Oxidative stress attenuates NO-induced modulation of sympathetic neurotransmission in the mesenteric arterial bed of spontaneously hypertensive rats

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Macarthur H, Westfall TC, Wilken GH. Oxidative stress attenuates NO-induced modulation of sympathetic neurotransmission in the mesenteric arterial bed of spontaneously hypertensive rats. Am J Physiol Heart Circ Physiol 294: H183–H189, 2008. First published October 26, 2007; doi:10.1152/ajpheart.01040.2007.—Current evidence suggests that hyperactivity of the sympathetic nervous system and endothelial dysfunction are important factors in the development and maintenance of hypertension. Under normal conditions the endothelial mediator nitric oxide (NO) negatively modulates the activity of the norepinephrine portion of sympathetic neurotransmission, thereby placing a “brake” on the vasoconstrictor activity of this transmitter. This property of NO is diminished in the isolated, perfused mesenteric arterial bed taken from the spontaneously hypertensive rat (SHR), resulting in greater nerve-stimulated norepinephrine and lower neuropeptide Y (NPY) overflow from this mesenteric preparation compared with that of the normotensive Wistar-Kyoto rat (WKY). We hypothesized that increased oxidative stress in the SHR contributes to the dysfunction in the NO modulation of sympathetic neurotransmission. Here we demonstrate that the antioxidant N-acetylcysteine reduced norepinephrine and increased NPY overflow in the mesenteric arterial bed taken from the SHR. Furthermore, this property of N-acetylcysteine was prevented by inhibiting nitric oxide synthase with N\(^{-}\)\(\text{G}\)-nitro-L-arginine methyl ester, demonstrating that the effect of N-acetylcysteine was due to the preservation of NO from oxidation. Despite a reduction in norepinephrine overflow, the nerve-stimulated perfusion pressure response in the SHR mesenteric bed was not altered by the inclusion of N-acetylcysteine. Studies including the Y\(_1\) antagonist BIBO 3304 with N-acetylcysteine demonstrated that this preservation of the perfusion pressure response was due to elevated NPY overflow. These results demonstrate that the reduction in the bioavailability of NO as a result of elevated oxidative stress contributes to the increase in norepinephrine overflow from the SHR mesenteric sympathetic neuroeffector junction.

antioxidant; hypertension; nitric oxide; norepinephrine; neuropeptide Y

SYMPATHETIC NERVES SYNTHESIZE and release the neurotransmitters norepinephrine, neuropeptide Y (NPY), and adenosine triphosphate (ATP) (30, 34, 37, 57). ATP mediates the rapid phase, norepinephrine the intermediate phase, and NPY the long-lasting phase of sympathetic nerve stimulation-induced vasoconstriction (24, 30, 43, 54). The postjunctional vasoconstrictive effects of norepinephrine, NPY, and ATP on blood vessels are mediated through vascular smooth muscle \(\alpha_1\), \(\gamma_1\), and \(\gamma_2\) receptors, respectively. In addition, norepinephrine, NPY, and ATP have prejunctional inhibitory actions on sympathetic neurotransmission through \(\alpha_2\), \(\gamma_2\), and \(\gamma_2\) receptors, respectively, that result in the negative regulation of their own release as well as the release of another (30, 43, 54, 55).

In addition to the sympathetic nervous system (SNS), the endothelium of the vasculature is also an extremely important component of vascular control. Among the many mediators released by the endothelium, one of the most important is the powerful vasodilator nitric oxide (NO). In addition to its direct effects on the vasculature, NO also modulates sympathetic neurotransmission (4, 8, 18, 51, 58). We have shown (27) that one mechanism by which NO modulates sympathetic neurotransmission is deactivation of a portion of the norepinephrine in the neuroeffector junction, thus placing a “brake” on both the post- and prejunctional activity of norepinephrine. An indirect consequence of this property of NO is a limit on the negative regulation of sympathetic cotransmission by norepinephrine. Indeed, we have shown (26, 27) that NPY release from sympathetic nerves is greater as a result of the deactivation of norepinephrine by NO. Together these findings demonstrate the importance of NO as a physiological modulator of the SNS.

The events that lead to the development of hypertension are still unclear, but increased activity of the SNS and development of endothelial dysfunction have both been implicated. There is considerable evidence for hyperactivity of the SNS in contributing to the initiation and maintenance of hypertension in a significant proportion of the hypertensive population (1, 12–14, 17, 40) as well as in various experimental models such as the spontaneously hypertensive rat (SHR) (21, 25, 46, 55). However, the precise causal mechanisms leading to increased sympathetic activity are still poorly understood.

Increased oxidative stress, evident in hypertension (3, 10, 20, 62), may be a factor in hyperactivity of the SNS since oxidative stress leads to the loss of the bioavailability of NO as a result of a reaction with superoxide anion (\(O_2^-\)) (31, 36, 49). This loss of NO would lead to an increase in the amount of norepinephrine within the neuroeffector junction and may explain our previous observations (26) of compromised NO modulation of sympathetic neurotransmission in the young (10–12 wk old) SHR. In this study we investigated the involvement of oxidative stress in changes observed in NO-mediated modulation of sympathetic neurotransmission in the SHR. As mentioned above, we have demonstrated (27) that by deactivating norepinephrine endogenous NO modulates sympathetic neurotransmission at the vascular neuroeffector junction of the rat mesenteric arterial bed. We further showed (26)

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that this modulation is compromised in mesenteric preparations taken from 10- to 12-wk-old SHR, contributing to the increased nerve-stimulated overflow of norepinephrine and concomitant decrease in NPY overflow observed in SHR compared with normotensive Wistar-Kyoto rat (WKY) controls. To examine the possible involvement of oxidative stress in the changes observed in the modulation of sympathetic neurotransmission in the SHR, we carried out experiments utilizing the antioxidant N-acetylcysteine. Our rationale was that if O$_2^*$ has increased in the vascular smooth muscle of the SHR as has been reported (19, 44, 60) then the addition of an antioxidant should protect NO from deactivation by O$_2^*$ and this should be reflected in a transition toward normal levels in the nerve-stimulated induced overflow of sympathetic neurotransmitters. Our results confirm that the hyperactivity of the SNS in hypertension may be driven, in part, by oxidative stress.

**MATERIALS AND METHODS**

**Materials.** N-acetylcysteine, N-nitro-l-arginine methyl ester (l-NAME), and Krebs buffer salts were all purchased from Sigma (St. Louis, MO). BIBO 3304 was a kind gift from Boehringer Ingelheim (Biberach, Germany). The NPY enzyme immunoassay (EIA) kit was purchased from Peninsula Laboratories (San Carlos, CA).

**Animals.** All procedures were carried out in accordance with National Institute of Health guidelines and were approved by the Institutional Animal Care and Use committee of Saint Louis University Health Sciences Center. Experiments were performed on 10- to 12-wk-old male WKY and SHR obtained from Harlan (Indianapolis, IN). Animals were housed two to four per cage in a constant-temperature 12:12-h light-dark cycle room.

**Isolated, perfused mesenteric preparation of the rat: surgery.** Rats were anesthetized with an intraperitoneal injection of pentobarbital sodium at a dose of 50 mg/kg. The abdomen was opened, and the associated intestine was excised by ligations of the descending colon proximal to the rectum, the duodenum distal to the stomach, and the superior mesenteric artery distal to the abdominal aorta. The superior mesenteric artery was cannulated with polyethylene-90 tubing, and heparinized saline was flushed through the mesenteric vascular bed. The four main branches of the mesenteric artery feeding the terminal ileum were located, and all remaining branches were ligated and severed. The remaining intestine was then separated from the vascular bed along the intestinal wall. The dissected vascular bed was placed in an organ bath maintained at 37°C and perfused and superfused with a modified Krebs buffer by a Gilson minipulse pump at rates of 2 ml/min and 0.5 ml/min, respectively. The Krebs buffer was composed of (in mM) 120 NaCl, 5.0 KCl, 1.2 MgSO$_4$, 2.4 CaCl$_2$, 0.027 EDTA, 11.1 glucose, and 25 NaHCO$_3$. The buffer was maintained at 37°C and aerated constantly with 95% O$_2$-5% CO$_2$. Nerve stimulation was allowed to equilibrate for 45 min before experimentation began. All drugs used were dissolved in the Krebs buffer and delivered by continuous infusion.

**Isolated, perfused mesenteric preparation of the rat: periaxial nerve stimulation.** Platinum ring electrodes were placed around the nerve, and the periaxial nerves were stimulated at 12 Hz for 30 s (for norepinephrine) or 90 s (for NPY) with a Grass S-88 stimulator. Perfusion from the bed was collected continuously in 1-min fractions by means of a fraction collector either into 1 ml of cold 0.1 N perchloric acid with 0.1% cysteine (for catecholamine studies) or into 1 ml of cold 0.1% trifluoroacetic acid (for NPY studies).

**Catecholamine measurements.** Norepinephrine in the mesenteric perfusate samples was identified and quantified by high-pressure liquid chromatography with electrochemical detection (HPLC-EC) as previously published (26, 27). The system consists of a Varian Pro-Star solvent delivery system and a model 9090 autosampler (Varian, Walnut Creek, CA) coupled to a C$_{18}$ column and an ESA Coulochem II detector. Separations were performed isocratically with a filtered and degassed mobile phase consisting of 12% methanol, 0.1 M sodium phosphate, 0.2 mM octyl sulfate, and 0.1 mM EDTA, adjusted to pH 2.8 with phosphoric acid. The HPLC system was coupled to a computer with which chromatograms were recorded and analyzed with Varian Star workstation software.

**NPY measurements.** NPY in the perfusate samples was purified by use of C$_{18}$ Sep-Pak columns and measured with a 96-well plate enzyme immunoassay kit (Peninsula Laboratories). The 96-well plate was read by a Powerwave X plate reader (Biotek Instruments, Winooski, VT), and the calculation of sample value was analyzed by KC Junior Software (Biotek Instruments).

**Lipid hydroperoxide levels.** Lipid hydroperoxide (LPO) formation was assessed in aortic tissue extractions by the use of a specific EIA kit (Cayman Chemical). Results were normalized to wet tissue weight for each tissue sample.

**Statistical analysis of data.** Data are expressed as means ± SE. Statistical analyses were carried out by Student’s t-test or by one-way analysis of variance followed by Newman-Keuls multiple-comparison tests. Statistical differences were accepted when $P < 0.05$.

**RESULTS**

All our experiments were carried out on mesenteric preparations taken from 10- to 12-wk-old SHR and compared with age-matched normotensive WKY genetic controls. This age group represents a young hypertensive animal, i.e., one in which hypertension is still developing. SHR have been extensively studied, and it is well established that these animals develop hypertension as they age. From 4 to 6 wk the blood pressure of the SHR is, for the most part, comparable to that of the WKY, although some reports indicate that there are already significant differences between the two strains (28, 32, 39, 48, 56). The blood pressure of SHR then begins to rise sharply until the rats are 10–12 wk old. The increase in blood pressure of SHR then slows and around 18–20 wk has stabilized (59). The SHR used in our experiments exhibited mean arterial pressures of 130 ± 4 mmHg, significantly greater than 88 ± 2 mmHg for WKY ($n = 6$; $P < 0.005$).

**Nerve-stimulated perfusion pressure responses are greater in mesenteric beds from SHR than from WKY controls.** The mean basal perfusion pressures of isolated, perfused mesenteric arterial beds taken from WKY and SHR were 22.4 ± 4.7 and 18.9 ± 3 mmHg, respectively. Periaxial nerve stimulation at 12 Hz increased the perfusion pressure to 71.6 ± 8.9 and 131 ± 16 mmHg for WKY and SHR, respectively. Figure 1 depicts these changes as percent increase over basal for both rat strains, with the increase for SHR significantly higher than that for WKY ($n = 8$; $P < 0.01$ vs. WKY).

**Nerve-stimulated overflow of norepinephrine from the mesenteric preparation is greater in SHR than WKY, but NPY overflow is greater in WKY than SHR.** In terms of neurotransmitter overflow, Fig. 2 depicts nerve-stimulated norepinephrine and NPY overflow for WKY and SHR preparations. The increase in norepinephrine was significantly higher for SHR preparations (163 ± 29% compared with WKY preparations (79.62 ± 20%; Fig. 2A; $n = 8$; $P < 0.05$ vs. WKY). Basal levels for norepinephrine were 1.86 ± 0.27 and 1.6 ± 0.28 ng/6 min for WKY and SHR, respectively. Conversely, the increase in NPY was significantly lower for SHR than for WKY (93 ± 16% vs. 218 ± 26.6%; Fig. 2B; $n = 8$; $P < 0.001$ vs. WKY). Basal levels for NPY were 0.21 ± 0.02 and 0.24 ± 0.01 ng/6 min for WKY and SHR, respectively.
N-acetylcysteine reduces nerve-stimulated mesenteric overflow of norepinephrine and increases NPY overflow from SHR preparations by increasing bioavailability of NO. In the presence of N-acetylcysteine (5 mM), nerve-stimulated norepinephrine overflow from the SHR mesenteric bed significantly decreased from 163 ± 29% to 30.6 ± 16.3% over basal (Fig. 3A; n = 8; **P < 0.01 vs. control). In contrast, nerve-stimulated NPY overflow from the mesenteric bed of the SHR significantly increased in the presence of N-acetylcysteine, from 93 ± 16% to 362 ± 87% over basal (Fig. 3B; n = 8; **P < 0.01 vs. control). The overflow of both norepinephrine and NPY from the WKY mesenteric bed was not significantly altered in the presence of N-acetylcysteine (Fig. 4; n = 8).

Since our hypothesis is that oxidative stress reduces the bioavailability of NO in the SHR, thus leading to increased norepinephrine overflow and, as a result, decreased NPY overflow compared with WKY, it appears likely that N-acetylcys-

Fig. 1. Perfusion pressure in the isolated, perfused mesenteric bed of the Wistar-Kyoto rat (WKY; n = 8) or the spontaneously hypertensive rat (SHR; n = 8) increases in response to periartrial nerve stimulation. **P < 0.01 compared with WKY.

Fig. 2. Nerve-stimulated norepinephrine overflow (A) from the 10- to 12-wk-old SHR mesenteric bed (n = 8) is greater than that from the age-matched WKY mesenteric bed (n = 8). Nerve-stimulated neuropeptide Y (NPY) overflow (B) from the 10- to 12-wk-old SHR mesenteric bed (n = 8) is less than that from the age-matched WKY mesenteric bed (n = 8). *P < 0.05, ***P < 0.001 compared with WKY.

Fig. 3. Nerve-stimulated norepinephrine overflow (A) decreases, whereas NPY overflow (B) increases from the mesenteric bed of the 10- to 12-wk-old SHR in the presence of N-acetylcysteine (NAC, 5 mM; n = 8). Inclusion of Nω-nitro-L-arginine methyl ester (L-NAME, 30 μM) with NAC (n = 5) reverses these effects. **P < 0.01 compared with control; †P < 0.05, ††P < 0.01 compared with NAC.

Fig. 4. Nerve-stimulated norepinephrine (A) or NPY (B) overflow from the mesenteric bed of the 10- to 12-wk-old WKY is not affected by the presence of NAC (5 mM; n = 8 for all).
Nerve-stimulated increase in perfusion pressure in the mesenteric bed of the 10- to 12-wk-old SHR (A) is unchanged in the presence of NAC (5 mM; n = 8) or in the presence of l-NAME (30 μM) with NAC (n = 8). Nerve-stimulated increase in perfusion pressure in the mesenteric bed of the 10- to 12-wk-old WKY (B) is decreased in the presence of NAC (5 mM; n = 8). *P < 0.05 compared with control.

Fig. 5. Nerve-stimulated increase in perfusion pressure in the mesenteric bed of the 10- to 12-wk-old SHR (A) is unchanged in the presence of NAC (5 mM; n = 8) or in the presence of l-NAME (30 μM) with NAC (n = 8). Nerve-stimulated increase in perfusion pressure in the mesenteric bed of the 10- to 12-wk-old WKY (B) is decreased in the presence of NAC (5 mM; n = 8). *P < 0.05 compared with control.

The 1 antagonist BIBO 3304 reduces nerve-stimulated perfusion pressure responses in SHR mesenteric preparation in presence of N-acetylcysteine. The lack of a difference in nerve-stimulated perfusion pressure responses in SHR mesenteric preparations in the presence of N-acetylcysteine alone or in conjunction with l-NAME was likely caused by the changes in contribution to postjunctional responses by norepinephrine and NPY. That is, in the presence of N-acetylcysteine NPY replaces norepinephrine as the major transmitter and therefore maintains postjunctional responses, whereas norepinephrine is restored to that role in the presence of l-NAME. To test this we carried out experiments in which we included the Y1 antagonist BIBO 3304 (300 nM) along with N-acetylcysteine (5 mM) in the perfusion buffer and found that under these circumstances the postjunctional increase in perfusion pressure in response to nerve stimulation was significantly decreased in the SHR mesenteric bed (729 ± 83% vs. 294 ± 88% over basal; Fig. 6; n = 8; P < 0.01 vs. N-acetylcysteine).

Oxidative stress is increased in SHR. Aortic tissue from the animals used in the above experiments was harvested in order to assess the general level of oxidative stress via levels of LPO in the vasculature. LPO levels in aortic tissue from WKY were significantly higher than in that from WKY (84 ± 18 vs. 13.8 ± 1.5 nmol/mg tissue; n = 6; P < 0.005 vs. WKY).

DISCUSSION

These results confirm our previous observations (26) on the differences that exist in sympathetic neurotransmission in mesenteric preparations taken from young (10–12 wk old) SHR and their normotensive age-matched WKY controls. Specifically, they demonstrate that whereas nerve-stimulated norepinephrine overflow is greater from the mesenteric arterial bed of 10- to 12-wk SHR than from WKY, nerve-stimulated NPY overflow is less. Prejunctional inhibitory activity by sympathetic cotransmitters on sympathetic neurotransmission is well established (30, 43, 54, 55). Therefore it is likely that elevated norepinephrine overflow in the SHR is negatively feeding back through prejunctional α receptors and reducing NPY overflow.

The present study was designed to investigate the role of increased oxidative stress in the increase in norepinephrine overflow in mesenteric preparations taken from 10- to 12-wk-old SHR. We previously showed that NO modulates norepinephrine activity under physiological conditions (27) and that this modulation is compromised in the SHR (26). NO bioavailability is known to be adversely affected by increased oxidative stress (31, 36, 49), and increased oxidative stress has been shown to occur in hypertension (3, 10, 20, 62). For that reason we focused this study on the role played by increased oxidative stress in the alterations in sympathetic neurotransmission in the SHR, with specific attention to NO bioavailability. Our results confirm that oxidative stress is indeed involved in the changes that occur in sympathetic neurotransmission in hypertension. Specifically, we show that the antioxidant N-acetylcysteine reduces the nerve-stimulated overflow of norepinephrine while...
Concomitantly increasing the nerve-stimulated overflow of NPY from the mesenteric arterial beds of the SHR. Furthermore, these changes were prevented by the NOS inhibitor l-NAME, demonstrating that the effects of N-acetylcysteine on transmitter overflow are indeed due to protection of the bioavailability of NO. In contrast, we show that neurotransmitter overflow in the normotensive age-matched WKY is not affected by N-acetylcysteine, which is not surprising given that NO-induced modulation of sympathetic neurotransmission is likely already optimal in normotensive rats (26, 27) and oxidative stress should not be a factor. Indeed, LPO analysis of aortic tissue taken from both sets of rats shows that SHR tissue has much greater levels of this lipid peroxidation marker than WKY tissue.

Perfusion pressure responses in mesenteric preparations taken from SHR, already significantly higher than their WKY normotensive controls, showed no net change in the presence of N-acetylcysteine alone or in combination with l-NAME. This was, on first analysis, somewhat surprising given the profound changes in neurotransmitter overflow. However, in the presence of N-acetylcysteine, nerve-stimulated norepinephrine overflow decreases and, at the same time, nerve-stimulated NPY overflow increases; thus it is probable that the NPY compensates for the loss of norepinephrine as far as perfusion pressure responses are concerned. Our experiments including the Y1 antagonist BIBO 3304 confirm that NPY is indeed responsible for preserving the elevation in perfusion pressure responses. Similarly, the presence of both l-NAME and N-acetylcysteine in combination causes nerve-stimulated norepinephrine overflow to increase while NPY overflow decreases, again resulting in no net effect on perfusion pressure responses. This again is likely a result of compensation by one transmitter for another. In contrast, perfusion pressure responses in WKY preparations were slightly, but significantly, reduced in the presence of N-acetylcysteine despite the lack of significant changes in neurotransmitter overflow. It is known that vascular NADPH oxidase, the major source of vascular O2- production (6, 11), has some constitutive activity, and the O2- produced by its activity is thought to play a physiological role in limiting the activity of the endothelium-derived vasodilator NO (7). Therefore, the decrease in perfusion pressure responses in the WKY preparations is likely due to heightened NO availability at the vascular smooth muscle, causing physiological antagonism to sympathetic vasoconstrictor. Although this must also be happening to some extent in the SHR preparations treated with N-acetylcysteine, it is likely masked by the overwhelming vasoconstrictor properties of the neurotransmitters. Indeed, perfusion pressure responses in the SHR preparations treated with both l-NAME and N-acetylcysteine, although not significantly different from SHR control, do show a moderate increase in perfusion pressure, possibly suggestive of a loss of this direct vasodilatory effect of NO as we demonstrated previously for l-NAME alone (26).

Overall our results confirm a primary role for oxidative stress in the compromise of NO-induced modulation of sympathetic neurotransmission in mesenteric preparations taken from 10- to 12-wk-old SHR, thus adding to the increasing body of evidence in recent years of the involvement of oxidative stress in disease states in general, and hypertension in particular. The presence of reactive oxygen species (ROS) in vascular disease contributes to vascular tissue damage, impaired vasodilation of blood vessels, and the modification of important proteins and signaling molecules (45, 62). The major source of ROS in the vasculature is vascular NADPH oxidase (6, 11), an enzyme that can be activated by a variety of stimuli including vascular growth factors, inflammatory cytokines, shear stress, metabolic factors, as well as the vasoactive peptide angiotensin (ANG) II, which is of particular interest as far as hypertension is concerned (7, 29). ANG II, acting via type 1 (AT1) receptors located on vascular smooth muscle cells, stimulates the activity of NADPH oxidase, leading to the production of O2- (20, 22, 38, 62). Indeed, a hallmark of the development of hypertension in the SHR [a renin-angiotensin system (RAS)-dependent model of hypertension] is increased oxidative stress (33, 35, 52, 61), and it has been shown that treating such animals with ANG-converting enzyme (ACE) inhibitors or AT1 receptor antagonists not only reverses the hypertensive response but also reduces oxidative stress markers (16, 38, 53, 61).

Thus it could be argued that the RAS deals a triple blow to the regulation of normal vascular tone in that it is a direct vasoconstrictor in its own right, it increases sympathetic neurotransmission, and it decreases the availability of NO as a result of increasing oxidative stress. All these factors undoubtedly factor into the success of ACE inhibitors and AT1 receptor antagonists as antihypertensive therapies. The loss in NO function as a result of the actions of O2- is further compounded by the fact that NO not only acts as a direct vasodilator but also modulates sympathetic neurotransmission. Given this latter point, it is possible that part of the positive modulatory effect of ANG II on sympathetic neurotransmission is due to the stimulation of oxidative stress.

Perhaps the most convincing evidence for the importance of oxidative stress in the pathogenesis of hypertension comes from studies showing that antioxidant treatment improves endothelial function and lowers blood pressure in experimental models of hypertension (5, 9, 15, 23, 41, 42, 47). Conversely, inducing oxidative stress in normotensive rats by depleting glutathione results in the development of hypertension (2, 50). The studies we have reported here are acute in nature. Future studies examining the long-term contribution to these changes in the SHR by ANG II and/or oxidative stress will be required in order to fully understand the contribution of oxidative stress to the changes in sympathetic activity in hypertension.

GRANTS
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