Modulation of neurotransmitter release by NO is altered in mesenteric arterial bed of spontaneously hypertensive rats

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Kolo, Lacy L., Thomas C. Westfall, and Heather Macarthur. Modulation of neurotransmitter release by NO is altered in mesenteric arterial bed of spontaneously hypertensive rats. Am J Physiol Heart Circ Physiol 287: H1842–H1847, 2004. First published June 17, 2004; 10.1152/ajpheart.00013.2004.—Nitric oxide (NO) reacts with catecholamines resulting in their deactivation. In the present study with the use of the perfused mesenteric arterial bed as a model of the sympathetic neuro-effector junction, the NO synthase (NOS) inhibitor \( \text{N}^\omega \)-nitro-L-arginine methyl ester (\( \text{l} \)-NAME) resulted in the enhancement of the perijateral nerve stimulation-induced increase in perfusion pressure and nitric oxide synthase (NOS) inhibition and can negatively regulate their own release as well as sympathetic neurotransmitter, vascular tone, nitric oxide synthase, and ATP.

It has been established that the neurotransmitters nitric oxide (NO), neuropeptide Y (NPY), and ATP are colocalized in and coreleased from many sympathetic neurons (7, 10, 35, 41). It has also been demonstrated that the application of each transmitter mimics a phase of sympathetic nerve stimulation and that each phase can be blocked with appropriate antagonists (7, 10, 35, 41). Moreover, norepinephrine, NPY, and ATP all have prejunctional inhibitory actions on sympathetic neurotransmission and can negatively regulate their own release as well as the release of each other (36, 43).

Nonneuronal mediators such as the well-characterized endothelium-derived vasodilator nitric oxide (NO) can also modulate sympathetic neurotransmission. Studies have shown that on sympathetic nerve stimulation, inhibition of NO synthase results in an increase in vasoconstriction in the rat tail artery (42), in the large coronary artery of anesthetized dogs (46), and in the vessels of the isolated adrenal medulla of the dog (1). We have observed that NO reacts with and deactivates catecholamines but not ATP and NPY (20, 24). For instance, in the isolated perfused mesenteric arterial bed of the rat, the ability of exogenous norepinephrine to increase perfusion pressure was attenuated by the incubation of the norepinephrine with the NO donor diethylamine NONOate (20). More importantly, the inclusion of the NO synthase (NOS) inhibitor \( \text{N}^\omega \)-nitro-L-arginine methyl ester (\( \text{l} \)-NAME) in the perfusion buffer, resulted in a concomitant increase in nitric oxide synthase overflow and perfusion pressure in response to perijeral nerve stimulation. This finding demonstrates that through deactivation of norepinephrine, NO modulates adrenergic neurotransmission under normal conditions at the sympathetic neuro-effector junction of the isolated perfused mesenteric arterial bed of the rat (20). From the fact that norepinephrine can negatively regulate sympathetic neurotransmission, it is possible that by deactivating norepinephrine, NO may indirectly modulate release of the sympathetic cotransmitters NPY and ATP.

The ability of NO to react with catecholamines may be important for understanding disease models such as hypertension where a decrease in NO availability has been reported (25, 26, 34). There are conflicting viewpoints as to the cause of this decrease in NO availability. It has been suggested that production of NO is altered; however, expression and activity of endothelial NOS have been found to be increased (32, 39), decreased (4), or unmodified (2) in hypertension. Others (18, 19, 23, 40) have suggested that NO production may not be altered, but its bioavailability may be reduced because of oxidative inactivation by excessive production of the superoxide anion in the vascular wall.

A decrease in available NO would not only result in a decrease in direct vasodilation but also, from previous observations, cause an increase in bioactive norepinephrine and other catecholamines. Indeed, there is an increase of plasma levels of norepinephrine in the presence of NOS inhibitors in the rat chronic renal failure model of hypertension (48). In addition, prolonged blockade of NOS caused by chronic \( \text{l} \)-NAME treatment resulted in arterial hypertension and elevated plasma levels of epinephrine in rats (22). This evidence also concurs with human studies. In humans with high blood pressure, the inhibition of NOS results in arterial hypertension and elevated plasma levels of epinephrine in rats (22). This evidence also concurs with human studies. In humans with high blood pressure and hypertension, the inhibition of NOS results in arterial hypertension and elevated plasma levels of epinephrine in rats (22).
pressure, plasma catecholamine content is increased by an average of 63% compared with normotensive humans (6). There is also support of such an increase of norepinephrine in a hypertensive animal model, the spontaneously hypertensive rat (SHR) (27, 30, 33, 44, 49). SHR animals develop hypertension with age.

Norepinephrine can negatively regulate sympathetic neurotransmission; therefore, a further consequence of the deactivation of norepinephrine by NO is likely to be an indirect modulation of the sympathetic cotransmitters NPY and ATP. Thus the decrease of NO availability in hypertension may also have consequences for the release of NPY and ATP. Alterations in plasma NPY levels have frequently been reported in hypertension models (12, 27, 44), but the results have been conflicting, possibly because of contamination with platelet-derived NPY (29). Responsiveness to ATP and the ATP metabolite adenosine has also been reported to be altered in the SHR (17, 21, 31).

In this study we investigated whether the reaction between endogenous NO and norepinephrine at the vascular neuroeffector junction can modulate sympathetic cotransmission in the isolated perfused mesenteric arterial bed of the rat. Furthermore, we investigated whether there is any alteration in the modulation of sympathetic neurotransmission by NO in the model of hypertension represented by the SHR.

MATERIALS AND METHODS

Materials. Norepinephrine (bitratrate salt); dopamine hydrochloride; 1H-[1,2,4]oxadiazolo[4,3-a]quinazolin-1-one (ODQ), L-NAME, yohimbine, S-nitroso-N-acyetylpenicillamine (SNAP), trifluoroacetic acid (TFA), and l-arginine were all purchased from Sigma (St. Louis, MO). NPY was purchased from American Peptide (Sunnyvale, CA). The NPY EIA kit was purchased from Peninsula Laboratories (San Carlos, CA).

Isolated perfused mesenteric preparation of the rat: surgery. 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. The perfused mesenteric bed of the rat was set up as previously described (15). All experiments were performed with 10–12 wk male Sprague-Dawley (SD), Wistar-Kyoto (WKY), or SHR rats. Animals were housed two to four animals per cage in a constant temperature 12:12-h light-dark cycle room. On the day of the experiment, the rats were anesthetized with pentobarbital sodium (50 mg/kg ip). The abdomen was opened, and the mesenteric arterial bed and associated intestines were removed after ligation of the descending colon proximal to the rectum and the duodenum proximal to the stomach. The superior mesenteric artery was cannulated with polyethylene-90 tubing connected to a syringe and flushed with heparinized saline. The four main branches of the mesenteric artery were ligated. The mesenteric vascular bed was then placed in an organ bath, maintained at 37°C, and perfused and superfused with Krebs buffer using a Grass S-88 stimulator. The perfusion buffer was collected in 1-min fractions into 0.1 N perchloric acid and then divided by basal transmitter over flow divided by basal transmitter over flow, and NPY over flow, and NPY over flow.

RESULTS

Neurotransmitter overflow in the presence of a NOS inhibitor in the perfused mesenteric bed of the Sprague-Dawley rat. Our previous results (20, 24) show that NO reacts with and deactivates catecholamines. Furthermore, NO-induced deactivation of dopamine released from the nerve growth factor-differentiated PC12 cell was accompanied by a concomitant increase in NPY release (unpublished observations). This suggests that exogenous NO in deactivating catecholamines indirectly modulates the release of NPY. However, the question remains as to the consequence of the deactivation of catecholamines by endogenous NO on sympathetic cotransmission at the vascular neuroeffector junction. To address this we used the isolated perfused mesenteric bed of the SD rat as a model of the sympathetic neuroeffector junction.

The SD mesenteric preparation of 10- to 12-wk-old animals was stimulated at 12 Hz, and perfusion pressure, norepinephrine overflow, and NPY overflow were measured. On periaxial nerve stimulation, NPY overflow (4.3 ± 0.6 increase over basal or 266 to 1,156 ng/6 min; n = 12; Fig. 1A) and norepinephrine overflow (2.3 ± 0.4-fold increase over basal or 1.8 to 4.14 ng/6 min; n = 8; Fig. 1B) significantly increased. The inclusion of L-NAME (3 × 10−5 M) decreased NPY overflow (2.2 ± 0.4-fold increase over basal or 225 to 503 ng/6 min; n = 8; Fig. 1A), whereas norepinephrine overflow was further increased (5.7 ± 1.3-fold increase or 1.2 to 6.8 ng/6 min; n = 8; Fig. 1B) on periaxial nerve stimulation. The effect of L-NAME was prevented by l-arginine (3 × 10−4 M), demonstrating that the effects of L-NAME inclusion were indeed caused by inhibition of NOS (n = 4; Fig. 1, A–B).
Modulation of neurotransmitter release in SHR

NOS inhibition increases perfusion pressure in the perfused mesenteric bed of SD rats. The mean basal perfusion pressure for all SD mesenteric preparation experiments was 12 ± 0.8 mmHg. Periarterial nerve stimulation at 12 Hz resulted in an increase in perfusion pressure (111 ± 12 mmHg; n = 20; Table 1). Inclusion of the NOS inhibitor L-NAME (3 × 10⁻⁵ M) did not affect the basal perfusion pressure; however, the nerve-stimulated perfusion pressure significantly increased to 174 ± 11 mmHg (n = 11; Table 1). This effect of L-NAME was prevented by the inclusion of l-arginine (3 × 10⁻⁴ M), demonstrating that the effects of L-NAME were indeed caused by inhibition of NOS (140 ± 9 mmHg; n = 4; Table 1).

α₂-Antagonism prevents the decrease in NPY overflow observed on NOS inhibition. The decrease in NPY observed in the presence of L-NAME is likely a result of the increase in bioactive norepinephrine in the absence of NO, and hence an increase in negative feedback by norepinephrine through the presynaptic α₂-receptor. To investigate this, we included the α₂-specific antagonist yohimbine in the perfusion buffer. On periarterial nerve stimulation, yohimbine alone did not significantly change NPY overflow (4.3 ± 0.6 to 4.9 ± 1.1-fold increase over basal; n = 5; Fig. 1, A and C). Conversely, yohimbine did increase norepinephrine overflow from 2.3 ± 0.2 to 5.3 ± 1.2-fold increase over basal (n = 5, respectively; Fig. 1, B and D).

In the presence of yohimbine, L-NAME (3 × 10⁻⁵ M) did not cause a decrease in NPY overflow (4.7 ± 0.8-fold release over basal; n = 5; Fig. 1C). However, L-NAME was still able to further increase norepinephrine overflow (19.3 ± 5.3-fold increase over basal; n = 5; Fig. 1D). This demonstrates that the increase in bioactive norepinephrine caused by NOS inhibition results in stimulation of the presynaptic α₂-receptor resulting in negative feedback on NPY release. Yohimbine (10⁻⁶ M) did not significantly change basal perfusion pressure (16.6 ± 1 vs. 18 ± 2 mmHg), perfusion pressure on stimulation (107 ± 7 vs. 113 ± 20 mmHg), or perfusion pressure on stimulation in the presence of L-NAME (205 ± 15 vs. 193 ± 32 mmHg; Table 1).

Modulation of neurotransmission in the perfused mesenteric bed of the SHR. To ensure that the SHR animals were hypertensive compared with the WKY, the femoral artery of the animals was cannulated and the blood pressure measured by a Grass recorder. The SHR had a significantly higher blood pressure compared with the WKY (134 ± 4 vs. 86 ± 2 mmHg; n = 4; data not shown). As with the SD, the mesenteric preparations from the WKY had a corresponding decrease in stimulated release of NPY (5.8 ± 0.8 to 3.6 ± 0.4-fold increase over basal; n = 7; Fig. 2A). And an increase in the release of norepinephrine (2.3 ± 0.7 to 6.5 ± 1.0-fold increase over basal; n = 7; Fig. 2B). In the presence of L-NAME (3 × 10⁻⁵

Table 1. Perfusion pressure of isolated mesenteric arterial beds from SD rats.

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<th>Basal, mmHg</th>
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<th>Stimulated + L-NAME, mmHg</th>
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<tr>
<td>−Yohimbine</td>
<td>12±0.8</td>
<td>111±12</td>
<td>174±11</td>
<td>140±9</td>
</tr>
<tr>
<td>+Yohimbine</td>
<td>18±2</td>
<td>113±20</td>
<td>193±32</td>
<td>ND</td>
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Values are means ± SE. L-NAME, N⁵-nitro-l-arginine methyl ester.
Hypertensive animals (SHR), L-NAME did not further increase norepinephrine in the 12-wk-old WKY rats (S, open bars; Fig. 2A). However, in the hypertensive spontaneously hypertensive rat (SHR), L-NAME inclusion did not alter NPY overflow compared with control stimulation in the normotensive 10- to 12-wk-old Wistar-Kyoto (WKY) rats (S, open bars; n = 7) where S/B is stimulated neurotransmitter divided by basal neurotransmitter overflow. However, in the hypertensive spontaneously hypertensive rat (SHR), L-NAME inclusion did not alter NPY overflow compared with control stimulation in the normotensive 10- to 12-wk-old WKY rats (S, open bars; n = 7; Fig. 2A). In addition, the SHR did not have a significant decrease in NPY overflow in the presence of L-NAME (3.8 ± 0.4 to 3.0 ± 0.6-fold increase over basal; n = 7; Fig. 2A). All changes observed in the presence of L-NAME were reversible by inclusion of L-arginine (data not shown).

The mean basal perfusion pressure for both the WKY and SHR mesenteric preparations was 26 ± 3 and 22 ± 1 mmHg, respectively (n = 16; Fig. 3). Periarterial nerve stimulation at 12 Hz resulted in an increase in perfusion pressure of both the WKY (94 ± 9 mmHg; n = 14; Fig. 3) and SHR animals (149 ± 11 mmHg; n = 16; Fig. 3). Upon inclusion of L-NAME (3 × 10⁻⁵ M) for 45 min, the perfusion response of the WKY significantly increased to 143 ± 10 mmHg (n = 14; Fig. 3), and the response of the SHR increased to 202 ± 13 mmHg (n = 16; Fig. 3). All changes observed in the presence of L-NAME were reversible by inclusion of L-arginine (data not shown).

**DISCUSSION**

It is well accepted that neurotransmission at the sympathetic nerve-effector junction is a finely tuned process controlled by the activation of both auto- and heteroreceptors located on the sympathetic nerve terminal (7, 10, 35, 41). The findings obtained in this study indicate that this fine-tuning also extends to factors released from other locations. First, we have confirmed that the inhibition of endogenous NO synthesis results in a simultaneous increase in nerve-stimulated perfusion pressure in, and norepinephrine overflow from, the isolated perfused mesenteric bed of the rat. These findings further support our previous work showing that endogenous NO modulates sympathetic neurotransmission via deactivation of catecholamines. Furthermore, NOS inhibition results in a concomitant decrease in NPY overflow. This decrease in NPY overflow was prevented in the presence of the α₂-adrenergic antagonist yohimbine. This new finding demonstrates that a consequence of the increase in norepinephrine upon NOS inhibition is an increased stimulation of the prejunctional α₂-receptors, thus resulting in a negative feedback of NPY release. In the hypertensive model of the SHR, norepinephrine overflow and perfusion pressure response are greater than the normotensive animals. Furthermore, the modulation of sympathetic neurotransmission by NO is compromised in that while NOS inhibition does cause a further increase in perfusion pressure, the neurotransmitter overflow is not altered.

Our previous results show that NO reacts with and deactivates catecholamines but not ATP or NPY, as found by the inability of catecholamines to increase the perfusion pressure of an isolated mesenteric arterial bed after incubation with a short-life NO donor (20, 24). Furthermore, our previous studies demonstrate that inhibition of endogenous NOS increases sti-
ulated norepinephrine overflow. However, it was not determined whether NOS inhibition would affect NPY overflow. The studies in this current paper demonstrate that in addition to increasing norepinephrine overflow, NOS inhibition results in a decrease in stimulated NPY overflow. Experiments with the \(\alpha_2\)-receptor antagonist yohimbine demonstrated that this decrease in NPY overflow was caused by the actions of norepinephrine. We can surmise from these results that under normal conditions there is a negative modulation of the bioactivity of norepinephrine by NO and that this in turn alters the cotransmission of NPY, and presumably ATP as well.

Before NOS inhibition, inclusion of yohimbine alone increased nerve-stimulated norepinephrine, but not NPY, overflow from the mesenteric bed, which is expected because antagonism of this receptor prevents norepinephrine from inhibiting neurotransmitter release per se (13). The ability of yohimbine to alter overflow of norepinephrine, but not NPY, may be due to differential modulation of sympathetic cotransmitters. There is emerging evidence that sympathetic cotransmitters can be differentially released and modulated. For instance, clonidine inhibits the release of norepinephrine but not ATP from the myenteric plexus of the guinea pig ileum (14). In addition, yohimbine produces a greater increase in the overflow of norepinephrine than ATP, suggesting that endogenously released norepinephrine has a greater influence on its own release than that of ATP (37). It is likely that this is also the case with the effect of norepinephrine on NPY release.

The ability of NO to react with catecholamines may be important for understanding disease models such as hypertension where a decrease in NO availability has been reported (25, 26, 34). Many types of hypertension are associated with an inadequate availability of NO either through decreased NO synthesis or increased NO metabolism (5, 9, 11). This loss of NO will lead to an increase in vasoconstriction both directly (by removal of a direct vasodilator) and indirectly (by increasing the availability of catecholamines). The increase in catecholamines should not only stimulate the vasculature but also alter the release of the sympathetic cotransmitters ATP and NPY through an increase in negative feedback mechanisms.

To examine how the modulation of sympathetic neurotransmission by NO may be altered in hypertension, the model of the SHR and their genetic controls the WKY was chosen. SHR have elevated sympathoadrenomodulatory activity, resulting in an increased efferent sympathetic outflow coupled with the development of hypertension (16, 38). All animals were age matched as the SHR blood pressure changes with age. From birth to 6 wk, the blood pressure of the SHR is comparable to the WKY (28, 45). The blood pressure of the SHR then begins to rise until the rats are 10–12 wk old. After this age, the blood pressure then stabilizes (47).

In the 10–12-wk-old WKY, the basal perfusion pressure was significantly higher than the SD. This may be due to differences in genetic strain. For this reason, all experiments were repeated in the WKY animal as a control for SHR. The perfusion pressure response and the level of norepinephrine overflow from the SHR mesenteric preparation upon peristaltic nerve stimulation was significantly greater than that of the age-matched normotensive SD and WKY animals. These data are consistent with studies examining catecholamine overflow from the perfused mesenteric arterial bed and the isolated caudal artery (44) and also in the plasma (49) of SHR animals.

More importantly, the neurotransmitter release from the SHR mesenteric preparation in the absence of l-NAME closely resembled the release from SD and WKY in the presence of l-NAME, suggesting that NO availability is compromised in the SHR preparation. Conversely, in the same SHR preparation there is an increased perfusion pressure response on nerve stimulation in the presence of l-NAME, suggesting that NO availability is not compromised, at least at the level of the vascular smooth muscle. From the fact that in the vasculature, endothelium-derived NO must first diffuse through the vascular smooth muscle layer to react with norepinephrine at the vascular neuroeffector junction, it is possible that NO (in addition to relaxing the smooth muscle) may react with substances released from this cell layer. Several studies have found that in hypertensive models, there is an excessive production of superoxide anion within the vascular smooth muscle (18, 19, 23, 40). Superoxide anion reacts swiftly with NO, thereby decreasing the bioavailability of NO. Therefore, a possible explanation for the fact that NOS inhibition had no effect on nerve-stimulated norepinephrine overflow from mesenteric preparations taken from the SHR but still increased nerve-stimulated perfusion pressure is that the NO released from the endothelial cells diffuses to the vascular smooth muscle and causes relaxation but is deactivated before it can reach the neuroeffector junction.

In conclusion, our results demonstrate that NO not only modulates vascular reactivity directly by vasorelaxation and indirectly by deactivating norepinephrine, but that the deactivation of catecholamines further results in modulation of the release of the sympathetic cotransmitter NPY. Our results also show that in the 10- to 12-wk-old SHR, the modulation of sympathetic neurotransmission by NO is compromised, although the ability of NO to cause direct vasorelaxation is not.

**GRANTS**

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