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Nitric Oxide Regulates c-*fos* Expression in Nucleus Tractus Solitarii Induced by Baroreceptor Activation via cGMP-Dependent Protein Kinase and cAMP Response Element-Binding Protein Phosphorylation

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ABSTRACT

Activation of the arterial baroreceptors induces expression of the proto-oncogene c-*fos* in the nucleus tractus solitarii (NTS), the terminal site of baroreceptor afferents in the medulla oblongata. This induced expression is an intracellular event that is crucial to long-term maintenance of stable blood pressure. Using Sprague-Dawley rats maintained under propofol anesthesia, we evaluated the role and delineated the underlying molecular mechanisms of nitric oxide (NO) in this process. Baroreceptor activation induced by 30 min of sustained hypertension significantly and sequentially increased the level of cyclic GMP-dependent protein kinase I (PKG-I), phosphorylated cyclic AMP response element-binding protein (pCREB), c-fos mRNA, and Fos protein in the NTS. All of these up-regulated expressions were significantly attenuated in animals that were pretreated immediately before baroreceptor activation with bilateral microinjection into the NTS of a selective neuronal nitric-oxide synthase (nNOS) inhibitor, 7-nitroindazole (2.5 pmol), or a soluble guanylyl cyclase (sGC) inhibitor, 1-H-[1,2,4]oxadiaolo[4,3-a]quinoxalin-1-one (1 nmol). Bilateral NTS microinjection of a cell-permeable cGMP analog, 8-bromoguanosine-3′,5′-cyclic monophosphate (10 nmol) significantly elevated the level of pCREB or c-fos mRNA in the NTS. On the other hand, the up-regulated CREB phosphorylation or c-*fos* induction evoked in the dorsomedial medulla by baroreceptor activation was significantly antagonized by NTS application of a cell-permeable cGMP antagonist, (*R*)p-8-bromoguanosine-3′,5′-cyclic monophosphorothioate (5 nmol), or a PKG inhibitor, (8*R*,9*S*,11*S*)-(−)-9-methoxy-carbamyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*,–2,7*b*,11a-trizadizo-benzo(*a*,*g*)cycloocta(*c*,*d*,*e*)-trinden-1-one (1 nmol). We conclude that NO derived from nNOS in the NTS on baroreceptor activation may participate in c-*fos* expression via phosphorylation of CREB in a process that engages the sGC/cGMP/PKG-I signaling cascade.

The cellular transcription factor Fos is the protein product of the immediate early gene c-*fos*. Activation of the arterial baroreceptors induces Fos expression (Chan and Sawchenko, 1998; Chan et al., 1998b, 1999, 2000) in the barosensitive neurons of nucleus tractus solitarii (NTS), the principal terminal site of primary baroreceptor afferents (Ciriello, 1983). This induced Fos expression represents an early intracellular event that leads to long-term inhibitory modulation of the baroreceptor reflex (BRR) response (Shih et al., 1996; Chan et al., 1997). The cellular signaling cascade triggered by the baroreceptor afferent inputs begins with activation of both *N*-methyl-D-aspartate (NMDA) and non-NMDA glutamate receptors in the NTS (Chan et al., 1998b) and ends with activation of the cyclic AMP response element (CRE) in the c-*fos* promoter region by the phosphorylated CRE-binding protein (pCREB) (Chan et al., 1999).

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ABBREVIATIONS: NTS, nucleus tractus solitarii; BRR, baroreceptor reflex; NMDA, *N*-methyl-D-aspartate; CRE, cyclic AMP response element; CREB, cyclic AMP response element-binding protein; NO, nitric oxide; sGC, soluble guanylyl cyclase; PKG, cyclic GMP-dependent protein kinase; CaM kinase, calcium/calmodulin-dependent protein kinase; nNOS, neuronal nitric-oxide synthase; pCREB, phosphorylated cAMP response element-binding protein; MSAP, mean systemic arterial pressure; NOS, nitric-oxide synthase; L-NMMA, N^G-monomethyl-L-arginine acetate; 7-NI, 7-nitroindazole; SMT, *S*-methylisothiourea; ODQ, 1-H-[1,2,4]oxadiaolo[4,3-*a*]quinoxalin-1-one; 8-Br-cGMP, 8-bromoguanosine-3′,5′-cyclic monophosphate; Rp-8-Br-cGMPS, Rp-8-bromoguanosine-3′,5′-cyclic monophosphorothioate; KT-5823, (8*R*,9*S*,11*S*)-(−)-9-methoxy-carbamyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*,−2,7*b*,11*a*-trizadizo-benzo(*a*,*g*)cycloocta(*c*,*d*,*e*)-trinden-1-one; aCSF, artificial cerebral spinal fluid; Fos-LI, Fos-like immunoreactivity; RT-PCR, reverse transcription-polymerase chain reaction; BP, blood pressure.

Nitric oxide (NO) is a gaseous messenger molecule that plays an important role in central cardiovascular regulation. In the NTS, NO induces hypotension and bradycardia (Tseng et al., 1996; Lin et al., 1999; Paton et al., 2001) and participates in cardiovascular responses induced by activation of glutamate receptors (Lin et al., 1999). These circulatory actions of NO are attributable to activation of soluble guanylyl cyclase (sGC) and subsequent increase in cGMP (Lin et al., 1999). The cellular targets for this augmented intracellular cGMP level include cGMP-regulated ion channels, cGMP-regulated phosphodiesterases, or cGMP-dependent protein kinases (PKGs) (Schmidt et al., 1993; Lucas et al., 2000). Of these targets, the cGMP/PKG signaling pathway has been reported to be involved in NO-induced regulation of c-*fos* expression in smooth muscle cells (Idriss et al., 1999; Gudi et al., 2000) or neuronal cells (Haby et al., 1994).

Activation of the glutamate receptors at the NTS by baroreceptor afferents results in activation of calcium/calmodulin-dependent protein kinases (CaM kinases), leading to phosphorylation of CREB and activation of CRE in the promotor of the c-*fos* gene (Chan et al., 1999, 2000). Calcium/calmodulin also activates the neuronal nitric-oxide synthase (nNOS) (Bredt and Snyder, 1990). It follows that NO produced by nNOS in the NTS on baroreceptor activation may participate in c-*fos* expression via phosphorylation of CREB in an sGC/cGMP/PKG-dependent manner. This hypothesis is validated in the present study. We demonstrated that NO derived from nNOS regulates induction of the immediate early gene c-*fos* in the NTS after baroreceptor activation. Such a regulation involves the sGC/cGMP pathway and requires transcriptional activation of CRE in the c-*fos* promotor region by pCREB in a PKG-I-dependent manner.

Materials and Methods

All experimental procedures were conducted in compliance with the guidelines of our institutional animal care committee and were in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. All efforts were made to minimize animal suffering and to reduce the number of animals used.

General Preparation. Adult male Sprague-Dawley rats (6 to 8 weeks, 200–223 g; $n = 238$) were purchased from the Experimental Animal Center of the National Science Council (Taipei, Taiwan). Rats were initially anesthetized with pentobarbital sodium (50 mg/kg, i.p.) for the performance of preparatory surgery. This surgery routinely included intubation of the trachea to facilitate ventilation and cannulation of the right femoral artery and vein to measure systemic arterial pressure and administer test agents. The left femoral vein was also cannulated for continuous infusion of propofol (Zeneca Pharmaceuticals, Macclesfield, UK) at 25 to 30 mg/kg/h. This scheme provides satisfactory anesthetic maintenance and preserves the capacity of central cardiovascular regulation (Yang et al., 1995).

The animal was thereafter fixed to a stereotaxic head-holder (Kopf Instruments, Tujunga, CA), and the rest of the body was placed on a heating pad to maintain rectal temperature at 37 ± 0.5°C. Pulsatile and mean systemic arterial pressure (MSAP) and heart rate were recorded on a polygraph (Gould Instrument Systems Inc., Cleveland, OH). The animal was mechanically ventilated (Harvard Apparatus Inc., Holliston, MA) to maintain end-tidal CO_2 within 4.0 to 4.5%, as monitored by a capnograph (Datex Normocap, Helsinki, Finland). This test was conducted to minimize possible confounding cardiovascular changes secondary to respiratory perturbation. All data were collected from animals with a steady baseline (MSAP ≥90 mm Hg) throughout the recording period.

Baroreceptor Activation. The arterial baroreceptors were activated by a sustained increase in MSAP evoked by i.v. infusion of phenylephrine (10 μg/kg/h) (Sigma-Aldrich, St. Louis, MO) for 30 min (Chan et al., 1998b, 1999, 2000). The infusion rate was adjusted (5–7 μl/kg/h) to maintain an average elevation in MSAP of 45 to 50 mm Hg. Systemic infusion of saline served as our volume and vehicle control, and animals that received preparatory surgery and maintained under propofol anesthesia served as our sham control.

Microinjection of Test Agents. Bilateral and sequential microinjection of test agents into the NTS was performed according to procedures reported in our recent studies (Chan et al., 1998b, 1999, 2000, 2003). The stereotaxic coordinates used were 0.5 to 0.9 mm below the surface of the fourth ventricle, 0 to −0.5 mm caudal from and 0.3 to 0.6 mm lateral to the obex. The test agents used included a nonselective NOS inhibitor, N^G-monomethyl-L-arginine acetate (L-NMMA; Reif and McCreedy, 1995) (Sigma-Aldrich); a selective nNOS inhibitor, 7-nitroindazole (7-NI) (Moore et al., 1993) (Sigma/RBI, Natick, MA); a selective inducible NOS inhibitor, *S*-methylisothiourea (SMT) (Southan et al., 1995) (Tocris Cookson Inc., Bristol, UK); an sGC inhibitor, 1-*H*-[1,2,4]oxadiaolo[4,3-*a*]quinoxalin-1-one (ODQ) (Hwang et al., 1998) (Tocris Cookson); a cell-permeable cGMP analog that preferentially activates PKG, 8-bromoguanosine-3′,5′-cyclic monophosphate (8-Br-cGMP) (Francis et al., 1988) (BIOMOL Research Laboratories, Plymouth Meeting, PA); a membrane-permeable, phosphodiesterase-resistant cGMP antagonist, (*R*)p-8-bromoguanosine-3′,5′-cyclic monophosphorothioate (Rp-8-Br-cGMPS) (Nakazawa and Imai, 1994) (BIOMOL Research Laboratories); or a membrane-permeable PKG inhibitor, (8*R*,9*S*,11*S*)-(−)-9-methoxy-carbamyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*,−2,7*b*,11*a*-triazadizo-benzo(*a*,*g*)cycloocta(*c*,*d*,*e*)-trinden-1-one (KT-5823) (Kase et al., 1987) (BIOMOL). The doses used were adopted from studies (Hwang et al., 1998; Murthy and Makhlouf, 1998; Trabace and Kendrick, 2000; Chan et al., 2001; Schmidtko et al., 2003) that employed the same test agents for the same purpose as in this study. The volume of each microinjection was restricted to 50 nl, and aCSF served as the vehicle and volume control for all agents. The only exception was ODQ or KT-5823, for which 0.2% dimethyl sulfoxide served as the vehicle control.

Fos-like Immunohistochemistry. At the conclusion of some physiological experiments, animals were processed for immunohistochemical staining of Fos-containing neurons (Chan et al., 1998b, 1999, 2000) using a sheep polyclonal antiserum (1:4000; Santa Cruz Biotechnology, Inc. Santa Cruz, CA). As a routine, sections from rats that received various treatment schedules from the same series of experiments were processed together. Sections incubated without the primary antiserum or with normal sheep or rabbit serum as a substitute for Fos antiserum served as negative controls. No specific immunoreactivity was observed in these control sections when they were processed together with the experimental tissues.

The criterion for identifying Fos-like immunoreactivity (Fos-LI) was a distinctly stained nucleus (Chan et al., 1998b, 1999, 2000). The caudal medulla oblongata was divided into six levels at 200-μm intervals between 0.6 mm caudal and 0.6 mm rostral to the obex. Five sections were selected randomly from each level, and the number of NTS neurons that displayed Fos-LI was counted bilaterally by two researchers in a single-blind fashion. The mean number of Fos-positive cells for each level of the NTS was averaged and tabulated.

Isolation of Total RNA and Reverse Transcription-Polymerase Chain Reaction Analysis. At the conclusion of some physiological experiments, tissues on both sides of the dorsomedial part of the medulla oblongata at the level of NTS (1 mm rostral or caudal from the obex) were collected by micropunches made with a stainless steel bore (1 mm i.d.) and frozen in liquid nitrogen. Medullary tissues thus obtained from six to eight rats were pooled for isolation of total RNA. Quantification of c-fos mRNA was conducted by reverse transcription-polymerase chain reaction (RT-PCR) (Chan et al., 1999,

2000). 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 (Amersham Biosciences AB, Uppsala, Sweden), and the amount of mRNA products for c-*fos* was expressed as the ratio to glyceraldehyde-3-phosphate dehydrogenase mRNA product.

Extraction of Protein and Western Blot Analysis. To prepare cytosolic and nuclear protein (Yeh et al., 2002), medullary tissues similarly obtained from six to eight rats were homogenized in lysis buffer (50 mM Tris buffer containing 0.25 M sucrose, 25 mM KCl, 10 mM $MgCl_2$, and 20 mM sodium molybdate, pH 7.4). After centrifugation at 12,000*g* at 4°C for 10 min, the cytosolic fraction was saved in liquid nitrogen until use. Pellets obtained after the first centrifugation were used to prepare purified nuclear protein. The pelleted nuclei were washed and resuspended in an extraction buffer containing 100 mM HEPES, pH 7.4, 1 mM EDTA, 3 mM $MgCl_2$, 1 M NaCl, 2 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 15 μg/ml leupeptin, 1 mM sodium orthovanadate, and 10% (v/v) glycerol. The nuclear suspension was centrifuged at 12,000*g* at 4°C for 30 min, and the supernatant was saved.

Western blot analysis of pCREB, CREB, or PKG-I protein at the dorsomedial medulla was performed using a rabbit polyclonal antiphospho-CREB antiserum (1:2000; Upstate Biotechnology), a rabbit anti-CREB antiserum (1:2000; Upstate Biotechnology), a rabbit anti-PKG-I antiserum (1:1000; Calbiochem, San Diego, CA), or a mouse monoclonal anti-α-tubulin antiserum (1:2000; Sigma-Aldrich). Incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) followed this procedure. Specific antibody-antigen complex was detected using an enhanced chemiluminescence Western blot detection system (PerkinElmer Life Sciences, Boston, MA). The amount of pCREB, CREB, or PKG-I protein was quantified by the Photo-Print Plus software (ETS Vilber-Lourmat, Torcy, France) and expressed as the ratio to α-tubulin protein, which served as the internal control to demonstrate equal loading of proteins.

Statistical Analysis. All values are expressed as mean ± S.E. One- or two-way analysis of variance with repeated measures was used, as appropriate, to assess the difference between experimental groups, followed by Scheffé's multiple range test for a posteriori comparison of individual means. $P < 0.05$ was considered statistically significant.

Results

Effects of NOS Inhibitors on Fos Expression in the NTS after Baroreceptor Activation. Similar to our previous observations (Chan et al., 1998b, 1999, 2000), baroreceptor activation resulted in an increase in the number of Fos-positive neurons in the NTS detected 2 h after stimulation (Fig. 1A). Likewise, this evoked Fos-LI also displayed a topographic distribution (Chan et al., 1998b, 1999, 2000) that concentrated primarily at levels of the NTS between 0.6 mm caudal and 0.2 mm rostral to the obex. Compared with the aCSF control, bilateral NTS microinjection immediately before baroreceptor activation of a nonselective NOS inhibitor, L-NMMA (5 nmol), or a selective nNOS inhibitor, 7-NI (2.5 pmol), significantly suppressed the evoked Fos expression in the NTS (Fig. 1A). Microinjection of a selective inducible NOS inhibitor, SMT (250 pmol), on the other hand, elicited minimal effect. It should be noted that the basal expression of Fos-LI detected in the NTS of animals that received saline infusion was not affected by pretreatment with bilateral NTS microinjection of L-NMMA (5 nmol), 7-NI (2.5 pmol), SMT (250 pmol), or aCSF (Fig. 1B).

Effects of NOS Inhibitors on c-fos mRNA Level in the Dorsomedial Medulla after Baroreceptor Activation. We next elucidated whether the suggested NO-induced modulation of Fos expression in the NTS after baroreceptor activation took place at the transcriptional level. RT-PCR analysis (Fig. 2) revealed a significantly increased c-fos mRNA level in the dorsomedial medulla 90 min after baroreceptor activation. Such an up-regulation of c-*fos* expression was significantly depressed in animals that received bilateral NTS microinjection of L-NMMA (5 nmol) or 7-NI (2.5 pmol), but not SMT (250 pmol), immediately before baroreceptor activation. The basal level of c-fos mRNA in the dorsomedial medulla of sham control animals or saline-infused animals was below our detection limit.

Effects of Soluble Guanylyl Cyclase Inhibitor on Fos Expression in the NTS after Baroreceptor Activation. Our third series of experiments delineated whether the NO/cGMP pathway is involved in the modulation of Fos expression evoked by baroreceptor activation in the NTS. Pretreatment with bilateral NTS microinjection of an sGC inhibitor, ODQ (1 nmol), discernibly suppressed the increase in the number of Fos-positive neurons in the NTS detected 120 min after baroreceptor activation (Fig. 3A). As a positive control, the same pretreatment also appreciably attenuated Fos expression in the NTS (Fig. 3B) in response to NTS application of the NO precursor L-Arg (100 nmol).

Effects of Blockade of NO/cGMP Signaling on Phosphorylation of CREB in the Dorsomedial Medulla after Baroreceptor Activation. We reported previously (Chan et al., 1999) that phosphorylation of CREB provides a crucial

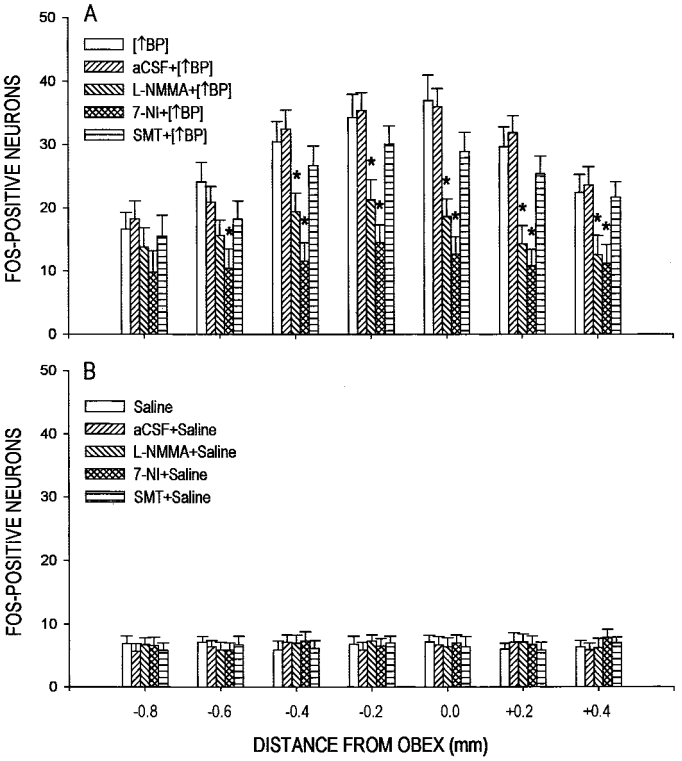

Fig. 1. Distribution of Fos-positive neurons at seven representative rostral/caudal levels of the NTS in animals that received bilateral NTS microinjection of aCSF, L-NMMA (5 nmol), 7-NI (2.5 pmol), or SMT (250 pmol) immediately before intravenous infusion of phenylephrine [↑BP] (A) or saline (B). Values are mean ± S.E. (n = 5 to 6 animals per group). ⋆, $p < 0.05$ versus [↑BP] group in Scheffé's multiple-range analysis.

link between baroreceptor activation and transcriptional activation of c-*fos* in the NTS. Our fourth series of experiments investigated whether this signaling process is involved in the modulation by NO of the evoked Fos expression in the NTS. We detected a significant degree of CREB phosphorylation in the nuclear fraction of extracts obtained from the dorsomedial medulla 60 min after baroreceptor activation (Fig. 4). Similar amounts of CREB were present in all samples, so these changes in CREB phosphorylation were not caused by uneven loading of nuclear protein. More importantly, this induced pCREB level was significantly attenuated in animals that received bilateral NTS microinjection of 7-NI (2.5 pmol) or ODQ (1 nmol) immediately before baroreceptor activation. Direct application of L-Arg (100 nmol) into the bilateral NTS also caused a significant increase in pCREB level detected 60 min after injection (data not shown).

Expression of cGMP-Dependent Protein Kinases in the Dorsomedial Medulla after Baroreceptor Activation. Basal levels of PKG-I were detected in the cytosolic faction of extracts from the dorsomedial medulla of sham control or saline-infused animals. A significant increase in PKG-I expression was observed 60 min after baroreceptor activation (Fig. 5). Notably, this up-regulation of PKG-I protein was significantly antagonized in animals that received microinjection bilaterally into the NTS of 7-NI (2.5 pmol) or ODQ (1 nmol) immediately before baroreceptor activation.

Effects of cGMP Analogs or Protein Kinase G Inhibitor on CREB Phosphorylation and c-*fos* Expression in the Dorsomedial Medulla after Baroreceptor Activation. Our final series of experiments established a causative relationship between the cGMP/PKG pathway, CREB phosphorylation, and c-*fos* expression in the NTS induced by baroreceptor activation. Similar to baroreceptor activation, bilateral NTS microinjection of a cell-permeable cGMP analog, 8-Br-cGMP (10 nmol), induced a significant increase in pCREB protein in the dorsomedial medulla 60 min postinjection (Fig. 6A). The same treatment also evoked appreciable c-fos mRNA expression 90 min after local application to the NTS (Fig. 6B). On the other hand, pretreatment with bilateral NTS administration of a membrane-permeable cGMP antagonist, Rp-8-Br-cGMPS (5 nmol), or a selective PKG inhibitor, KT-5823 (1 nmol), significantly blunted CREB phosphorylation (Fig. 6A) or c-fos mRNA expression (Fig. 6B) detected 60 or 90 min after baroreceptor activation.

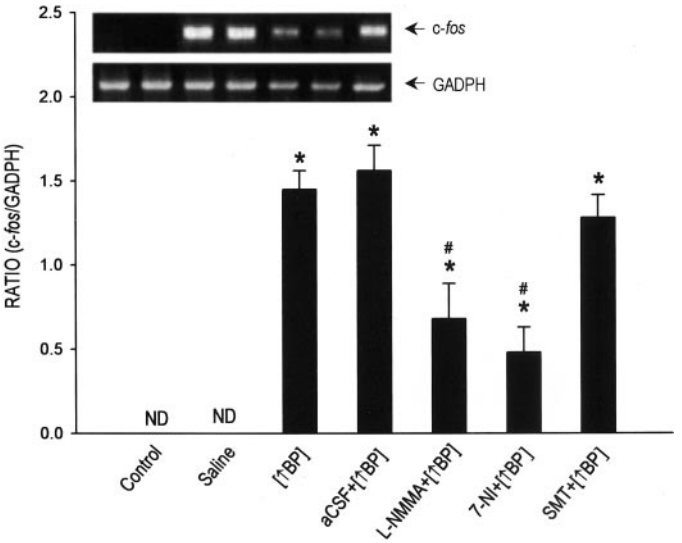

Fig. 2. Representative gels for RT-PCR products (inset) or the amount of c-fos mRNA relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA detected from the caudal dorsomedial medulla 90 min after animals were subjected to sustained hypertension (lane 3, [↑BP]), or in animals that also received bilateral NTS microinjection of aCSF (lane 4), L-NMMA (5 nmol; lane 5), 7-NI (2.5 pmol; lane 6), or SMT (250 pmol; lane 7) immediately before intravenous infusion of phenylephrine [↑BP]. Lanes 1 and 2, extracts from the caudal dorsomedial medulla of sham and saline controls. Values are mean ± S.E. of quadruplicate analysis on samples obtained from 6 to 8 animals in each group. ⋆, $p < 0.05$ versus saline group; #, $p < 0.05$ versus [↑BP] group in Scheffé's multiple-range analysis. ND, nondetectable.

Discussion

We reported previously (Chan et al., 1998b, 1999, 2000) that activation of glutamate receptors at the NTS by baroreceptor afferents induces Fos expression via the CaM kinase/pCREB/CRE signaling cascade. The present study revealed that another cellular consequence of baroreceptor activation in the NTS entails production of NO derived from nNOS, which leads to transcriptional activation of c-*fos* gene via phosphorylation of CREB in a process involving the sGC/cGMP/PKG pathway.

Several lines of evidence support the presence of a short-term interplay between glutamate and NO in the NTS. Whereas microinjection of glutamate (Lin et al., 1999) or NO donors (Tseng et al., 1996; Lin et al., 1999; Paton et al., 2001) into the NTS elicits hypotension and bradycardia, the effects

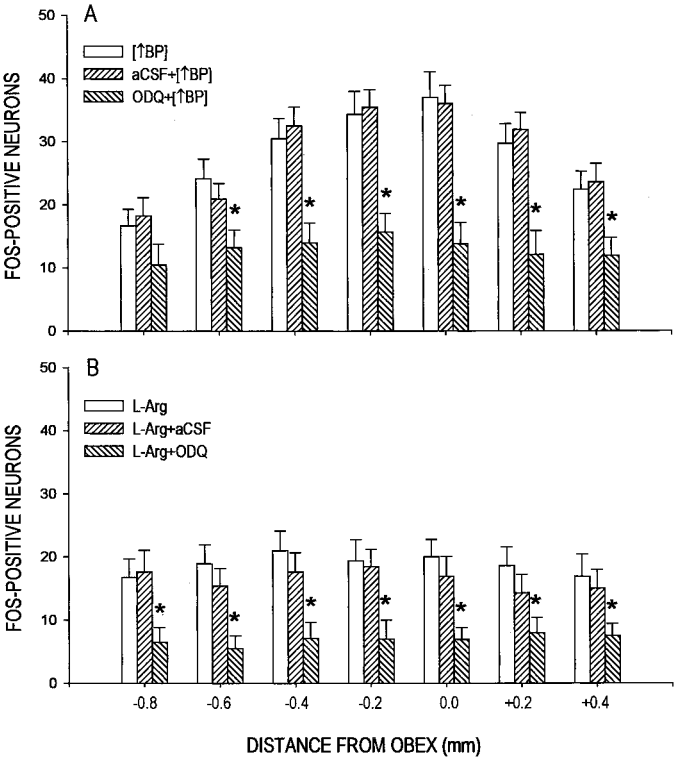

Fig. 3. A, distribution of Fos-positive neurons in seven representative rostral/caudal levels of the NTS in animals that received bilateral NTS microinjection of aCSF or ODQ (1 nmol) immediately before intravenous infusion of phenylephrine ([↑BP]). B, microinjection of L-Arg (100 nmol) given alone or together with aCSF or ODQ (1 nmol). Data on [↑BP] and aCSF + [↑BP] from Fig. 1A were duplicated for comparison. Values are mean ± S.E. ($n = 5$ to 6 animals per group). ⋆, $p < 0.05$ versus [↑BP] or L-Arg group in Scheffé's multiple-range analysis.

of NO depend on the integrity of the ionotropic glutamate receptors (Lin et al., 1999). Intriguingly, the present study identified a long-term consequence of activation of NMDA and non-NMDA receptors by the glutamatergic baroreceptor afferents at the NTS in the form of nNOS induction that eventually leads to transcriptional expression of c-fos mRNA and protein. Anatomically, all nNOS-immunoreactive neurons in the NTS contain both NMDA and non-NMDA receptor subunits (Lin and Talman, 2002). In addition, a majority of nNOS immunoreactivity and nNOS mRNA is distributed in functionally identified barosensitive NTS neurons that exhibit Fos-LI in response to baroreceptor activation (Chan and Sawchenko, 1998). As one of the most potent activators of nNOS (Garthwaite, 1991; Bhardwaj et al., 1997), it is likely that, by acting on the ionotropic glutamate receptors on NTS neurons, glutamate released from the baroreceptor afferent terminals activates nNOS via binding of the elevated Ca^{2+} to calmodulin (Bredt and Snyder, 1990).

Another novel finding of the present study is that baroreceptor activation resulted in up-regulation of PKG in the cytosol, followed by phosphorylation of CREB in the nucleus and induction of c-fos mRNA in the NTS. Because blockade of nNOS or sGC activity in the NTS significantly attenuated

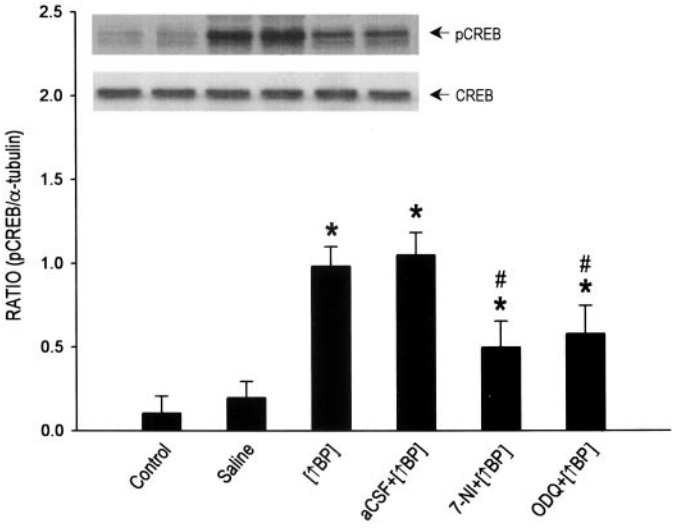

Fig. 4. Representative gels for CREB or pCREB protein (inset) or the amount of pCREB protein relative to α-tubulin protein detected from the caudal dorsomedial medulla 60 min after animals were subjected to sustained hypertension (lane 3, [↑BP]), or in animals that also received bilateral NTS microinjection of aCSF (lane 4), 7-NI (2.5 pmol; lane 5) or ODQ (1 nmol; lane 6) immediately before intravenous infusion of phenylephrine ([↑BP]). Lanes 1 and 2, extracts from the caudal dorsomedial medulla of sham and saline controls. Values are mean ± S.E. of quadruplicate analysis on samples obtained from 6 to 8 animals in each group. ⋆, $p < 0.05$ versus saline group; #, $p < 0.05$ versus [↑BP] group in Scheffé's multiple-range analysis.

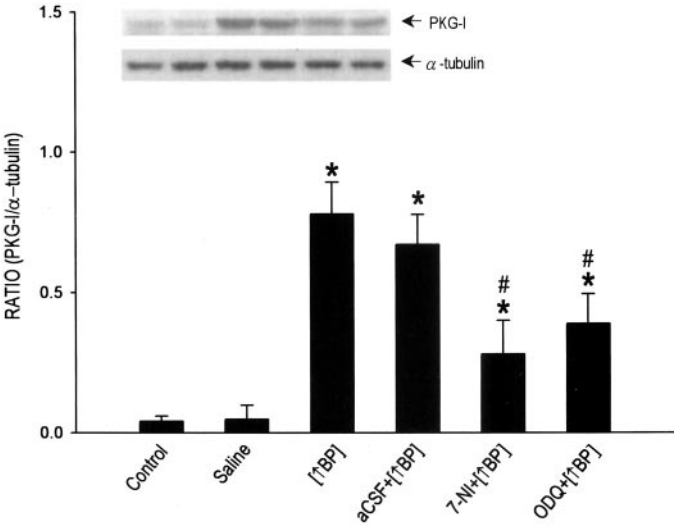

Fig. 5. Representative gels (inset) for PKG-I or the amount of PKG-I protein relative to α-tubulin protein detected from the caudal dorsomedial medulla 60 min after animals were subjected to sustained hypertension (lane 3, [↑BP]), or in animals that also received bilateral NTS microinjection of aCSF (lane 4), 7-NI (2.5 pmol; lane 5), or ODQ (1 nmol; lane 6) immediately before intravenous infusion of phenylephrine ([↑BP]). Lanes 1 and 2, extracts from the caudal dorsomedial medulla of sham and saline control animals. Values are mean ± S.E. of quadruplicate analysis on samples obtained from six to eight animals in each group. ⋆, $p < 0.05$ versus saline group; #, $p < 0.05$ versus [↑BP] group in Scheffé's multiple-range analysis. ND, nondetectable.

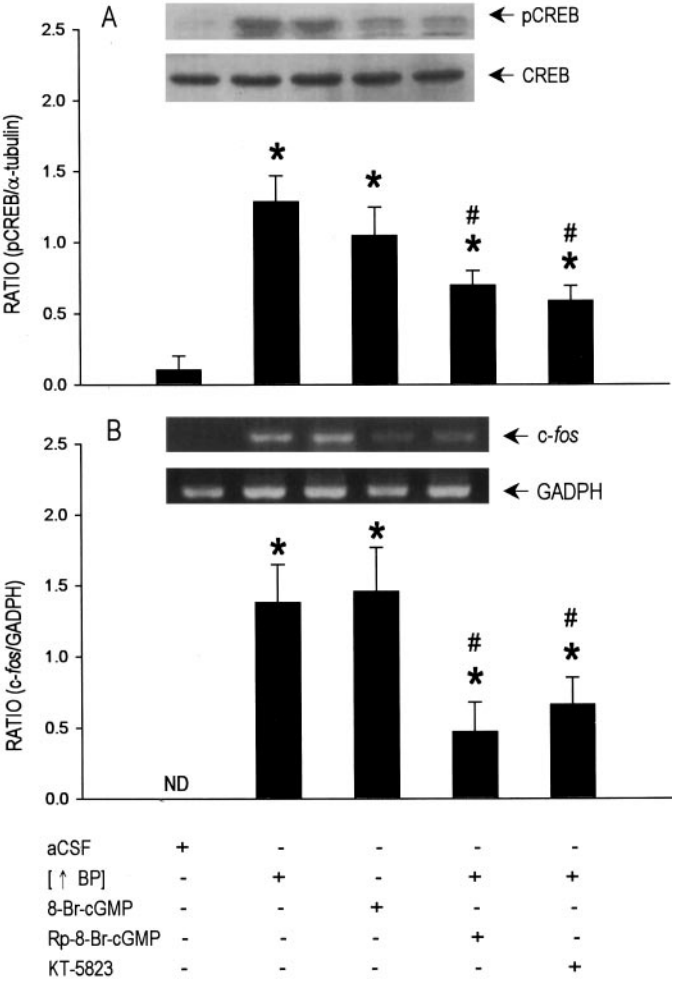

Fig. 6. Representative gels for pCREB protein (A) or c-fos mRNA (B) (inset) or the amount of pCREB protein relative to α-tubulin protein or c-fos mRNA relative to GADPH mRNA detected from the caudal dorsomedial medulla 60 or 90 min, respectively, after animals received bilateral NTS microinjection of 8-Br-cGMP (10 nmol; lane 3), or in animals that were subjected to sustained hypertension (lane 2, [↑BP]) or, in addition, bilateral NTS microinjection of Rp-8-Br-cGMPS (5 nmol; lane 4) or KT-5823 (1 nmol; lane 5) immediately before intravenous infusion of phenylephrine [↑BP]. Lane 1, extracts from the caudal dorsomedial medulla of saline controls. Values are mean ± S.E. of quadruplicate analysis on samples obtained from 6 to 8 animals in each group. ⋆, $p < 0.05$ versus saline group; #, $p < 0.05$ versus [↑BP] group in Scheffé's multiple-range analysis. ND, nondetectable.

these cellular responses, it is likely that activation of sGC/cGMP/PKG pathways by nNOS-derived NO in the NTS is primarily responsible. We reported previously (Chan et al., 1999) that phosphorylation of CREB in the NTS occurs at 15 min and lasts for at least 90 min after baroreceptor activation. This process is followed by peak induction of c-fos mRNA at 90 min and expression of Fos protein at 120 min after baroreceptor activation (Chan et al., 2000). The kinetics of pCREB phosphorylation and c-fos mRNA induction detected in the present study after baroreceptor activation essentially coincides with this temporal profile. These intracellular events, however, seem to take place at a slower rate compared with those in response to direct activation of the NO pathway (Ohki et al., 1995; Wu et al., 2000).

Many of the cellular effects of NO are mediated via changes in gene expression (Chiche et al., 1998). Thus, NO induces nuclear translocation of PKG-I in cGMP-treated cells (Gudi et al., 2000). Activated PKG-I has also been demonstrated to translocate into the nucleus to activate the c-*fos* promoter (Idriss et al., 1999; Gudi et al., 2000). Furthermore, the presence of pCREB in the nucleus is required for activation of the CRE site on the c-*fos* promoter region (Chan et al., 1999). It is therefore of interest that 7-NI or ODQ suppressed the up-regulation of PKG-I in the dorsomedial medulla induced by baroreceptor afferent inputs. Together, these observations lend credence to the notion that PKG-I plays an active role in phosphorylation of CREB and subsequent induction of c-fos mRNA in the NTS after baroreceptor activation.

Our results with cGMP analogs or PKG inhibitor established a causative relationship between the cGMP/PKG pathway, CREB phosphorylation, and c-*fos* induction in the NTS after baroreceptor activation. We recognize that although KT-5823 has been used as an effective membrane-permeable inhibitor for PKG (Kase, 1987), it is reportedly ineffective against PKG-mediated response in intact human platelets and rat mesangial cells (Burkhardt et al., 2000). Nonetheless, we also demonstrated that, whereas 8-Br-cGMP induced a significant increase in pCREB and c-fos mRNA level at the dorsomedial medulla, those same intracellular events evoked after baroreceptor activation were blunted by Rp-8-Br-cGMPS. These corroborative observations therefore support the notion that the regulation by NO on c-*fos* expression in the NTS after baroreceptor activation is mediated by PKG.

In smooth muscle cells, NO-releasing agents and cGMP analogs down-regulate PKG-I expression (Soff et al., 1997). These observations are contrary to our present demonstration of an up-regulation in PKG-I expression in the dorsomedial medulla after baroreceptor activation of NTS neurons. We suspect that the discrepancy may reside in the duration of an experimental stimulus. Whereas PKG-I suppression was observed after NO-releasing agent was given continuously for 4 days (Soff et al., 1997), our animals received only 30 min of baroreceptor activation. In addition, PKG-I expression in the spinal cord is significantly increased after formalin-induced hyperalgesia (Schmidtko et al., 2003).

Studies on the c-*fos* promoter revealed that this proto-oncogene is transcriptionally regulated by signaling pathways that include mitogen-activated protein kinases (Schinelli et al., 2001; Kukushkin et al., 2002), CaM kinases, and cyclic nucleotide-dependent protein kinases (Gudi et al., 2000). Our laboratory reported previously (Chan et al., 2000) that activation of CaM kinases in the NTS represents an important step in the cascade of intracellular events that leads to c-*fos* expression after baroreceptor activation. We further established in the present study the significance of the NO/cGMP/PKG-I signal transduction pathway in the regulation of c-*fos* expression in the NTS evoked by baroreceptor activation. It follows that multiple intracellular signaling pathways are engaged in transcriptional regulation of c-*fos* induction in the NTS after baroreceptor activation. The participatory role of mitogen-activated protein kinases in this mode of Fos expression in the NTS, however, awaits further elucidation.

In view of our demonstrated regulatory action of NO on Fos expression in the NTS, it is conceivable that an enhanced NO formation rather than an elevated Fos expression in this nucleus plays a key role in the reduced BRR control of circulation during hypertension. In this regard, it should be noted that c-*fos* expression (Chan et al., 1999) and nNOS activation (Bredt and Snyder, 1990) are two independent intracellular events downstream of activation of CaM kinases that results from activation of the glutamate receptors at the NTS by baroreceptor afferents (Chan et al., 1998b, 2000). Furthermore, Fos expression in the NTS represents an early intracellular event that leads to long-term inhibitory modulation of BRR response (Shih et al., 1996; Chan et al., 1997). Moreover, a heightened Fos expression is associated with reduced BRR response in spontaneously hypertensive rats (Chan et al., 1998a, 2002). Intriguingly, this heightened Fos expression is causally related to the augmented expression of angiotensin subtype 1 receptor in the NTS of spontaneously hypertensive rats after baroreceptor activation (Chan et al., 2002). As such, it is the interplay rather than relative importance of NO, c-*fos*, and angiotensin receptors in the NTS that is crucial to BRR control of circulation.

In conclusion, the present study demonstrated that induction of the immediate early gene c-*fos* in the NTS after baroreceptor activation is regulated by NO derived from nNOS. Such a regulation involves the sGC/cGMP pathway and requires transcriptional activation of CRE in the c-*fos* promotor region by pCREB in a PKG-I-dependent manner. Regulation of c-*fos* induction by the NO/cGMP/PKG signaling pathway contributes to long-term changes in synaptic plasticity that are critical for learning and memory (Lu et al., 1999; Rose, 2000). We also proposed previously (Shih et al., 1996; Chan et al., 1997) that Fos expression in the NTS represents an early intracellular event that leads to long-term inhibitory modulation of BRR response. The present study provides novel evidence to further reveal that transcriptional regulation of the c-*fos* gene by the NO/cGMP/PKG-I signaling cascade plays an important role in this long-term synaptic plasticity at the NTS in central cardiovascular regulation. Paton et al. (2001) reported that microinjection of either NO donor or NO precursor into the NTS reduces the short-term cardiac baroreceptor reflex response. Blockade of endogenous NO production in the NTS, on the other hand, enhances the same reflex (Waki et al., 2003). As such, the identified regulation by NO/cGMP/PKG-I pathway on Fos expression after baroreceptor activation is functionally significant because it provides a crucial link that extends the short-term depressive actions of NO on BRR response to long-term inhibitory modulation of Fos expression in the NTS.

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