Noradrenergic Cell Specific Gene Transfer With Neuronal Nitric Oxide Synthase Reduces Cardiac Sympathetic Neurotransmission in Hypertensive Rats

Dan Li, Lijun Wang, Chee-Wan Lee, Tom A. Dawson, David J. Paterson

Abstract—Nitric oxide–cGMP pathway can inhibit cardiac norepinephrine (NE) release. Sympathetic hyperresponsiveness in hypertension may result from oxidative stress impairing this pathway. We tested the hypothesis that the gene transfer of neuronal NO synthase (nNOS) could restore sympathetic balance in the spontaneously hypertensive rat (SHR). An adenovirus (5×10^10 particles) constructed with a noradrenergic neuron-specific promoter (PRS ×8) encoding nNOS (Ad.PRS-nNOS) or enhanced green fluorescence protein (Ad.PRS-eGFP) was targeted to the right atrial wall by percutaneous injection in age-matched male SHRs and Wistar-Kyoto (WKY) rats. Five days after transduction, right atria were removed, and evoked [3H] norephinephrine (NE) release, NO activity, and cGMP were measured. In the Ad.PRS-eGFP treated group, tissue levels of cGMP were significantly lower in the SHR compared with the WKY atria. NE release was also greater in the SHR, and soluble guanylate cyclase inhibition did not alter evoked [3H] NE release in the Ad.PRS-eGFP–treated SHR. All atria treated with Ad.PRS-nNOS had enhanced NO activity when compared with Ad.PRS-eGFP atria. Ad.PRS-nNOS in WKY rats reduced NE release compared with the Ad.PRS-eGFP group. Guanylate cyclase inhibition enhanced NE release in both Ad.PRS-nNOS– and Ad.PRS-eGFP–treated WKY atria. Ad.PRS-nNOS restored cGMP levels in the SHR to those seen in the WKY atria. In the SHR, Ad.PRS-nNOS also attenuated NE release compared with Ad.PRS-eGFP group. This was reversed by guanylate cyclase inhibition. We conclude that artificial upregulation of sympathetic nNOS via gene transfer with a noradrenergic promoter may provide a novel approach for correcting peripheral sympathetic hyperactivity in hypertension. (Hypertension. 2007;50:1-6.)

Key Words: nitric oxide gene transfer autonomic nervous system norepinephrine hypertension cyclic GMP

In hypertension, cardiac autonomic impairment is an important contributor to the pathophysiological phenotype of the disease. Much evidence supports the observation that the activity of the sympathetic nervous system increases dramatically both in hypertensive patients and in the spontaneously hypertensive rat (SHR). The mechanism causing enhanced neurohumoral activation is unknown, but may be directly related to oxidative stress caused by hypertension disrupting nitric oxide (NO)–cGMP modulation of norepinephrine (NE) release in central autonomic nuclei and peripheral varicosities. Neuronal production of NO affects cardiac excitability through its action as a neuromodulator of postganglionic sympathovagal transmission. Both endogenous and exogenous NO inhibits evoked NE release during cardiac sympathetic nerve stimulation in the rat in vitro. Functionally, NO or the membrane-permeable cGMP analogue 8-bromo-cGMP decreases the heart rate response to sympathetic nerve stimulation in vitro, whereas NO synthase (NOS) inhibitors causes the opposite effect. In addition, oxidative stress caused by intermittent hypoxia decreases cardiac sympathetic neuronal NOS (nNOS) expression, which is associated with an enhanced heart rate response to sympathetic activation. Moreover, soluble guanylate cyclase (sGC), the key precursor of cGMP-dependent effects of NO, is downregulated in the aorta and the atria of the SHR. Tetrahydrobiopterin (BH₄) and total biopterin levels are not different between SHR and Wistar-Kyoto (WKY) rat atria where sympathetic nerves innervate the heart, suggesting that BH₄ is not uncoupled from NOS. Therefore strategies aimed at increasing bioavailability of NO could be advantageous in rescuing this impaired neural phenotype.

Gene transfer with adenoviral vectors is highly effective in modifying gene expression and has been used with good effect to understand whether changes in protein expression or enzyme activity play a key role in physiological function. Because adenoviruses transduce a broad range of cells, the gene of interest can be placed into cells that may not constitutively express them, thereby complicating the interpretation of the data. To minimize this limitation, we have recently developed a novel adenoviral vector constructed...
with a noradrenergic neuron-specific promoter (PRS ×8) to drive nOES or enhanced green fluorescence protein (eGFP) expression, and showed that nOES targeted to only cardiac sympathetic neurons can inhibit noradrenergic neurotransmission and increase nOES activity in normotensive rats.19

We therefore examined the hypothesis that a significant component of cardiac sympathetic hyper-responsiveness in hypertension occurs at the end organ level because of impaired NO-cyclic nucleotide signaling. From this we further hypothesized that gene transfer with a noradrenergic cell specific promoter coupled to nOES could increase the bioactivity of nOES and the production of cGMP that results in restoration of sympathetic transmission in the SHR to similar responses seen in the WKY rat.

**Materials and Methods**

An extended methods and results section can be found in the online data supplement available at http://hyper.ahajournals.org.

**Animals**

Age-matched (16 to 20 weeks old) male SHR (n = 28) and WKY rats (n = 28), young Sprague-Dawley (SD) rats (8 to 13 days old; n = 6) were purchased from Harlan (Bicester, UK) and housed under standard laboratory conditions. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the Animals (Scientific Procedures) Act 1986 (UK) and were performed under British Home Office license requirements (PPL 30/2130).

**Gene Transfer Procedure**

Replication-deficient adenoviral vectors encoding recombinant eGFP or nOES under control of the noradrenergic neuron-specific promoter (PRS ×8) were generated as described previously.19 Targeted percutaneous gene transfer to the right atrium was performed under isoflurane (Isocare, Animalcare Ltd) anesthesia (4% for induction and 2% to 3% for maintenance, in 100% O2), using a technique similar to that described previously for the guinea pig.9 SHR or WKY rats received an injection of 5×109 particles of Ad.PRS-nOES or Ad.PRS-eGFP in 300 μL of phosphate-buffered saline. Experiments were performed ~5 days after injection.

**[3H]Norepinephrine Release Experiments on the Isolated Right Atrium**

An isolated right atrium preparation was generated by the method described previously20 with some modification. Details and experimental protocols may be found in the online data supplement.

**Immunohistochemistry**

Cardiac sympathetic neuron isolation and transduction was prepared by the method described previously.19 Detailed methods for immunohistochemistry may be found in the online data supplement.

**Measurement of Tissue cGMP Levels and NOS Activity**

Details may be found in the online data supplement.

**Statistical Analysis**

The results are given as the means±SEM. Analysis was performed using the paired or unpaired Student t test as appropriate. For all experiments, statistical significance was accepted at P<0.05.

**Results**

**Norepinephrine Release Was Increased in the SHR**

Field stimulation-evoked release of [3H] NE from right atria was significantly enhanced at 5-Hz in the SHR (S1: +2.22±0.13%, S2: +2.19±0.14%, n = 5) compared with the WKY rats (S1: +1.68±0.04%, S2: +1.55±0.04, n = 6, P<0.01, unpaired t test; Figure 1). S1 and S2 represent the...
first and second field stimulation, respectively, where there was no difference between S1 and S2 in both strains indicating no significant time dependent changes.

Effects of SNP and ODQ on Release of Norepinephrine in Field Stimulation

To determine whether the sympathetic activated NO–cGMP pathway was impaired in the SHR, the exogenous NO donor SNP and the sGC inhibitor ODQ were applied to preparations. SNP (20 \( \mu \)mol/L) significantly decreased the release of \[^{3}H\] NE in response to 5-Hz field stimulation in the WKY (from 1.63\%±0.09\% to 1.28\%±0.10\%, \( n=6 \), \( P<0.01 \), paired \( t \) test, Figure 2), whereas \[^{3}H\] NE release from SHR atria (\( n=6 \)) was unaffected (\( n=6 \); \( P<0.05 \), WKY vs SHR, unpaired \( t \) test). Conversely, ODQ (10 \( \mu \)mol/L) enhanced the release of \[^{3}H\] NE in the WKY (from 1.60\%±0.07\% to 1.82\%±0.11\%, \( n=5 \), \( P<0.01 \), paired \( t \) test; Figure 3), but did not affect release in the SHR (\( n=6 \)).
Production of cGMP in Atria After Ad.PRS-nNOS
We observed cGMP concentration was significantly reduced in SHR compared with WKY tissue both in untreated tissue (3.57±0.11 versus 4.50±0.36 pmol/mg protein, *P<0.05, unpaired t test, n=6 in each strain) and after gene transfer with Ad.PRS-eGFP (3.70±0.11 versus 4.37±0.17 pmol/mg protein, †P<0.05, unpaired t test, n=6 in each strain; Figure 6). However, overexpression of Ad.PRS-nNOS reversed the attenuated production of cGMP in SHR (5.06±0.52 pmol/mg protein, n=6, †P<0.05, unpaired t test) compared with untreated or Ad.PRS-eGFP–treated SHR.

Effects of Gene Transfer With Ad.PRS-nNOS on Release of Norepinephrine
Previously, we showed that the Ad.PRS-nNOS gene transfer significantly reduced norepinephrine release evoked by field stimulation of isolated right atria in the normotensive SD rat.19 In the present study, we observed similar results in the WKY rats (Figure 7A). Ad.PRS-nNOS (1.49±0.05%, n=6) caused 16.1±3.1% reduction in the evoked NE release compared with Ad.PRS-eGFP–treated tissue (1.77±0.02%, n=5, *P<0.01, unpaired t test). sGC inhibitor ODQ (10 μmol/L) also significantly enhanced the sympathetic responsiveness in the WKY rats treated with either the Ad.PRS-nNOS (1.67±0.06%) or Ad.PRS-eGFP–treated tissues (2.09±0.08%) (*P<0.01 respectively, unpaired t test). In the SHR (Figure 7B), overexpression of Ad.PRS-nNOS (1.59±0.08%, n=6) significantly decreased the [3H] NE release by 21.2±4.0% compared with Ad.PRS-eGFP (2.02±0.17%, n=5, †P<0.05, unpaired t test). There was no effect of ODQ on Ad.PRS-eGFP–treated SHR atria. However, ODQ significantly increased the NE release from Ad.PRS-nNOS–transfected SHR atria (1.89±0.11%, n=6, †P<0.05, paired t test).

Discussion
In the present study, we report 3 novel findings. First, a significant component of the sympathetic hyper-responsiveness in the SHR occurs postganglionically at the end organ level with enhanced evoked norepinephrine release. Secondly, this response was associated with impaired sGC-cGMP signaling in the SHR compared with the WKY rat. Thirdly, targeted gene transfer of nNOS with a noradrenergic cell specific promoter increased the bioactivity of nNOS and cGMP levels in cardiac sympathetic nerves. This resulted in decreased NE release in the SHR to levels seen in the normotensive control.

Peripheral Sympathetic Activity in the SHR
Autonomic imbalance in the central and peripheral nervous system has been implicated in the etiology of hypertension, and is also seen after myocardial infarction and heart failure.21-24

Impaired cardiac parasympathetic regulation and enhanced sympathetic activity characterize cardiac disease states, and are regarded as an independent predictor of mortality.25-26 Bradycardia and acetylcholine release in response to vagal nerve stimulation is reduced in the SHR at the level of the cardiac postganglionic neurons.27 Here we observed that the norepinephrine release from right atria in response to field stimulation in the SHR was significantly higher compared with the WKY rat. This gives direct evidence that the sympathetic nervous system is hyper-reactive in the SHR at the level of heart, and is in general agreement with whole animal studies that report a global sympathetic phenotype in this model of hypertension.28,29

NO–cGMP Signaling in the SHR
Defective NOS signaling via eNOS has been widely implicated in the vascular aspect of hypertension, although cumu-
ative evidence now suggests that neuronally synthesized NO may also play a role in the pathophysiology of hypertension.\textsuperscript{30} The signaling pathway responsible for nNOS-derived NO inhibiting sympathetic neurotransmission is not completely understood. However, it could involve NO modifying cell physiology through activation of sGC and subsequent induction of cGMP production, which in turn activates cGMP-dependent protein kinase and phosphodiesterases that decrease cAMP-dependent phosphorylation of neuronal Ca\textsuperscript{2+} channels resulting in decreased exocytosis of neurotransmitter.\textsuperscript{31}

Emerging data indicates that sGC, the main target protein for NO, is markedly desensitized/downregulated in hypertension.\textsuperscript{14,32–35} In particular, downregulation of the α\textsubscript{1} subunit of sGC in the atria and aorta of the SHR compared with WKY rat has recently been reported.\textsuperscript{15} We observed that guanylate cyclase inhibition increased NE release in the WKY but was without effect in the SHR, suggesting functional uncoupling of NO to its second messenger. Consistent with this idea we confirmed that tissue levels of cGMP were significantly lower in the SHR compared with the WKY. Transiently, decreased sGC expression and cGMP production has been proposed to account for impaired vasodilatation in the Afro-Caribbean population\textsuperscript{36} (susceptible to hypertension), fetal programming of hypertension,\textsuperscript{37} and in pulmonary hypertension.\textsuperscript{38} Similarly, downregulation of the sGC-dependent pathway\textsuperscript{34,35} has been demonstrated in aged and spontaneously hypertensive rats where superoxide production has been reported to trigger desensitization of vascular sGC in hypertension.\textsuperscript{34} Because nNOS activity and tetrahydrobiopterin levels in the SHR are not different compared with the WKY, this suggests uncoupling of nNOS from its main cofactor is unlikely to be the mechanism of peripheral sympathetic dysfunction in the SHR, and that the defect resides at the level of sGC-cGMP linkage.

**Effect of Noradrenergic Neuron-Specific Overexpression of nNOS on Sympathetic Function in the SHR**

Administration of the NO donor SNP did not decrease NE release in the SHR. It is possible that acute pharmacological administration of NO donors at a relatively low concentration (20 μmol/L) may not fully mimic the action of NO generated intracellularly by the nNOS transgene, given its labile and highly reactive properties. In particular, a relatively high local concentration of NO may be required to increase the bioavailability of NO under conditions of increased oxidative stress that have been demonstrated previously in hypertension.\textsuperscript{39,40} In addition, a much longer period of incubation may be needed to achieve the effect than that can be provided in a stable in vitro experimental environment. However, overexpression of Ad.PRS-nNOS in the SHR significantly decreased the [3H] NE release compared with Ad.PRS-eGFP, and the sGC inhibitor ODQ significantly increased the NE release from Ad.PRS-nNOS-transduced SHR atria. The noradrenergic promoter is highly specific with no detectable leakage of gene expression into other cell types.\textsuperscript{19} Transduction with Ad.PRS-nNOS increased nNOS activity in the atrial extracts and enhanced atrial cGMP levels compared with Ad.PRS-eGFP.

Importantly, gene transfer of adenovirus nNOS can also increase the expression of the defective α\textsubscript{1} subunit of sGC in the SHR,\textsuperscript{27} which appears to be the key step for the functional changes. The mechanism underpinning this observation is not known, but when results are taken together they show that nNOS gene transfer provided a more potent NO signal than the NO donor.

**Perspectives**

Enhanced presynaptic postganglionic release of NE is not the only site of defective autonomic signaling in hypertension. nNOS gene transfer into the paraventricular nucleus can also decrease sympathetic outflow in the rat.\textsuperscript{41} In addition, our group\textsuperscript{15} recently found that the atria of the SHR were hyper-responsive to β-adrenergic stimulation which was associated with elevated cAMP levels. This was accompanied by an increased basal and norepinephrine-stimulated L-type calcium current (I\textsubscript{Ca,L}) in single sinoatrial node cells. Gene transfer of nNOS decreased cAMP and the norepinephrine stimulated I\textsubscript{Ca,L} in the SHR. Combined with the present study, these results support the role of nNOS-sGC-cGMP in modulating peripheral noradrenergic activity at both presynaptic and postsynaptic levels, and that gene transfer of nNOS into either neurons or into pacemaking tissue can rescue the peripheral sympathetic hyperactivity seen in the SHR. Given the autocrine and paracrine action of NO in the heart, targeting both myocytes and sympathetic neurons with nNOS could be beneficial where this gaseous messenger may behave like a “smart beta blocker” that decreases overall adrenergic drive at these 2 sites. Whether this approach is viable to treat hyper-responsive sympathetic and beta adrenergic phenotypes remains to be established.

**Acknowledgment**

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**Disclosures**

None.

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**Methods**

**[^3]H]Norepinephrine release experiments on the isolated right atrium**

**Experiment set up**

SHRs and WKY rats were used. An isolated right atrium preparation was generated by the method described previously\(^1\) with some modification. Briefly, animals were euthanatized with intraperitoneal injection of pentobarbitone. The right atria were removed and transferred to preheated organ bath containing Tyrode’s solution. After a 45 minute equilibration, the atrium was incubated with 5 \(\mu\)Ci l-[7,8-\[^3\]H]Norepinephrine (Amersham UK) for 30 min with field stimulations at 5Hz for 10 seconds every 30 seconds to incorporate the \[^3\]H NE into the sympathetic transmitter stores. Excess \[^3\]H NE was washed from the preparation by superfusing for 60 minutes at a rate of 3 ml/min with Tyrode’s solution. Following the wash period, superfusion was stopped and the bath solution was collected by replacing 3 ml Tyrode’s solution every 3 minutes.

**Experimental protocols**

Experiments were carried out by using three groups of atria to define neurotransmitter release (Fig. S1). Control cycles (30 min) were followed by using the NO donor sodium nitroprusside (SNP, 20 \(\mu\)mol/L, group 2; see Fig. 2 and Fig. S2) or the soluble guanylate cyclase (sGC) inhibitor 1H-(1,2,4)oxadiazolo(4,3-a)quinoxaline-1-one (ODQ, 10 \(\mu\)mol/L, group 3; see Figs. 3, 7 and Fig. S3). Time control experiments (group 1; see Fig. 1) lasted 99 min. The atrium was stimulated at 5Hz for 1 minute at the beginning of the 16\(^{th}\) (S1) and 76\(^{th}\) (S2) minutes (in group 2, stimulated at the 46\(^{th}\) (S2) minutes). At the end of the experiment, the atrium was immersed overnight in 3 ml Tyrode’s solution containing 4 U/ml papain (Sigma) and the radioactivity contained in the extract was determined.
Measurement of radioactive content

A 0.5 ml aliquot of each collected fraction was transferred to scintillation vials containing 4.5 ml of scintillation fluid (Ecoscint A, National Diagnostics) and the amount of radioactivity in each sample (disintegrations per minute) was measured using a liquid scintillation counter (Tri-carb 2800TR, Packard). $[^{3}H]$ NE outflow was expressed as a ratio of NE radioactivity at a particular time point over the total radioactivity. Total radioactivity is the sum of NE radioactivity at all time points and the remaining radioactivity in the atrium. Percentage changes in NE release was expressed as a ratio of increase in NE radioactivity following electrical stimulation over the total radioactivity.

Immunohistochemistry

Cardiac sympathetic neuron isolation and transduction was prepared by the method described previously. 2 8-13 days SD rats were used. The cells were infected with 0.5-1×10$^9$ adenoviral particles (VP) per well. Cultured primary neurons were fixed with 4% Paraformaldehyde and permeabilized with 0.1% Triton X100 and 1% BSA. Cells were then processed for immunoreactivity with mouse anti tyrosine hydroxylase (TH), 1:200 (Sigma) or rabbit anti mouse nNOS, 1:200 (Invitrogen). Fixed cells were incubated sequentially with 10% normal horse serum, primary antibody and biotinylated secondary antibody. Finally, the immunofluorescent signals were detected by Texas Red Streptavidin (SA-5006, Vector Labs) and Fluorescein Streptavidin (SA-5001, Vector Labs) for TH and nNOS detection respectively.

Measurement of tissue cGMP levels

Atria were frozen in liquid nitrogen for subsequent analysis of atrial cGMP using a radioimmunoassay kit. Tissues were minced in 800 μL ice-cold trichloroacetic acid (6%) and homogenized at 4°C. The homogenates were centrifuged at 2000g for 10 minutes at 4°C, and
the supernatant was extracted with water-saturated ether 3 times and then dried using a SpeedVac concentrator (Savant). The dried samples were resuspended and a $^{125}\text{I}$-cGMP kit (Amersham UK) was used to measure the amount of cGMP level, with the bound form separated from the free form using magnetic separation. The pellet was treated with 500 µL NaOH (1N) and used for protein quantification.

**Measurement NOS activity**

NOS activity in atria was quantified by measuring the conversion of $[^3\text{H}]$-L-arginine to $[^3\text{H}]$-L-citrulline using a modified procedure as previously described.\(^3\),\(^4\) A specific eNOS inhibitor (L-N\(^5\)-(1-Iminoethyl) ornithine, Dihydrochloride, Calbiochem Ltd.) was added to the assay buffer at a concentration of 10 µg/ assay. Radioactivity was quantified by liquid scintillation counting. The results are expressed in fmol citrulline /mg protein /min.

**References**


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**Figure S1:**
Protocols for $[^3]$HNorepinephrine release experiments. Diagonal bars show the period of sample collections to be compared with control or each other. For time control, corresponding period was compared. S1 and S2 respectively represent the first and second field stimulation (5Hz, 1 minute).

**Figure S2:**
Raw data trace showing effect of the NO donor, SNP (20 µmol/L) on $[^3]$H NE release during 5 Hz field stimulation in WKY (A) and SHR (B).

**Figure S3:**
Raw data traces showing the effect of sGC inhibition, (ODQ; 10 µmol/L) on $[^3]$H NE release in response to 5 Hz field stimulation in WKY (A) and SHR (B).
**Table S1.** Raw data of percentage changes of $[^3]$HNE release from isolated atria in response to 5Hz field stimulation in WKY and SHR. S1 and S2 represent the first and second stimulation.

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Table S2. Effect of SNP (20 µmol/L) and ODQ (10 µmol/L) on percentage changes of [³H]NE release during 5Hz field stimulation in WKY and SHR.

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Means ± SE.
**Table S3.** Effect of ODQ (10 µmol/L) on percentage changes of $[^3]$H]NE release during 5Hz field stimulation in gene transferred WKY and SHR.

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<td>± 0.080</td>
<td>± 0.056</td>
<td>± 0.066</td>
<td>± 0.174</td>
<td>± 0.137</td>
<td>± 0.080</td>
<td>± 0.118</td>
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Fig. S1

Time (minute)

0  15  30  45  60  75  90

Group 1
(Fig.1)
WKY (n=6)
SHR (n=5)

Group 2
(Fig.2 & Fig.S2)
WKY (n=6)
SHR (n=6)

Group 3
(Figs.3 & 7 & Fig.S3)
WKY (n=5)
WKY+Ad.PRS-eGFP (n=5)
WKY+Ad.PRS-nNOS (n=6)
WKY+Ad.PRS-nNOS (n=5)
SHR+Ad.PRS-eGFP (n=5)
SHR+Ad.PRS-nNOS (n=6)
Fig. S2

A. WKY

- Control
- SNP (20 µmol/L)

B. SHR

% Total NE Release % Total NE Release

Time (min)
Fig. S3

A  WKY

- Control
- ODQ (10 µmol/L)

B  SHR

% Total NE Release

Time (min)

% Total NE Release

0 15 30 45 60 75 90

0 1 2 3 4 5