123,319 (1.6 nmol). Control treatments with artificial cerebrospinal fluid, sense cDNA, or antisense oligonucleotide with a scrambled sequence were ineffective. We conclude that under minimal cardiovascular perturbation, Fos expression mediated via activation of AT₁ subtype receptors may underlie the inhibitory modulation of beat-to-beat baroreflex control of blood pressure by Ang II at the nucleus tractus solitarii. (Hypertension. 2001;38:130-135.)

Key Words: angiotensin II receptors, angiotensin II genes nucleus tractus solitarii

As the principal terminal site of the primary baroreceptor afferents,₁ the caudal nucleus tractus solitarii (NTS) in the medulla oblongata has become a major focus in recent studies²–⁹ on the anatomic and temporal specificity of Fos expression after cardiovascular perturbations. In terms of function, workers at our laboratory reported that both basal and induced Fos expression in the caudal NTS may represent an early step in the cascade of intracellular events that leads to long-term inhibitory modulation of baroreceptor reflex (BRR) response. Microinjection of angiotensin (Ang) II into the NTS reduces BRR sensitivity.¹⁰ The endogenous Ang II exerts a tonic inhibitory modulation on BRR response via an action on Ang II subtype 1 receptors (AT₁R) but not subtype 2 receptors (AT₂R) at the caudal NTS.¹¹ Furthermore, Ang II induces the expression of c-fos gene via activation of the AT₁R on non-neuronal cells¹²,¹³ and brain neurons.¹⁴–¹⁶ It follows that Fos protein expressed via activation of AT₁R may underlie the inhibitory modulation of BRR response by Ang II in the caudal NTS. The present study was undertaken to test this hypothesis. We are cognizant that the level of c-fos is low in quiescent cells but is rapidly induced within minutes of extracellular stimulation.¹⁷ Thus, spontaneous BRR response, which is responsible for beat-to-beat maintenance of resting systemic arterial pressure (SAP) in the absence of evoked cardiovascular perturbation,₁⁸ provides a suitable experimental model. In particular, advances in computer application allow for the evaluation of spontaneous BRR response in the form of transfer function analysis of simultaneous fluctuations in resting SAP and heart rate (HR).¹⁹,²⁰

Methods

General Preparation

Experiments were performed in compliance with the guiding principles on the care and use of animals endorsed by our institutional animal care committee. Ninety-eight specific pathogen-free, male, adult (14 to 20 weeks old, 275 to 300 g) Sprague-Dawley rats purchased from the Experimental Animal Center of the National Science Council (Taiwan, Republic of China) were used. Animals were anesthetized initially with pentobarbital sodium (50 mg/kg IP) for preparative surgery. They received thereafter an intravenous infusion of pentobarbital sodium (15 to 20 mg · kg⁻¹ · h⁻¹), which provides satisfactory anesthetic maintenance while preserving the capacity of central cardiovascular regulation, including the spontaneous BRR response.²⁰ During the experiment, animals were allowed to breathe spontaneously with room air, and body temperature was maintained with a heating pad at 37°C.
Spontaneous BRR Response

The procedures for cross-spectral analysis of SAP, HR, and spontaneous BRR response were similar to those in our previous studies.\textsuperscript{5,20} For this study, the magnitude of transfer function between SAP and HR signals determined at the frequency range for the low-frequency spectral component (0.25 to 0.8 Hz)\textsuperscript{20} was taken as the index for spontaneous BRR response.\textsuperscript{19,20} We also ascertained that the corresponding phase of transfer function exhibited negative values, which indicates that the SAP fluctuation at this frequency range precedes that of HR.

Microinjection of Test Chemicals

Microinjection bilaterally of test chemicals into the caudal NTS\textsuperscript{4–8,11,21} was performed stereotaxically and sequentially with a glass micropipette (50- to 70-μm tip) connected to a 0.5-μL Hamilton microsyringe. The stereotaxic 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. The volume of microinjection was limited to 50 nL on each side to restrict the extent of diffusion and to minimize the confounding effect of volume artifact.

Test Chemicals

Ang II was purchased from Sigma Chemical Co. PD 123,319 was purchased from RBI. Losartan was a gift from Du Pont-Merck. Three 15-mer oligonucleotides,\textsuperscript{4,6,21} which were phosphorothioated in all positions (Quality Systems), were used. The key antisense oligonucleotide was designed to target a region of the c-fos mRNA that flanks the initiation codon (5′-129 to 143-3′). A sense c-fos oligonucleotide that is complementary to the antisense sequence and an antisense c-fos oligonucleotide with a scrambled sequence were used as the specificity controls. All test chemicals were prepared with artificial cerebrospinal fluid (aCSF) at pH 7.4. Animals that received microinjection of aCSF bilaterally into the caudal NTS served as the vehicle control. Animals that were surgically prepared, placed in the stereotaxic headholder without subsequent experimental treatments, and maintained with intravenous infusion of pentobarbital sodium for 4 hours served as the sham control.

Histology

The brain stem was removed after each experiment and fixed in 30% sucrose in 10% formaldehyde-saline solution for ≥72 hours. Histological verifications of the tip of micropipettes used in microinjection of test chemicals were carried out on 25-μm frozen sections stained with neutral red; 1% Evans blue was added to the injection solution to facilitate histological verifications.

Immunohistochemical Staining and Quantification of Fos-Like Immunoreactivity

In some experiments, animals were processed for immunohistochemical staining of Fos protein\textsuperscript{4–9,21} at the conclusion of the physiological experiments. A goat polyclonal antiserum (1:4000 SC-52-G; Santa Cruz) generated against the amino terminus of c-fos was used. Fos-like immunoreactivity (Fos-LI) was visualized by the Vectastain ABC kit (Vector PK4005), based on diaminobenzidine with nickel chloride intensification. No specific immunoreactivity was observed in control sections, which were incubated without the anti-Fos antisemur or with substitution for Fos antisemur with normal goat serum, when they were processed together with the experimental tissues. The criterion for the identification of Fos-positive neurons was a distinctly stained nucleus.\textsuperscript{4–9,21} The total number of Fos-positive neurons thus identified in the caudal NTS, spanning 1.0 mm caudal and 0.4 mm rostral to the obex, was counted bilaterally by 2 separate individuals in a single-blind manner, with each separately tabulated and subsequently averaged.

Statistical Analysis

All values are expressed as mean±SEM. Two-way ANOVA with repeated measures or 1-way ANOVA was used as appropriate. Both analyses were followed by either the Scheffé or Dunnett multiple range test for a posteriori comparison of mean values. \textit{P}<0.05 was considered to be statistically significant.

Results

Baseline Spontaneous BRR Response

Under the experimental condition of the present study, the baseline SAP-HR transfer magnitude obtained before treatment from all animals studied was 0.56±0.01 bpm/mm Hg (n=94). With 95% confidence values at 0.02 bpm/mm Hg, these animals as a group showed a highly stable spontaneous BRR response.

Temporal Effect of Microinjection Bilaterally of Ang II Into the Caudal NTS on Spontaneous BRR Response

Contrary to the minimal effect exerted by aCSF (vehicle), microinjection bilaterally into the caudal NTS of Ang II (5, 10,
20, or 40 pmol) resulted in significant and dose-dependent inhibition of the spontaneous BRR response (Figures 1 and 2A). These suppressive actions exhibited an approximate latency of 5 minutes and lasted for the 95-minute observation period. In preliminary experiments, this Ang II–induced inhibition of spontaneous BRR response persisted over 120 minutes.

Effect of Comicroinjection Bilaterally of Ang II and Its Antagonist Into the Caudal NTS on Spontaneous BRR Response

Simultaneous microinjection into the bilateral NTS of Ang II (10 pmol) and the nonpeptide AT1R antagonist losartan (1.6 nmol) appreciably reversed the reduction in SAP-HR transfer magnitude induced by the octapeptide (Figures 1 and 2B). However, an equimolar dose of the nonpeptide AT2R antagonist PD 123,319 was ineffective against the suppressant action of Ang II on the spontaneous BRR response (Figures 1 and 2B).

Effect of Antisense c-fos Oligonucleotide Treatment on the Inhibition of Ang II on Spontaneous BRR Response

As we reported previously, a trend of enhancement of spontaneous BRR response (Figure 4) began 60 minutes after comicroinjection into the bilateral caudal NTS of aCSF and an antisense c-fos oligonucleotide (20 pmol) that targets the initiation codon of the c-fos mRNA, became significant at 90 minutes, and persisted for $\geq 120$ minutes (Figures 4 and 5A at $-5$ minutes). Intriguingly, a trend of reversal of the spontaneous BRR response from Ang II–induced inhibition began 35 minutes after treatment and became significant at 65 minutes (Figure 5B). The SAP-HR transfer magnitude approached baseline level by 95 minutes. Apart from this difference in trend, the magnitude of the reversed Ang II–induced inhibition at 65 and 95 minutes (Figure 5B) was invariably less than the
algebraic sum of the individual effects of the octapeptide or antisense oligonucleotide on spontaneous BRR response.

**Effect of Antisense c-fos Oligonucleotide Treatment on Fos Expression in the Caudal NTS**

Microinjection bilaterally of an antisense c-fos oligonucleotide (20 pmol) into the NTS, delivered 120 minutes before the application of Ang II (Figure 6A), significantly reduced Fos expression induced by the octapeptide at the caudal NTS. The level of Fos-LI detected was in fact appreciably lower than that in sham-control animals (330 ± 25 neurons, n = 4). The same trend was observed when antisense c-fos oligonucleotide was comicroinjected with Ang II into the bilateral NTS (Figure 6B). The number of Fos-positive neurons in the caudal NTS was comparable to that in aCSF-treated control animals (cf Figure 3). It should be mentioned that the decrease in Fos-LI at the caudal NTS after treatment with antisense c-fos oligonucleotide was not due to a false-negative reaction. Fos-LI was still demonstrated in the caudal and rostral ventrolateral medulla in the treated animals.

**Effects of Control c-fos Oligonucleotides on Spontaneous BRR Response and Fos Expression in the Caudal NTS**

We verified the specificity of the observed biologic activity of the antisense c-fos oligonucleotide by evaluating the effects of 2 control sequences of oligonucleotide. Microinjection bilaterally into the caudal NTS of the sense c-fos oligonucleotide (20 pmol) resulted in no discernible alteration in baseline magnitude of SAP-HR transfer function (Figure 4). It also elicited minimal effects on Ang II–induced inhibition of spontaneous BRR response or Fos expression, either as a pretreatment (Figures 5A and 6A) or together with the octapeptide (Figures 5B and 6B). Comparable observations (Figures 4 to 6) were obtained from treatment with an antisense oligonucleotide with a scrambled sequence (20 pmol).

**Microinjection Sites**

Histological verifications indicated that loci in the medulla oblongata on which test chemicals were delivered were distributed in the caudal NTS, at 0 to −0.6 mm from the obex. Microinjection of the same amount of test chemicals into a more rostral part of the NTS (0.8 to 1.0 rostral to the obex, n = 3) or areas adjacent to the caudal NTS (n = 3) elicited no discernible alterations in spontaneous BRR response or Fos-LI in the caudal NTS.

**Discussion**

The present study established in animals under minimal circulatory perturbation that Ang II at the caudal NTS exerted an inhibitory modulation on the spontaneous BRR response. We further provided the first demonstration that Fos protein expressed in the caudal NTS via activation of AT1 R may underlie this beat-to-beat cardiovascular regulation. The caudal NTS is a logical central site at which our identified linkage among Ang II, AT1 R, and Fos expression acts to exert an inhibitory modulation on spontaneous BRR response. Binding sites for AT1 R predominate in areas of the brain thought to mediate most of the hemodynamic effects of Ang II, including the NTS.22 Sustained hypertension induces Fos expression primarily in a subpopulation of NTS neurons located ventromedial to the solitary tract.5 This subpopulation of NTS neurons is the site at which mRNA for AT1 R, but not AT2 R, is localized.23

Based on evaluation of the magnitude of transfer function, a novel finding in the present study is that in addition to evoked BRR response,11 inhibitory modulation by Ang II operates at the level of beat-to-beat variability of SAP and HR signals. We further showed that the octapeptide was already efficacious at 5 pmol and that the inhibitory effect optimized at 10 pmol. That comparable inhibition on evoked BRR response requires an optimal dose of 40 pmol further attested to the sensitivity of this modulatory action of Ang II on the spontaneous BRR response.
Another intriguing finding in the present study is that activation of the AT₁R by Ang II is upstream to the induced expression of Fos protein at the NTS. We found that selective blockade of AT₁R in the caudal NTS by losartan significantly reversed the inhibition of spontaneous BRR response and the increase in Fos-LI at the NTS induced by Ang II. More importantly, when co-microinjected bilaterally into the caudal NTS with an antisense c-fos oligonucleotide, a discernible reversal of the inhibition by Ang II on spontaneous BRR response began 65 minutes after treatment. We found, however, that this reversal was not due simply to an algebraic sum of the individual effects of Ang II or antisense oligonucleotide on the spontaneous BRR response.

Our results suggest that the initial phase of the AT₁R-mediated inhibition by Ang II of spontaneous BRR response does not require the engagement of Fos protein. It is generally contended that AT₁R are coupled via G protein or proteins to stimulation of inositol phospholipid hydrolysis, followed by an increase in calcium mobilization or protein kinase C. Speculatively, the same signal transduction pathways may also underlie the inhibitory action of Ang II via AT₁R at the NTS. Workers at our laboratory demonstrated recently that induction of the c-fos gene in the NTS after baroreceptor stimulation involves activation of calcium/calmodulin kinases and phosphorylation of the transcription factor cAMP response element binding protein. Other authors pointed out that transcriptional regulation of c-fos by Ang II is achieved via the protein kinase C pathway and involves the serum response element, the binding site for the dimeric serum response factor. An association of AT₁R with calcium mobilization or protein kinase C pathways thus provides a logical link between activation of this subtype receptor and c-fos induction at the caudal NTS.

Figure 5. Time course alterations in spontaneous BRR response after microinjection bilaterally into the caudal NTS of Ang II (All) (10 pmol), 120 minutes after pretreatment with a sense (20 pmol), scrambled (20 pmol), or antisense (20 pmol) c-fos oligonucleotide (A), or of Ang II (10 pmol) administered with 1 of the 3 oligonucleotides (B). Values presented are mean±SEM (n=6 to 8 animals per group). *P<0.05 vs pre-Ang II control group in the Dunnett test. P<0.05 vs Ang II plus sense or scrambled group in the Scheffé multiple range test.

Figure 6. Total Fos-like immunoreactivity in the caudal NTS in rats that received pretreatment with microinjection bilaterally into the caudal NTS of a sense (20 pmol), scrambled (20 pmol), or antisense (20 pmol) c-fos oligonucleotide for 120 minutes, followed by Ang II (All) (10 pmol) (A), or of Ang II (10 pmol), administered with 1 of the 3 oligonucleotides (B). Values presented are mean±SEM (n=6 to 8 animals per group). *P<0.05 vs Ang II plus sense or scrambled group in the Scheffé multiple range test.

NTS. Workers at our laboratory demonstrated recently that induction of the c-fos gene in the NTS after baroreceptor stimulation involves activation of calcium/calmodulin kinases and phosphorylation of the transcription factor cAMP response element binding protein. Other authors pointed out that transcriptional regulation of c-fos by Ang II is achieved via the protein kinase C pathway and involves the serum response element, the binding site for the dimeric serum response factor. An association of AT₁R with calcium mobilization or protein kinase C pathways thus provides a logical link between activation of this subtype receptor and c-fos induction at the caudal NTS.

Another interesting finding in the present study is that pretreatment with an antisense c-fos oligonucleotide essentially blunted the Ang II–induced inhibition of spontaneous BRR response in the caudal NTS. Among the candidate late-responsive genes that contain the octomer activating protein-1 consensus sequence in their regulatory domain is the AT₁R gene. The possibility therefore exists for Fos expression induced by Ang II to in turn upregulate the molecular synthesis of AT₁R. This possibility, however, must await further elucidation.

We are confident that our observed blunting effect of the antisense oligonucleotide on Ang II–induced inhibition of
spontaneous BRR response and Fos expression in the caudal NTS was related to its complementarity with c-fos mRNA. Microinjection into the bilateral caudal NTS of the same antisense oligonucleotide, which is designed to target a region of the c-fos mRNA that flanks the initiation codon (5'-129 to 143-3'), blunts both basal and evoked Fos expression in the caudal NTS. Application of a sense oligonucleotide complementary to the antisense c-fos sequence or an antisense oligonucleotide with random sequence minimally affected the efficacy of Ang II in inhibition of the spontaneous BRR response or Fos expression at the NTS.

We were concerned that pentobarbital anesthesia may have confounded our results by influencing the spontaneous BRR response. As we reported previously, our scheme of anesthesia minimally affected the spontaneous BRR response or Fos expression at the NTS. Perturbations, Fos expression mediated via activation of AT1R, may underlie the inhibitory modulation of beat-to-beat BRR control of blood pressure by Ang II at the caudal NTS. We previously demonstrated that an elevated expression in basal Fos protein at the caudal NTS is associated with the dysfunction in BRR response in spontaneously hypertensive rats. Exaggerated angiotensin-mediated synaptic transmission at the caudal NTS was demonstrated in hypertensive animals, along with an increase in the density of angiotensin receptors in the caudal NTS. It follows that our identified causative relationship among Ang II, AT1R, and Fos expression at the caudal NTS represents a subtle cascade of intracellular events to ensure that the octapeptide may exert a long-term inhibitory modulation on beat-to-beat BRR control of blood pressure. An exaggeration of this linkage may lead to further impairment of the BRR response during hypertension.

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References

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