Nitric oxide decreases the biological activity of norepinephrine resulting in altered vascular tone in the rat mesenteric arterial bed

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Kolo, Lacy L., Thomas C. Westfall and Heather Macarthur. Nitric oxide decreases the biological activity of norepinephrine resulting in altered vascular tone in the rat mesenteric arterial beds. Am J Physiol Heart Circ Physiol 286: H296–H303, 2004; 10.1152/ajpheart.00668.2003.—Nitric oxide (NO) reacts with catecholamines resulting in their deactivation. In this study, we demonstrated that coincubation of NO donors with sympathetic neurotransmitters decreased the amount of norepinephrine detected but not ATP or neuropeptide Y (NPY). Furthermore, we found that the ability of norepinephrine to increase perfusion pressure in the isolated perfused mesenteric arterial bed of the rat was attenuated by the incubation of norepinephrine with the NO donor diethylamine NONOate. Conversely, the vasoconstrictive ability of NPY and ATP was unaffected by incubation with NONOate. Periaxial nerve stimulation in the presence of the NO synthase (NOS) inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) resulted in an increase in both perfusion pressure response and norepinephrine levels. This was prevented by L-arginine, demonstrating that the effects of L-NAME were indeed specific to the inhibition of NOS. To confirm that NO was not altering the release of norepinephrine from the sympathetic nerve via presynaptic activation of guanylate cyclase, we repeated the experiments in the presence of the guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-one (ODQ). Unlike L-NAME, ODQ infusion did not increase norepinephrine overflow, demonstrating that modulation of norepinephrine by NO at the vascular neuroeffector junction of the rat mesenteric vascular bed is not the result of presynaptic guanylate cyclase activation. These results demonstrate that, in addition to being a direct vasodilator, NO can also alter vascular reactivity at the sympathetic neuroeffector junction in the rat mesenteric bed by deactivating the vasoconstrictor norepinephrine.

sympathetic neurotransmitters; nitric oxide synthase; adenosine 5′-triphosphate, neuropeptide Y; vascular tone

IT HAS BEEN ESTABLISHED that the neurotransmitters norepinephrine, neuropeptide Y (NPY), and ATP are colocalized in and coreleased from many sympathetic neurons (10, 16, 47, 50). 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 (10, 16, 47, 50). Moreover, norepinephrine, NPY, and ATP all have presynaptic inhibitory actions on sympathetic neurotransmission and can negatively regulate their own release as well as the release of each other (49, 53).

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 synthesis results in an increase in vasoconstriction in the rat tail artery (51), in the large coronary artery of anesthetized dogs (55), and in the vessels of the isolated adrenal medulla of the dog (1).

The enhancement of vasoconstriction may be due in part to the removal of the relaxation normally caused by NO; however, there is evidence that in the absence of NO there is an increase in the amount of norepinephrine released from sympathetic nerves. Endogenous NO has been shown to have an inhibitory effect on the stimulated release of catecholamines from the sympathetic nerves and the adrenal medulla. For instance, blocking NO synthase facilitates the release of catecholamines into the plasma (39, 51, 52), facilitates the release of norepinephrine from adrenergic nerves in canine and guinea pig pulmonary blood vessels (4, 21), and increases adrenal catecholamine release in pithed rats (37). It has been proposed that the inhibitory effect of NO on the action of norepinephrine is due to a postjunctional physiological antagonism (3, 4). An alternative explanation involves a prejunctional activation of the cGMP second messenger pathway by NO within the nerve terminal, leading to either an inhibition (21) or potentiation (56, 58) of norepinephrine release. Furthermore, NO may be involved in altering the release of ATP and NPY, which are cotransmitters with norepinephrine at the sympathetic vascular neuroeffector junction. NO is known to inhibit responses to NPY (33) and in hypertensive animals, a disease state characterized by a lack of available NO (13, 18), NO potentiated responses to ATP and its nonhydrolyzable analog α,β-methyl ATP (24, 38).

Our laboratory (32) has observed that NO chemically reacts with catecholamines resulting in a decrease in catecholamine levels measured by high-performance liquid chromatography coupled to electrochemical detection (HPLC-EC). More importantly, we (32) have also seen a decrease in the biological activity of catecholamines after incubation with NO. Other investigators (9, 11) have demonstrated that in a test tube incubation, NO or NO donors almost completely converted dopamine, epinephrine, and norepinephrine to their 6-nitro derivatives.

The literature suggests that NO may modulate the action or release of norepinephrine from adrenergic nerves. However, NO may well be altering the biological activity of norepinephrine, which in turn will alter norepinephrine levels and ultimately vascular tone. This characteristic of NO may be important for understanding disease models such as hypertension where a decrease in NO availability has been reported (35, 36, 41).

In this study, we investigate whether exogenous NO alters the biological activity of norepinephrine or its cotransmitters.

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NPY or ATP. In addition, we examine whether endogenous NO acts as a modulator of sympathetic neurotransmission at the vascular neuroeffector junction of the isolated perfused mesenteric arterial bed of the rat.

MATERIALS AND METHODS

Materials. Norepinephrine (bitartrate salt); diethyelamine HCl; 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalino-one (ODQ); Nω-nitro-l-arginine methyl ester (l-NNAME); 5-nitro-N-acetylpenicillamine (SNAP); ATP; and l-arginine were all purchased from Sigma (St. Louis, MO). Diethyelamine NONOate (NONOate) was purchased from Calbiochem (San Diego, CA). NPY was purchased from American Peptide (Sunnyvale, CA).

Test tube experiments. SNAP (10⁻⁶·3 × 10⁻⁴ M) was incubated with synthetic norepinephrine (20 ng/ml), ATP (100 ng/ml), or NPY (1–10⁻⁴ M) for 5 min in a test tube. The reaction was stopped by the addition of 0.1 N perchloric acid + 0.1% cysteine and placement on ice. In some experiments, hemoglobin (Hb; 10⁻⁴ M) was included in the incubate. Norepinephrine levels were measured by HPLC-EC, ATP by HPLC-fluorometric detection, and NPY by radioimmunoassay.

Isolated perfused mesenteric preparation of the rat: surgery. The perfused mesenteric bed of the rat was set up as described previously (25). All procedures were carried out in accordance with National Institute of Health guidelines and were approved by the the Institutional Animal Care and Use Committee of Saint Louis University Health Sciences Center. All experiments were performed with 10- to 12-wk-old male Sprague-Dawley rats. Animals were housed two to three per cage in a constant temperature 12-h light/12-h 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-bicarbonate buffer using a Gilson minipump at a rate of 3 and 0.5 ml/min, respectively. The Krebs buffer was composed of (in mM) the following: 120 NaCl, 5.0 KCl, 1.2 MgSO4, 2.4 CaCl2, 0.027 EDTA, 11.1 glucose, and 25 NaHCO3.

Mesenteric perfusion system, Walnut Creek, CA). Buffer A (pH = 6.0) consisted of a 0.1 M phosphate buffer and buffer B (pH = 6.0) consisted of 75% 0.1 M phosphate buffer and 25% methanol. The fluorescent purine derivatives were detected at an excitation wavelength of 300 nm and an emission wavelength of 420 nm (Varian 9070 fluorescence detector). Identification of the purine peaks was carried out by comparison of retention times of purine standards. Sample purine content was quantified by peak integration (Varian Star Workstation Software).

Catecholamine measurements. Catecholamines were identified and quantified by HPLC-EC as previously published (6, 12). The system consists of a Varian model 2510 solvent delivery system and a Varian model 410 Prostar autosampler coupled to a C18 column and an ESA Col chromatography detector. Separations were performed isocratically using a filtered and degassed mobile phase consisting of 10% methanol, 0.1 M sodium phosphate, 0.2 mM sodium octyl sulfate, and 0.1 mM EDTA, adjusted to pH 2.8 with phosphoric acid. The HPLC system is coupled to a computer with which chromatograms were recorded and analyzed with Varian Star workstation software.

Statistical analysis of data. Data are expressed as means ± SE. Statistical analysis of variance was carried out using one-way analysis of variance followed by Newman-Keuls multiple comparison tests. Statistical differences were accepted when P < 0.05.

RESULTS

NO decreases amount of catecholamine measured in test tube. Incubating norepinephrine (20 ng/ml) with the NO donor SNAP (10⁻⁶·3 × 10⁻⁴ M) significantly decreased the chemical detection of norepinephrine by HPLC-EC (n = 6; Fig. 1A). Coincubation with Hb prevented the decrease in norepinephrine levels. Incubation of the cotransmitter ATP (100 ng/ml) or NPY (325 pg/ml) with SNAP (10⁻⁶·3 × 10⁻⁴ M) had no effect on the amount of this transmitter as measured by HPLC-fluorometric detection or by radioimmunoassay, respectively (n = 6; Fig. 1, B and C).
they were incubated with NONOate without the risk of our results being affected by physiological antagonism due to any NO still being released by the donor. Whereas fresh NONOate (0 h; $10^{-7}$ M) significantly relaxes mesenteric arterial beds preconstricted with methoxamine (60 ± 9% relaxation; $n = 7$; Fig. 2), 3-h-old NONOate was unable to relax a preconstricted bed (6 ± 5% relaxation; $n = 4$; Fig. 2). This confirms that NONOate has lost all NO-releasing ability by 3 h, and therefore, we chose this as the length of incubation time with norepinephrine, NPY, and ATP. Time control incubations of these vasoconstrictors with saline were also carried out.

Dose-response curves were constructed for the pressor response and the EC$_{50}$ of the vasoconstrictor determined ($n = 6$; Fig. 3, A–C). The EC$_{50}$ concentration of norepinephrine, NPY or ATP was then incubated with the NONOate ($10^{-7}$ M). The ability of NPY ($5 \times 10^{-7}$ M) and ATP ($10^{-4}$ M) to constrict the mesenteric preparation was unchanged after 3 h of incubation with NONOate ($125 \pm 18$ vs. $117 \pm 12$; $n = 4$ and $66 \pm 5$ vs. $65 \pm 10$ mmHg; $n = 6$, respectively; Fig. 4, B–C). Conversely, after incubation with NONOate, norepinephrine ($10^{-6}$ M) no longer constricted the mesenteric bed (0 vs. $89 \pm 14$ mmHg; $n = 14$; Fig. 4A). The chemical backbone of NONOate, diethylamine hydrochloride, had no effect on the constrictor ability of norepinephrine, confirming that the effect observed was indeed due to NO (Fig. 4A). In addition, the NO donor NONOate ($10^{-7}$–$10^{-3}$ M) was incubated with norepinephrine in a test tube and shown to decrease the amount of norepinephrine measured by HPLC-EC as shown for SNAP earlier ($n = 12$; data not shown).

NO synthase inhibition increases perfusion pressure and norepinephrine overflow in perfused mesenteric bed of rats. It is clear that exogenous NO in the form of NO donors alters the biological activity of norepinephrine. However, the question remains as to the effect of endogenous NO on sympathetic neurotransmission at the vascular neuroeffector junction. To answer this question, we used the isolated perfused mesenteric bed of the rat. The mean basal perfusion pressure at 12 Hz resulted in an increase in perfusion pressure ($98 \pm 19.8$ mmHg) and a corresponding increase in the stimulated release of norepinephrine ($2.3 \pm 0.3$ mmHg). The NO donor NO still being released by the donor. Whereas fresh NONOate (0 h; $10^{-7}$ M) significantly relaxes mesenteric arterial beds preconstricted with methoxamine (60 ± 9% relaxation; $n = 7$; Fig. 2), 3-h-old NONOate was unable to relax a preconstricted bed (6 ± 5% relaxation; $n = 4$; Fig. 2). This confirms that NONOate has lost all NO-releasing ability by 3 h, and therefore, we chose this as the length of incubation time with norepinephrine, NPY, and ATP. Time control incubations of these vasoconstrictors with saline were also carried out.

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At this time, the mesenteric bed was perfused with the NO synthase inhibitor L-NAME (3 × 10⁻⁵ M) for 45 min. The basal tone of the vascular bed was unchanged by L-NAME perfusion. However, on periarterial nerve stimulation in the presence of L-NAME (30 s, 12 Hz), the perfusion pressure significantly increased to 191 ± 35 mmHg (n = 8; Fig. 5A). The effect of L-NAME was prevented by L-arginine (3 × 10⁻⁴ M), demonstrating that the effects of L-NAME were indeed caused by inhibition of NO synthase (n = 4; Fig. 5A and B).

Inhibition of guanylate cyclase has no effect on norepinephrine overflow in mesenteric bed of rats. Our data show that inhibiting NO synthase results in an increase in the bioactivity and relative levels of norepinephrine. However, we wanted to determine whether any changes in norepinephrine overflow were due to the classic ability of NO to activate cGMP. Therefore, we carried out further experiments in the presence of the guanylate cyclase inhibitor ODQ (10⁻⁵ M) (19). We reasoned that if NO was activating cGMP presynaptically to inhibit norepinephrine release, then inhibiting guanylate cyclase with ODQ should be identical to treatment with L-NAME, i.e., norepinephrine overflow in the perfusate should be increased on stimulation of the periarterial nerves. ODQ treatment did not increase the stimulatedoverflow of norepinephrine (2.3 ± 0.2- vs. 1.9 ± 0.2-fold increase; n = 14; Fig. 6A). Conversely, treatment with L-NAME again increased the amount of norepinephrine overflow measured in the perfusate (2.3 ± 0.2- to 5.7 ± 1.4-fold increase; n = 8). Furthermore, treatment with both L-NAME and ODQ did not further increase the stimulated norepinephrine overflow levels (5.4 ± 1.6-fold increase; n = 7), supporting our hypothesis that NO is modulating norepinephrine directly (Fig. 6A). ODQ treatment did cause an increase in nerve-stimulated perfusion pressure in this preparation equivalent to that caused by L-NAME (176 ± 10 vs. 191 ± 35 mmHg; Fig. 6B). Treatment with both agents simultaneously had no additional effect on perfusion pressure, possibly because the maximum perfusion pressure for this preparation had been reached (Fig. 6B). These results show that modulation of norepinephrine by NO at the vascular neuroeffector junction of the mesenteric vascular bed under normal conditions is not the result of presynaptic activation of cGMP. However, the classic action of NO on the vascular smooth muscle is indeed via this pathway.

DISCUSSION

It is well accepted that neurotransmission at the sympathetic neuroeffector junction is a finely tuned process controlled by the activation of both auto- and heteroreceptors located on the sympathetic nerve terminal (reviewed in Ref. 31). The findings obtained in this study indicate that this fine tuning also extends to factors released from other locations. First, we have shown that exogenous NO reacts with and deactivates norepinephrine as indicated by the loss of its vasoconstrictor ability. Furthermore, we have confirmed that this action of NO is specific to the catecholaminergic portion of sympathetic neurotransmission because the biological activity of both NPY and ATP are unaffected by exogenous NO. More significantly, we have demonstrated that the inhibition of endogenous NO synthesis results in an increase in the amount of norepinephrine overflow.

Fig. 3. Dose-response curves depicting the vasoconstrictor effects of norepinephrine (NE; 10⁻⁸–10⁻⁴ M), NPY immunoreactivity (ir) (10⁻¹⁰–10⁻⁶ M), and ATP (10⁻³–10⁻² M) were constructed to find the EC₅₀ of each neurotransmitter. 

0.2-fold increase; n = 30; Fig. 5A). At this time, the mesenteric bed was perfused with the NO synthase inhibitor L-NAME (3 × 10⁻⁵ M) for 45 min. The basal tone of the vascular bed was unchanged by L-NAME perfusion. However, on periarterial nerve stimulation in the presence of L-NAME (30 s, 12 Hz), the perfusion pressure significantly increased to 191 ± 35 mmHg (n = 8; Fig. 5A). In addition, norepinephrine overflow also significantly increased 5.7 ± 1.3-fold (n = 8; Fig. 5B). The effect of L-NAME was prevented by L-arginine (3 × 10⁻⁴ M), demonstrating that the effects of L-NAME were indeed caused by inhibition of NO synthase (n = 4; Fig. 5, A and B).
measured after periarterial nerve stimulation from the sympathetic vascular neuroeffector junction of the isolated perfused mesenteric bed of the rat. This finding reveals that endogenous NO does indeed modulate sympathetic neurotransmission at this junction. Moreover, the manner in which this occurs is likely to be by direct chemical interaction with the catecholamine as it is released rather than prejunctional activation of guanylate cyclase because inhibition of guanylate cyclase did not alter the amount of norepinephrine measured in the overflow after periarterial nerve stimulation.

These findings are consistent with previous work from our lab (32) showing that incubation of dopamine with the NO donor SNP not only decreased the amount of catecholamine measured by HPLC, but more importantly, resulted in a loss in ability of dopamine to increase cAMP in rat pheochromocytoma (PC12) cells.

The source of the endogenous NO in the preparation we used here could be either endothelial or neuronal in nature (or indeed both) because the rat mesenteric bed has been shown to possess both endothelial and neuronal NO synthase (27, 42). However, because the mesenteric preparation is a vascular bed, the endothelium is likely to be the major source of NO. Periarterial nerve stimulation in the presence of the NO synthase inhibitor L-NAME resulted in an increase in both perfusion pressure in the isolated perfused mesenteric bed, as well as norepinephrine overflow. These increases in perfusion pressure and norepinephrine were prevented by the addition of L-arginine, demonstrating that the effects of L-NAME were indeed specific to the inhibition of NO synthase. A major part of this increase in perfusion pressure is likely to be directly due to the removal of a powerful vasodilator. However, the fact that there is now more active norepinephrine in the absence of NO probably influences perfusion pressure as well. Indeed, the literature suggests that the increase in the nerve-stimulated pressor response in the presence of L-NAME is in part dependent on increased norepinephrine. For instance, in the pithed vagotomized rat, the α1-adrenoceptor antagonist prazosin prevented the increase in the nerve-stimulated pressor response in the presence of L-NAME to levels below that of vehicle-treated rats (17). Furthermore, in the isolated mesenteric preparation, prazosin prevented the increase in perfusion pressure caused by nerve stimulation but did not significantly reduce the measurement of norepinephrine overflow in the presence of L-NAME (14). Thus we believe we can reasonably conclude that in the presence of L-NAME, the increase in perfusion pressure is caused primarily by the increased availability of NE acting on its postjunctional receptor.

The role played by NO in modulating the catecholaminergic portion of sympathetic neurotransmission is perhaps more noteworthy when considering the events leading to the development of disease states such as hypertension. Many types of hypertension are associated with an inadequate availability of NO either through decreased NO synthesis, increased NO metabolism, or decreased NO bioavailability (8, 13, 20). 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 norepinephrine and other catecholamines. This increase in norepinephrine will not only stimulate the vasculature but also affect the release and action of the sympathetic cotransmitters ATP and NPY. Indeed, alterations in plasma NPY levels have frequently been reported in hypertensive animals and humans (15, 26, 48, 54). Furthermore, responses to ATP and adenosine are also reported to be altered in hypertensive models (28, 29). The effect of NO-induced catecholamine deactivation on ATP and NPY release is currently under investigation.

As already stated, our results indicate that NO is modulating sympathetic neurotransmission in the rat mesenteric bed by

![Figure 4. NONOate (10^{-6} M; closed bars) or DiHCl (10^{-6} M; hatched bars) was incubated with NE (10^{-6} M), NPY (5 \times 10^{-7} M), or ATP (10^{-4} M) for 3 h. At this time, the incubates were perfused through the mesenteric arterial bed. NONOate did not alter the ability of NPY or ATP to constrict the mesentery compared with control (B; n = 4 and C; n = 6). Whereas NONOate inhibited the biological activity of NE significantly (A; n = 14) *P < 0.05.](http://ajpheart.physiology.org/DownloadedFrom/10.220.33.2/onJune28,2017)
direct inactivation of norepinephrine rather than through activation of cGMP pathways in the nerve terminal. However, it has been shown by other investigators that NO can indeed alter catecholamine release through cGMP-dependent mechanisms in preparations such as bovine adrenal chromaf

Fig. 5. Periarterial nerve stimulation of the mesenteric bed increases perfusion pressure (A) and stimulated overflow (B) of norepinephrine (S; closed bars; n = 30). In the presence of N\(^{-}\)nitro-L-arginine methyl ester (L-NAME, 3 \times 10^{-5} M), both the perfusion pressure and NE overflow significantly increased (S + L-NAME; gray bars; n = 8). Effect of L-NAME was prevented by L-arginine (3 \times 10^{-5} M; hatched bars; n = 4). *P < 0.05.

deferred release through cGMP-dependent mechanisms in preparations such as bovine adrenal chromaffin cells (5), the rat tail artery (44), as well as from rat striatal nerves (23). Whereas these studies found that NO donors decreased catecholamine levels or adrenergic vasoconstriction, these studies did not take into account any alternative modulatory actions by NO. It is possible that both types of actions by NO play a role in the modulation of sympathetic neurotransmission at different junctions.

It is likely that removal of NO by L-NAME has a dual effect on perfusion pressure in that vasodilation (direct effect of NO) is reduced and at the same time vasoconstriction is increased (effect of increased norepinephrine). Although there was no effect on norepinephrine overflow, inhibition of guanylate cyclase within the mesenteric preparation did increase the nerve-stimulated perfusion pressure to a level that was comparable with L-NAME treatment. This is not surprising because ODQ treatment prevents the classic vasodilatory action of NO via activation of soluble guanylate cyclase, preventing the increase of cGMP in vascular smooth muscle cells that results in vasorelaxation (22). As the maximum increase in pressor response for this preparation has been reached, an additive effect of both agents was not seen.

In contrast to our findings, studies by Yamamoto et al. (56) found that NO synthase inhibition in the rat mesenteric arterial bed led to a decrease in norepinephrine overflow in response to transmural field stimulation. Interestingly, this same group reported that the NO donor sodium nitroprusside had no effect on stimulated norepinephrine overflow (57). The disparity between our results and theirs could be due to a major difference in the exact preparation used. The mesenteric bed in their studies was still attached to the intestines, which could have complicated the results. Moreover, we stimulated only the periarterial sympathetic nerves of this vascular bed rather than stimulating all the nerves in the preparation by transmural field stimulation (2, 43, 45).

Our findings highlight that NO alters the biological activity of norepinephrine. One possible way that NO can affect catecholamines is by converting them to their 6-nitro-derivatives
(9, 11). This is particularly interesting because 6-nitrocatecholamines have been found in several parts of the body, including the rat paraventricular nucleus (46) and the rat spinal cord (7), and have a number of possible biological properties. Furthermore, both 6-nitronorepinephrine and 6-nitrodopamine have been suggested to interfere with neuronal NO synthase activity (40), suggesting a possible path by which NO can indirectly modulate its own availability.

In conclusion, our results demonstrate that in addition to being a direct vasodilator, NO can also alter vascular reactivity by deactivating norepinephrine thus reducing the availability of this vasoconstrictor at the sympathetic vascular neuroeffector junction of the isolated perfused mesenteric bed of the rat.

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