Nitric oxide inhibits the expression of AT₁ receptors in neurons

Neeru M. Sharma,¹ Hong Zheng,¹ Yi-Fan Li,² and Kaushik P. Patel¹

¹Department of Cellular and Integrative Physiology, University of Nebraska Medical Center, Omaha, Nebraska; and²Division of Basic Biomedical Science, College of Medicine, University of South Dakota, Vermillion, South Dakota

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Sharma NM, Zheng H, Li YF, Patel KP. Nitric oxide inhibits the expression of AT₁ receptors in neurons. Am J Physiol Cell Physiol 302: C1162–C1173, 2012. First published January 4, 2012; doi:10.1152/ajpcell.00258.2011.—We have previously observed an increased expression of angiotensin II (ANG II) type 1 receptor (AT₁R) with enhanced AT₁R-mediated sympathetic outflow and concomitant downregulation of neuronal nitric oxide (NO) synthase (nNOS) with reduced NO-mediated inhibition from the paraventricular nucleus (PVN) in rats with heart failure. To test the hypothesis that NO exerts an inhibitory effect on AT₁R expression in the PVN, we used primary cultured hypothalamic cells of neonatal rats and neuronal cell line NG108-15 as in vitro models. In hypothalamic primary culture, NO donor sodium nitroprusside (SNP) induced dose-dependent decreases in mRNA and protein of AT₁R (10⁻⁵ M SNP, AT₁R protein was 10 ± 2% of control level) while NOS inhibitor N⁵-monomethyl-L-arginine (L-NAME) induced dose-dependent increases in mRNA and protein levels of AT₁R (10⁻³ M L-NAME, AT₁R protein was 148 ± 8% of control level). Similar effects of SNP and L-NAME on AT₁R expression were also observed in NG108-15 cell line (10⁻⁶ M SNP, AT₁R protein was 30 ± 4% of control level while at the dose of 10⁻³ M L-NAME, AT₁R protein was 171 ± 15% of the control level). Specific inhibition of nNOS, using antisense, caused an increase in AT₁R expression while overexpression of nNOS, using adenoviral gene transfer (Ad.nNOS), caused an inhibition of AT₁R expression in NG108 cells. Antisense nNOS transfection augmented the increase while Ad.nNOS infection blunted the increase in intracellular calcium concentration in response to ANG II treatment in NG108 cells. In addition, downregulation of AT₁R mRNA as well as protein level in neuronal cell line in response to S-nitroso-N-acetyl penicillamine (SNAP) treatment was blocked by protein kinase G (PKG) inhibitor, while the peroxynitrite scavenger deferoxamine had no effect. These results suggest that NO acts as an inhibitory regulator of AT₁R expression and the activation of PKG is the required step in the regulation of AT₁R gene expression via cGMP-dependent signaling pathway.

sympathetic nerve activity; angiotensin II; cGMP; protein kinase G

ALTERED EXPRESSION OF NEUROTRANSMITTERS plays an important role in the regulation of sympathetic activity in central nervous system (CNS). Among these modulators of neuronal activity within the CNS are nitric oxide (NO) and angiotensin II (ANG II). The interactions between NO and ANG II have been observed in peripheral circulation (3, 31, 36, 37) as well as in the CNS (2, 22, 27). ANG II exerts its biological activity by binding to G protein-coupled receptor, ANG II type 1 (AT₁R), which is expressed on CNS neurons, astrocytes, and microglia (5) and is known to play an important role in cardiovascular diseases like heart failure, hypertension, and diabetes (15, 23). Multiple intracellular signal-transduction cascades, phospholipases A₂, C, and D, adenylyl cyclase, and voltage-dependent Ca²⁺ channels are linked to AT₁R activation (12). AT₁R overexpression and activation are related to the risk factors such as hypertension, hypercholesterolemia, and diabetes leading to heart failure (49). Components of the renin-angiotensin system including angiotensinogen, angiotensin-converting enzyme, and AT₁R are well known to exist in the CNS (26, 38, 51).

NO is a gaseous molecule produced from L-arginine by a family of NADPH-dependent enzymes named NO synthase (NOS). NO is produced by neuronal isoform of NOS (nNOS) functions as an atypical neurotransmitter eliciting multiple effects in the CNS and peripheral nervous system. Two distinct mechanisms of action of NO were documented in the literature: first, it activates the soluble guanylate cyclase/3', 5'-guanosine monophosphate/protein kinase G (sGC/cGMP/PKG) pathway that leads to phosphorylation on PKG consensus sites of target proteins affecting gene transcription. Second, generation of peroxynitrite, through reaction of NO with superoxide, directly modifies protein function by conformational modification via nitration of tyrosine or S-nitrosylation of free cysteine residues (14, 16). These mechanisms of cellular responses to NO are not mutually exclusive, even though they may preferentially occur at different concentrations of NO (48). NO activates heterodimeric enzyme sGC through reaction with its heme center. Activation of PKG by cGMP through increased production of cGMP was the first cellular target for transduction of NO-mediated signals (18).

The paraventricular nucleus (PVN) of the hypothalamus is a site where a number of neurotransmitters, excitatory as well as inhibitory, converge to influence its neuronal activity. Protein expression of nNOS is decreased in the PVN of rats with heart failure, indicating a reduced NO-mediated inhibitory effect on sympathetic outflow (28). At the same time there is an increase in the levels of AT₁R in the PVN of rats with heart failure (52). Whether the overexpression of AT₁Rs is due to a reduction in NO during heart failure is not clear. In our previous studies we showed that AT₁R-mediated functional excitatory effects induced by ANG II stimulation on renal sympathetic nerve activity, mean arterial pressure, and heart rate within the PVN are inhibited by NO (29). The basic mechanisms for the interaction between NO and AT₁Rs are not well established. The purpose of present study was to elucidate the basic molecular mechanism of the interaction between NO and AT₁R expression using primary hypothalamic cell culture and neuronal cell culture, NG108 cell line, that has both AT₁Rs and endogenous nNOS.

METHODS

Animals

The present study conformed to the guidelines for the care and use of laboratory animals of the National Institutes of Health and the American Physiological Society and was approved by the Nebraska Medical Center Institutional Animal Care and Use Committee.
In the present study we have used two in vitro cell culture models. Primary culture. Primary hypothalamus cells were dissociated from 1- to 5-day-old Sprague-Dawley rats. Rats were decapitated and the brain was rapidly removed to iced (4°C) Hanks’ buffer from GIBCO (nominally Ca\(^{2+}\) and Mg\(^{2+}\) free). The hypothalamus was dissected free of surrounding tissue and then placed in 2.5 ml of the same Hanks’ buffer solution warmed to 37°C containing 2.5 mg/ml trypsin (Sigma 25 mg/ml, 100 μl). During incubation for 30 min, the hypothalamus was triturated utilizing a 1 ml glass pipette to promote the release of individual neurons into solution (for 1 min every 10 min of incubation). Following incubation, the neurons were transferred to Hanks’ solution containing BSA (10 mg/ml) and incubated for another 30 min at 37°C and pipetted over a cell strainer into a 50-ml conical tube. The resultant pellet was cultured in Neurobasal Medium A (GIBCO) plus 10% FBS (GIBCO) and 2% of B27 supplement (GIBCO) at 37°C in a humidified atmosphere of 95% air-5% CO\(_2\) until use (3-5 days following isolation).

Cell line culture. The NG108-15 cells (neuroblastoma X glioma) were purchased from American Type Culture Collection (ATCC HB-12317). Stock culture of hybrid cells was grown in high-glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin G, and streptomycin in a humidified atmosphere of 5% CO\(_2\) at 37°C. Cells (10\(^4\) cells/ml) were seeded in six-well plates and grown until 60–70% confluent before experiment.

Cell Treatments

Cells, primary hypothalamic, or NG108, were treated with the NO donor sodium nitroprusside (SNP; 0–10\(^{-5}\) M) or the NO blocker \(N^\gamma\)-monomethyl-L-arginine (L-NMMA; 0–10\(^{-5}\) M) for 24 h. NG108 cells were also treated with the NO donor S-nitroso-N-acetyl penicillamine (SNAP) in the absence or presence of the PKG inhibitor 8-bromoguanosine-3’,5’-cyclic monophosphorothioate (Rp-Br-cGMP).
final concentration 100 nM) or the peroxynitrite scavenger deforxamine (Def; final concentration 100 μM) for 24 h.

**Inhibition of nNOS With Antisense to nNOS**

Antisense (5’-ACGTGTTCTCTTCCATG-3’) targeting rat nNOS (GenBank accession no. NM 052799) was designed and modified with phosphorothiate oligodeoxynucleotides to improve the stability. The target site chosen for nNOS antisense oligonucleotide (nNOS-AS-ODN) is the AUG translational initiation codon and some nearby downstream bases of the mRNA sequence of nNOS. Specificity of nNOS-AS-ODN was checked by BLAST search of GenBank. A mismatched ODN (MS-ODN) consisting of the same number of bases in random order was used as negative control. Transient transfection using nNOS-AS-ODN or MS-ODN was performed in NG108 cells using Lipofectamine 2000 as per the manufacturer’s instructions. Cells were lysed for Western blotting or used for immunostaining after 48 h of transfection.

**Overexpression of nNOS Via Adenoviral Infection**

Adenoviral nNOS (Ad.nNOS) vector carrying the rat nNOS gene sequence and adenoviral vector encoding enhanced green fluorescent protein (Ad.EGFP) were constructed, isolated, and purified by double cesium chloride density centrifugation as previously described (4, 30). Ad.nNOS contains rat nNOS cDNA under the control of the cytomegalovirus immediate/early promoter and expresses functional nNOS protein (30) and was successfully used in our lab to upregulate the expression of nNOS (28 –30). Cells were exposed to Ad.nNOS vector in a dose-dependent manner, 1 × 10⁶ to 1 × 10⁹ plaque-forming units (pfu)/ml final concentration in standard medium for 24 h before the experimentation.

**Semiquantitative RT-PCR for the Measurement of AT1R mRNA**

Total RNA was extracted from cell line by Tri-Reagent (MRC) method as per the manufacturer’s instructions. Briefly, the homogenate was separated into organic and aqueous phases by the addition of isopropanol, washed with ethanol, and solubilized in 10 μl nuclease free water. Following extraction of RNA, samples underwent reverse transcription for 40 min at 37°C in the presence of 1.5 M random hexamers and 100 units of Moloney murine leukemia virus (MMLV)-RTase. One microliter of cDNA product was used for polymerase chain reaction (PCR) amplification. β-Actin was amplified with each cDNA template as internal control. For AT1R, the sequences of primers used for RT-PCR were 5’-AGAGGATTCTCGT-GCTTGAAG-3’ and 3’-AGGGATCATGACAAATATG-5’, and for β-actin, the primers were 5’-GGGAAAATCGTGCGTGACATT-3’ and 3’-CGGATGTCAACGTCACACTT-5’. The PCR products were fractionated on 1% agarose-ethidium gel and the bands were visualized by UV-transillumination. The intensities of the bands were quantified using Kodak 1D image software and the values are expressed as percent change from the control group.

**Immunodetection of AT1R**

Primary hypothalamic cell culture and NG108 cells were washed three times with ice-cold PBS and lysed in protein extraction buffer (10 mM Tris, 1 mM EDTA, 1% SDS, 0.1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride). Protein content of lysate was estimated using the bicinchoninic acid method with bovine serum albumin as standard (Pierce, Rockford, IL). Cell lysates were matched for protein using 1% SDS, and then equal volumes of 2× 4% SDS sample

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**Fig. 3.** A: mRNA expression of AT1R with increasing doses of N⁵-monomethyl-l-arginine (L-NMMA) in primary cultured hypothalamic cells measured by RT-PCR. Top: a representative gel. Bottom: densitometry analyses of AT1R mRNA level normalized to actin and calculated as percentage of control without any treatment. B: Western blot analysis of AT1R relative to tubulin in primary cultured hypothalamic cells treated with different concentrations of L-NMMA. Top: a representative gel. Bottom: densitometry analyses of AT1R protein level normalized to tubulin and calculated as percentage of control without any treatment. Values are means ± SE from three independent experiments. *P < 0.05 vs. control.
buffer were added. Protein lysates (20–30 μg) were electrophoresed on 7.5% sodium dodecyl sulfate-polyacrylamide gel in Tris-glycine running buffer and transferred to a polyvinylidene difluoride membrane (Millipore). Nonspecific binding sites were blocked by incubating the membrane with 5% nonfat dry milk (wt/vol) in TBST buffer (10 mM Tris, 150 mM NaCl, 0.05% Tween 20) at ambient temperature for 1 h. The membrane was probed with a primary anti-AT1R rabbit polyclonal (sc-1173) (0.4 μg/ml) or anti-mouse monoclonal nNOS (sc-5302) (0.4 μg/ml) from Santa Cruz Biotechnology at 4°C overnight followed by incubation with a corresponding peroxidase-conjugated secondary antibody. The signals were visualized using an enhanced chemiluminescence substrate (Pierce Chemical) and detected by Work lab digital image system. AT1R protein level was normalized using tubulin. To confirm the absence of cross nonspecific binding of AT1R antibody, we used a human embryonic kidney (HEK) cell line lacking AT1R as negative control and same cell line transfected with human AGTR1 cloned in pcDNA 3.1 expression vector (UMR cDNA Resource Center, Univ. of Missouri, Rolla) as positive control. Using immunoblot detection, we were able to detect AT1R signal in HEK-293 cells transfected with AGTR1 expression plasmid only but not in the empty cells (Fig. 1).

Immunofluorescence Staining of AT1R

Adherent NG108 cells grown on laminin-coated 6-mm Transwell-Clear inserts (Corning, Costar) were fixed in 4% paraformaldehyde in PBS for 10 min. After permeabilization with 0.2% Triton X-100 for 20–30 min, 10% goat serum was used for blocking. Following blocking, the cells were incubated with primary antibodies against anti-mouse monoclonal nNOS (sc-5302) or anti-AT1R rabbit polyclonal (sc-579) from Santa Cruz Biotechnology, respectively, overnight at 4°C. After washing with PBS, the cells were incubated with fluorescence-conjugated secondary antibodies for 1 h at room temperature. Coverslips were then mounted onto frosted glass microscope slides using Fluoromount G (Southern Biotechnology) and observed under Leica-DMR microscope with corresponding filters. ImageJ software (National Institutes of Health, Bethesda, MD) was used to scale the images.

Calcium Concentration Measurement

Subconfluent NG108 cells were grown on Nunc Lab-Tek chamber and were transfected with MS-ODN or nNOS-AS-ODN, 48 h before experimentation. In a different set of cultures, cells were infected with recombinant adenoviral vectors Ad.EGFP or Ad.nNOS, 24 h before the final experiments. ANG II-stimulated changes in intracellular calcium concentration ([Ca2+]i) were assessed by using fluo-3 (Invitrogen) fluorescence imaging using a Zeiss LSM 510 META confocal microscope with a ×63 oil immersion objective. Briefly, cells were loaded with fluo-3 (5 μM) for 30 min at 37°C and then subsequently washed with fresh medium three times. The chamber was placed on the stage of the confocal microscope and scanned every 2 s using confocal microscopy. An argon laser provided fluorescence excitation at 488 nm, and the emitted light (515 nm) captured along with transmitted images. After acquisition of the baseline pictures for 30 s, ANG II was gently added into the chamber and the calcium fluorescent images (green) were captured in a time series. All analyses of [Ca2+]i were processed at a single-cell level and were calculated as mean pixel values ± SE; values are expressed as the percent change in relative fluorescence intensity of control versus ANG II treatment.

Statistics

The data are expressed as means ± SE, and statistical significance was set at P < 0.05. Statistical comparisons of two groups were made using Student’s t-test.

Fig. 4. A: protein expression of AT1R with increasing concentrations of SNP in NG108 cell line. Top: a representative gel. Bottom: densitometry analyses of AT1R protein level normalized to tubulin and calculated as percentage of control without any treatment. B: expression of AT1R in NG108 cells treated with different doses of l-NMMA. Top: a representative gel. Bottom: densitometry analyses of AT1R protein level normalized to tubulin. Values are means ± SE from four independent experiments. *P < 0.05 vs. control.
RESULTS

Expression of AT1R mRNA and Protein in Primary Culture of Hypothalamic Cells Incubated With SNP

Hypothalamic primary cell line was treated with different concentrations of the NO donor SNP, and changes in AT1R mRNA expression were analyzed by RT-PCR after 24 h (Fig. 2). The SNP-mediated increase in NO dose dependently decreased AT1R mRNA expression, with maximum decrease observed at the highest concentrations (10⁻⁵ M) (Fig. 2A). Moreover, stimulation with SNP also induced a dose-dependent decrease in AT1R protein expression (at the dose of 10⁻⁵ M SNP, AT1R protein was 10 ± 2% of the control level) (Fig. 2B).

Expression of AT1R mRNA and Protein in Primary Culture of Hypothalamic Cells Incubated With the NOS Inhibitor L-NMMA

On the contrary to overproduction of NO, blocking of NO production using the NOS inhibitor L-NMMA significantly increased the mRNA level of AT1R expression even at lower doses (Fig. 3A) and peaked at hypothalamic cells treated with 10⁻⁶ M L-NMMA. Similar to mRNA, the expression of AT1R protein had a progressive increase, with an approximate 50% increase at the dose of 10⁻⁵ M L-NMMA (Fig. 3B).

Expression of AT1R Protein in NG108 Cell Line Incubated With SNP or L-NMMA

Parallel to the primary cell culture, stimulation of NG108 cell line with increasing concentrations of SNP also revealed a decrease in AT1R/tubulin ratio compared with untreated control (Fig. 4A). At the dose of 10⁻⁵ M SNP, AT1R protein was significantly decreased by 90% to control level. In contrast to SNP, AT1R protein expression increased with L-NMMA stimulation in a dose-dependent manner, being statistically significant at 10⁻⁷ M (40%), 10⁻⁶ M (71%), and 10⁻⁵ M (88%) (Fig. 4B).

Effect of Antisense to nNOS on Expression of AT1R in NG108 Cell Line

AT1R expression was studied in NG108 cells transfected with antisense nNOS to specifically inhibit the nNOS expression using Western blotting and immunofluorescence microscopy (Fig. 5). Transient transfection with nNOS-AS-ODN effectively silenced the nNOS expression and upregulated the AT1R expression as evidenced by Western blotting and immunostaining (Fig. 5, A and B), suggesting that decreased production of NO upregulated the expression of AT1R in NG108 cell line.

Effect of Overexpression of nNOS Using Adenoviral Transfection With Ad.nNOS on Expression of AT1R in NG108 Cell Line

Conversely, to determine the effect of overexpression of nNOS on AT1R expression, nNOS was overexpressed using adenoviral gene transfection in NG108 cell line. The changes in nNOS protein and AT1R expression were analyzed by Western blotting and immunofluorescence microscopy. In
Ad.nNOS-infected cells, AT1R expression was significantly downregulated at 1 × 10^9 pfu/ml dose compared with uninfected and Ad.EGFP-infected controls (Fig. 6). Moreover, immunostaining of nNOS was increased while AT1R staining was significantly attenuated compared with uninfected and Ad.EGFP-infected controls (Fig. 7), suggesting the downregulation of AT1R expression with upregulation of nNOS expression.

Confocal Ca^{2+} Imaging in NG108 Cell Line

To evaluate the functional significance of changes in expression of AT1R, changes in [Ca^{2+}]i influx to ANG II were measured using confocal live cell imaging technique (50, 55). The treatment of ANG II (1 μM) increased the intensity of Ca^{2+} staining in all four experimental groups of cells (Figs. 8A and 9A). Quantitative analysis of cell images showed that transient transfection with nNOS-AS-ODN augmented the increase in [Ca^{2+}]i compared with MS-ODN-transfected cells (8.5 ± 0.7 vs. 21.6 ± 6.1) (Fig. 8B), suggesting a greater effect of ANG II via AT1R in antisense nNOS-transfected cells. In contrast, Ad.nNOS-infected NG108 cells demonstrated a blunted increase in [Ca^{2+}]i response to ANG II treatment compared with the Ad.EGFP-infected group of cells (5.6 ± 0.8 vs. 10.5 ± 2.6) (Fig. 9B).

Effect of SNAP and Rp-Br-cGMP on AT1R Expression in NG108 Cell Line

Most of the effects attributed to NO are mediated by the stimulation of NO-sensitive sGC and the resulting intracellular increase in cGMP. To further investigate the molecular mechanisms underlying the NO-dependent AT1R downregulation, we evaluated the potential role of the sGC/cGMP/PKG pathway and peroxynitrite radicals. NG108 cells were incubated with the NO donor SNAP alone or with the PKG inhibitor Rp-Br-cGMP. Figure 10, A and B, shows that treatment with SNAP (10^-6 mol/l) reduced AT1R mRNA levels corresponding to 44% of control cells (Fig. 10A); however, in the presence of Rp-Br-cGMP, the SNAP-induced decrease in AT1R mRNA expression levels was restored ~90% compared with control nontreated cells (Fig. 10Aii). Consistent with mRNA levels, the NO-induced decreased protein level of AT1R was also restored in the presence of PKG inhibitor Rp-Br-cGMP (Fig. 11, Ai and Aii), suggesting that the effect of SNAP was PKG dependent.

Effect of SNAP and Deforxamine on AT1R Expression in NG108 Cell Line

Furthermore, to verify whether the decreased levels of AT1R by SNAP were a consequence of nitrosylation of a sulfhydryl group of downstream effectors protein via increased peroxynitrite levels, NG108 cells were treated with SNAP in the presence or absence of peroxynitrite scavenger Def. mRNA and protein levels of AT1R were measured as well (Figs. 10Aiii and 11Aiii). The effect of SNAP on mRNA and protein expression of AT1R was not modified in the presence of Def, suggesting that the effect of SNAP is independent of peroxynitrite levels.

DISCUSSION

In the present study we observed that NO donors inhibit AT1R expression in primary cultures of the hypothalamus and neuronal cell line in a dose-dependent manner. Conversely, NO blockade leads to increased AT1R expression in these cell cultures. Manipulations of nNOS expression also induced alterations of AT1R expressions. Decreased expression of nNOS (using antisense to nNOS) leads to increased AT1R expression, while increased expression of nNOS (using Ad.nNOS) leads to decreased AT1R expression. Consistent with these observations, the functional responses of [Ca^{2+}]i influx to ANG II were potentiated with antisense treatment while overexpression with Ad.nNOS caused a blunting of the [Ca^{2+}]i influx to ANG II. Furthermore, NO-mediated inhibition of AT1R gene expression is regulated in part by increased signaling through the cGMP-dependent pathway. These data suggest that NO appears to exert an inhibitory influence in the regulation of AT1R expression via a PKG pathway in neuronal cells.

The neuroblastoma X glioma hybrid NG108-15 cell line used in the present study has been generated by Dr. B. Hamprecht’s laboratory (13). NG108 cells have neuronal properties, are immunoreactive to renin, and have angiotensin, angiotensinogen, angiotensin converting enzyme as well as AT1 and AT2 receptor subtypes (10, 24, 44). The NG108 cells also have endogenous expression of nNOS (40). Therefore, NG108 cells have been an extensively used model of cellular action of ANG II in neurons (21, 40, 53).

AT1R levels are downregulated in the hypothalamic primary culture following treatment with NO donor SNP. This downregulation most likely occurs at the level of transcription as suggested by parallel changes in AT1R mRNA level that are consistent with changes in protein expression level. Previously, it has been established that in vascular smooth muscle cells,
chronic treatment (18–24 h) with a potent NO donor, SNAP, decreases the expression of the AT1R (3, 17) while treatment with an NO synthase inhibitor [nitro-L-arginine methyl ester (l-NAME)] upregulates the expression of the AT1R in adrenal glomerulosa cells (46). This effect of NO was also associated with a regulatory mechanism at the transcriptional level because the gene transcription inhibitor actinomycin D did not affect the decrease of AT1R mRNA levels by NO (17). Different posttranslational modifications such as N-glycosylation (8) and phosphorylation (39, 45) are known to regulate the functional properties of AT1Rs. N-glycosylation is important for the maturation of AT1Rs while phosphorylation at Ser/Thr is required for the internalization of the receptors. S-nitrosylation of cysteine thiol by NO is another posttranslational modification that regulates protein functionality (41, 42). Studies by Leclerc et al. (25) showed that acute treatment (30 min) with SNP reduced the binding affinity of AT1R in HEK-293 cells without affecting total binding capacity, which provides evidence that NO regulates the binding affinity of the AT1R via S-nitrosylation, suggesting posttranslational modification.

ANG II is known to increase the cytosolic Ca2+ in sympathetic neurons (9), area postrema neurons (6), and in neurons and astrocytes of rat circumventricular organs (11). In the present study, antisense-mediated decrease in expression of nNOS potentiated the increase in [Ca2+]i influx to ANG II stimulation. Furthermore, overexpression of nNOS using nNOS adenoviral vector markedly attenuated the ANG II-induced increases in [Ca2+]i. These data provide the functional evidence of AT1R regulation via an NO mechanism, which may be important in the regulation of central neurons such as those in the PVN that have these mechanisms and are involved in the regulation of cardiovascular function.

NO is an unstable and diffusible molecule having a very short half-life. Long-term effects of NO in regulating the expression of gene occur through regulation of transcription factors (7). The regional concentration as well as availability

Fig. 7. Representative immunofluorescence photomicrographs from the NG108 cells transfected with Ad-EGFP and Ad-nNOS (1 × 10⁸ plaque-forming units/ml) and stained for nNOS (green) and AT1R (red) (A). Triple staining in control and Ad-nNOS-treated cells (B). Blue spots show the nucleus stained by 4′,6′-diamidino-2-phenylindole (DAPI).
and the nature of downstream target molecules is central for the physiological roles of NO. Low nanomolar concentrations of NO activate sGC, leading to an increase in intracellular second messenger cGMP levels (48). cGMP in turn activates downstream effectors, such as PKG, cyclic nucleotide gated channels (CNG), and cyclic nucleotide phosphodiesterases, thus regulating the activities of a number of proteins that are implicated in regulating distinct cellular and physiological processes (32). In the present study, downregulation of AT1R mRNA as well as protein levels in neuronal cell line was blocked by the specific PKG inhibitor Rp-Br-cGMP in response to SNAP treatment, indicating that activation of PKG is the required step in the regulation of AT1R gene expression by the cGMP-dependent signaling pathway. In contrast to this observation, specific inhibitor of sGC, ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinazolin-1-one), did not modify the effect of SNP on the binding affinity of the AT1R in HEK-293, cells suggesting a cGMP/PKG-independent pathway (25). NO at micromolar concentrations reacts with reactive oxygen species to form peroxynitrate radicals, which regulate functions of cellular proteins via posttranslational modifications. NO signaling in a cGMP-dependent manner or through nitrosative conformational changes regulates various transcription factors, such as cAMP response element-binding protein (CREB), c-fos, and c-Jun, and zinc-finger transcription factors, such as egr-1, and NF-κB (7). AT1 gene expression in neuronal cell line CATHa stimulated with ANG II is increased by activation of NF-κB, AP-1, c-Jun, and c-Jun amino terminal kinase (JNK) (34). In PC12 cells also, NO donors lead to increased DNA binding of AP1 complex and promote activation of c-Jun and c-fos in a cGMP/PKG-dependent pathway. A role of these transcription factors downstream of PKG in regulation of NO mediated AT1R expression remains to be determined.

Increased levels of ANG II in the circulation during disease states such as heart failure (20), hypertension (47), and diabetes (15) are known to exert effects on the CNS to increase sympathoexcitation (29). These physiological and pathological actions of ANG II are mediated through AT1R (1). Activation of AT1R instigates hypertension and myocardial infarction, two predominant conditions leading to chronic heart failure. AT1R-mediated increase in reactive oxygen species is also associated with reduced bioavailability of NO, leading to increased sympathoexcitation (49). Angiotensin converting enzyme inhibitor (19) as well as ANG II receptor blockers (29) are effective in reducing sympathoexcitation during chronic heart failure. ANG II also has more long-term or chronic actions in CNS tissues that are manifested through changes in gene and protein expression and enzyme activities (43). Various
Fig. 9. [Ca^{2+}]_i changes in response to ANG II in NG108 cells of two experimental groups (Ad.EGFP and Ad.nNOS). A: representative pictures before and after ANG II treatment. B: cumulative data represented as percent change in [Ca^{2+}]_i from the basal to 1 min of ANG II treatment in two groups of cells (n = 15–20 cells from three coverslips in each group). *P < 0.05 vs. Ad.EGFP.

Fig. 10. mRNA expression of AT_{1R} in NG108 cells treated with S-nitroso-N-acetyl pencillamine (SNAP) in the presence or absence of 8-bromoguanosine-3',5'-cyclic monophosphorothioate (Rp-Br-cGMP) or deforxamine (Def) measured by RT-PCR. A: representative gel of AT_{1R} mRNA levels. B: densitometry analyses of AT_{1R} mRNA level normalized to actin. Values are means ± SE from three independent experiments. *P < 0.05 vs. corresponding control without SNAP treatment.
kinases, including MAP kinases (ERK 1/2, JNK, p38MAPK), receptor tyrosine kinases (platelet-derived growth factor, epidermal growth factor, insulin receptor), and nonreceptor tyrosine kinases [Src, JAK/STAT, focal adhesion kinase (FAK)] were known to be involved in AT\(_1\)R mediated ANG II action (33). Because of the central role played by AT\(_1\)R in regulating the effect of ANG II, it is important to explore the mechanisms that control the expression of these receptors.

AT\(_1\)R expression has been always known to be upregulated in the CNS centers responsible for the regulation of sympathetic outflow in diseased conditions characterized by the increase sympathoexcitation such as hypertension and chronic heart failure. Previously, we have shown a decrease in expression of nNOS (28) and enhanced expression of AT\(_1\)R (52) within the PVN of rats with chronic heart failure. Decreased levels of NO were also proposed to potentiate the ANG II to signal to increase the sympathetic activity in conscious rabbits (31). In the present study, nNOS-AS-ODN transfection in neuronal cell line significantly increased \([\text{Ca}^{2+}]_i\) in response to ANG II, suggesting that decreased expression of nNOS leads to increased expression of AT\(_1\)R expression. These results demonstrate an important modulating effect of NO on AT\(_1\)R expression at the cellular level, suggesting a possible mechanism for the increase in AT\(_1\)R and subsequent increase in sympathetic outflow in disease states viz. chronic heart failure and hypertension. It is of interest to note that angiotensin-(1–7) increases nNOS-derived NO which influences neuronal potassium current to inhibit neuronal excitation (50). In the present study, nNOS-AS-ODN transfection in neuronal cell line significantly increased \([\text{Ca}^{2+}]_i\) in response to SNAP treatment, suggesting that activation of PKG is the required step in the regulation of AT\(_1\)R gene expression by the cGMP-dependent signaling pathway. Moreover, \([\text{Ca}^{2+}]_i\) changes in response to ANG II are more pronounced with antisense nNOS treatment while ameliorated with nNOS upregulation using Ad.nNOS in NG108 cells.

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**Fig. 11.** Protein expression of AT\(_1\)R in NG108 cells treated with SNAP in the presence or absence of Rp-Br-cGMP or Def measured by Western blotting. A: representative gel of AT\(_1\)R protein levels. B: densitometry analyses of AT\(_1\)R protein levels normalized to tubulin. Values are means ± SE from three independent experiments. *\(P < 0.05\) vs. corresponding control without SNAP treatment.

**Fig. 12.** Schematic diagram illustrating intracellular signaling cascade that may mediate feedback inhibition of AT\(_1\)R expression via inhibition of protein kinase G (PKG) pathway. Nitric oxide (NO) stimulates the soluble guanylate cyclase (sGC) to generate the second messenger 3,5' guanosine monophosphate (cGMP) from guanosine triphosphate (GTP). The soluble cGMP activates cyclic nucleotide-dependent PKG, which leads to phosphorylation on PKG consensus sites of target proteins affecting gene transcription. On the other hand, superoxide generated by NADPH oxidase reacts with NO to form peroxynitrite radicals, which affect the expression of target genes via post-translational modifications viz. nitration or cysteinylation. In the present study, the PKG inhibitor Rp-Br-cGMP downregulates the AT\(_1\)R mRNA as well as protein levels while the peroxynitrite scavenger deforxamine (DFO) has no effect in neuronal cell line in response to SNAP treatment, suggesting that activation of PKG is the required step in the regulation of AT\(_1\)R gene expression by the cGMP-dependent signaling pathway. Moreover, \([\text{Ca}^{2+}]_i\) changes in response to ANG II are more pronounced with antisense nNOS treatment while ameliorated with nNOS upregulation using Ad.nNOS in NG108 cells.
therapeutic target in some disease conditions such as hypertension and heart failure.

In conclusion, the evidence presented here suggests that NO utilizes a PKG-dependent mechanism to downregulate the expression of AT1R in neurons (Fig. 12). This NO-mediated decrease in AT1R expression via the PKG pathway in neurons may provide the basis for the development of new therapeutic agents with enhanced specificity for the treatment of complications in the levels of nNOS and AT1R associated with pathophysiological conditions such as chronic heart failure and hypertension. Further studies are required to clarify the detailed mechanism of AT1R downregulation by NO and the possible relationship with nNOS/NO signaling.

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AUTHOR CONTRIBUTIONS
N.M.S., H.Z., and Y.-F.L. performed the experiments; N.M.S., H.Z., Y.-F.L., and K.P.P. analyzed the data; N.M.S., H.Z., Y.-F.L., and K.P.P. drafted the manuscript; N.M.S., H.Z., Y.-F.L., and K.P.P. edited and revised the manuscript; N.M.S., H.Z., Y.-F.L., and K.P.P. approved the final version of the manuscript; Y.-F.L. and K.P.P. conception and design of the research.

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