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NITRIC OXIDE FROM ENDOTHELIUM AND SMOOTH MUSCLE MODULATES RESPONSES TO SYMPATHETIC NERVE STIMULATION: IMPLICATIONS FOR ENDOTOXIN SHOCK

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SUMMARY: The influence of nitric oxide (NO) on vascular responses to transmural stimulation (TNS) of noradrenergic nerves was studied in isolated rings of rat iliac arteries. TNS produced frequency-dependent contractions in all vessels. The NO synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA) significantly enhanced TNS responses in intact vessels, but not in those in which the endothelium had been removed. However, in endothelium-denuded rings incubated for 8 hours, L-NMMA increased the contractions induced by nerve stimulation, an effect which was prevented by treatment with dexamethasone or cycloheximide, and enhanced by incubation with lipopolysaccharide and γ-interferon. Addition of L-arginine reversed the effect of L-NMMA in intact rings; however, it significantly decreased below control values TNS-induced contractions in vessels without endothelium. The results indicate that a) the arterial response to noradrenergic nerve stimulation is modulated by NO originating either in endothelial cells or in smooth muscle cells after induction of NO synthase activity, and b) once NO synthase is induced, the limiting step in NO production is the availability of the substrate L-arginine. An overproduction of vascular NO in the presence of endotoxin or other inflammatory stimuli may prevent the vascular response to sympathetic stimuli and contribute to the vasodilation observed in inflammation or endotoxic shock.

Vascular tone is controlled by the simultaneous influence of neurogenic mechanisms (1) and the release of nitric oxide (NO) by the endothelial cell layer (2). Circulating factors can also affect vascular resistance, either by acting directly on smooth muscle cells or by modulating NO release (3). Although in some vascular beds, such as cerebral (4) or mesenteric vessels (5), both constrictor and dilator perivascular nerves have been described, most vessels are innervated only by

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sympathetic vasoconstrictor fibers (1), and, therefore, the endothelium provides the counteracting dilator mechanism.

An interaction between NO and sympathetic regulation of vascular resistance has been previously shown. In perfused isolated arteries from several species, contractile responses to electrical stimulation of perivascular nerves (transmural nerve stimulation, TNS) are enhanced when the endothelium is removed (6-8). The endothelial inhibitory effect on the vascular responses to TNS is abolished by hemoglobin (8), suggesting that NO is the mediator involved. Also, in guinea-pig pulmonary arteries, the NO synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA) has been shown to enhance TNS-induced contractions (9).

It has recently been reported that vascular smooth muscle can also synthesize and release NO when stimulated by endotoxin lipopolysaccharide (LPS), or some cytokines (10,11). These substances induce in smooth muscle cells the expression of a NO synthase, whose properties differ from those of the physiologically active endothelial enzyme in that a) it is not constitutively expressed, b) its activity is not regulated by Ca^{2+}-calmodulin, and c) it is dependent on tetrahydrobiopterin (11,12).

On the other hand, it has been suggested that in some vascular beds, such as cerebral arteries (13), NO can be released directly from perivascular nerve terminals. In these cases, NO would be acting as an atypical neurotransmitter, which may contribute to non-adrenergic, non-cholinergic (NANC) relaxations.

The purpose of this work was to analyze the modulator effect of both endothelial and non-endothelial NO on the rat iliac artery response to stimulation of the perivascular noradrenergic nerves.

MATERIALS AND METHODS

Iliac arteries from male Sprague-Dawley rats (250-350 g) were isolated and cut into cylindrical segments, 2 mm in length. The rings were suspended on two intraluminal parallel wires, introduced into an organ bath containing a physiological solution, and connected to a Pioden strain gauge (Pioden UF1, UK) for isometric tension recording. The composition of the physiological saline solution (PSS) was as follows (mM): NaCl, 115; KCl, 4.6; $CaCl_2$, 1.25; $NaHCO_3$, 25; KH_2PO_4, 1.2; $MgSO_4$, 1.2; EDTA, 0.01; and glucose, 11. Rings were allowed to equilibrate for 90 minutes at a tension of 1.5 g. TNS (200 mA, 0.2 ms, trains of 100 pulses at 1 to 16 Hz) was carried out using two parallel platinum electrodes, one on each side of the ring, connected to a CS-20 stimulator (Cibertex, Madrid, Spain). Stimuli were applied at least 8 minutes apart. When the effects of NO synthase inhibitors or L-arginine were analyzed, TNS was applied 15 minutes after addition of the drugs. LPS (100 ng/ml), γ-interferon (γ-IFN, 100 u/ml), dexamethasone (8×10^{-7}M), and cycloheximide (10^{-5}M), were added to the vessels immediately after isolation from the animal. All experiments were performed in the presence of 5×10^{-6}M indomethacin. In some of the rings the endothelium was mechanically removed. The effect of acetylcholine (ACh) was tested

in each vessel to assess the functional integrity (relaxation > 80% of the active tone) or the absence (relaxation < 10% of the active tone) of the endothelial layer.

Results are expressed as mean ± SE. Data were analyzed using the Wilcoxon test. A p value less than 0.05 was considered significant.

Prostaglandin $F_{2\alpha}$ tetrodotoxin, phentolamine, prazosin, N^G-nitro-L-arginine methyl ester (L-NAME), dexamethasone, cycloheximide, endotoxin, γ-IFN and acetylcholine were purchased from Sigma. N^G-monomethyl-L-arginine (L-NMMA) was synthesized by Wellcome Laboratories.

RESULTS

TNS produced in the rat iliac artery frequency-dependent contractions, which were completely blocked by tetrodotoxin, phentolamine or prazosin (all at 1 μM), indicating that they were mediated by stimulation of perivascular noradrenergic nerves (data not shown).

Contractions induced by TNS in intact rings, immediately after the equilibration period was completed, were significantly enhanced in the presence of the NO synthase inhibitor L-NMMA (100 μM). This effect was observed at all the frequencies used, and was reversed by L-arginine (Figure 1). A similar result was obtained with L-NAME (30 μM, data not shown). When rings denuded of endothelium were assayed in the same experimental conditions, contractions induced by TNS were not significantly different in the presence or absence of L-NMMA (Figure 2), suggesting that in intact vessels, neurogenic vascular responses were modulated by NO originating from endothelial cells.

However, among endothelium-denuded vessels, 3 out of 10 arteries were still sensitive to the NO synthase inhibitor, in spite of a complete functional removal of endothelial cells, which was established by the absence of the dilator response to ACh. This results suggested that a non-endothelium-derived NO was also released in rat iliac arteries, and was modulating the response to perivascular nerve activity. The origin of such non-endothelial NO was next investigated.

Rings with or without endothelium, incubated in the presence of the adrenoreceptor blockers phentolamine and propranolol, and precontracted with prostaglandin $F_{2\alpha}$ did not relax upon TNS, thus ruling out the possibility of a neural production of NO in the rat iliac artery.

In order to know whether the enzyme responsible for NO production in vessels without endothelium could be of the inducible type, the equilibration period for denuded rings was extended from 90 min to 8 hours, and then TNS was applied. TNS-induced contractions observed after 8 hours were significantly reduced (48.4 ± 5 %), as compared with the values recorded after the standard 90 min equilibration period in

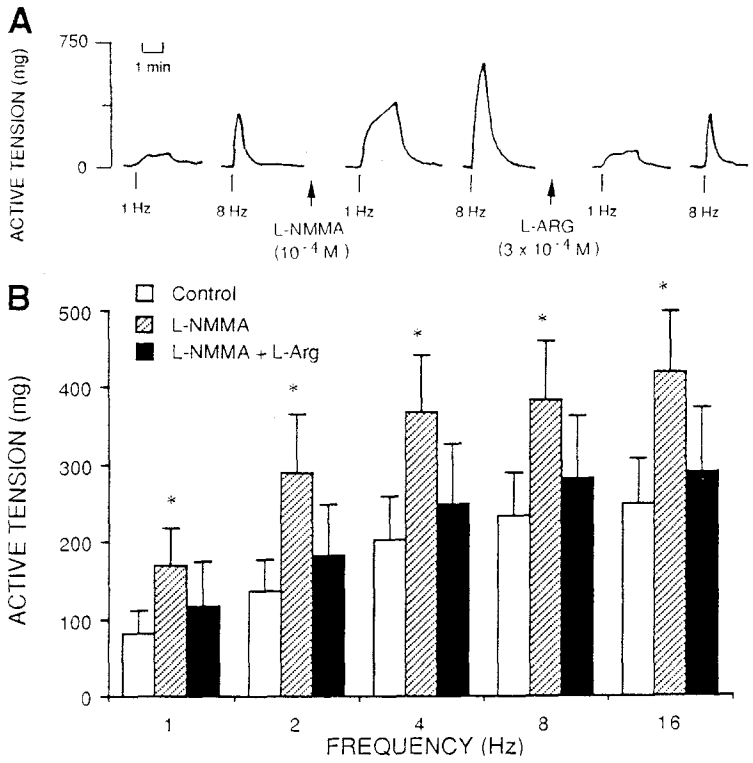

Figure 1, A. Representative recording of the contractions induced by TNS on endothelium-containing rat iliac arteries, in control conditions and after addition of L-NMMA (100 μM) and L-arginine (300 μM).
B. Summary of the effects of TNS (1-16 Hz) on endothelium-containing rat iliac arteries in control conditions and in the presence of L-NMMA (100 μM), or L-NMMA plus L-arginine (300 μM). Experiments were performed 90 min after mounting the tissues in the organ bath. Data represent the mean ± SE of the values obtained in 14 arteries isolated for 9 animals. * $p < 0.05$.

the same vessels. At this time, the contractions induced by TNS in endothelium-denuded vessels were significantly enhanced in the presence of L-NMMA (Figure 2). The mean increase in the contractile response to TNS in the presence of the NO synthase inhibitor was more evident when LPS and γ-IFN were present (Figure 2). On the contrary, dexamethasone, which inhibits the induction of NO synthase (14), reduced the effect of L-NMMA on TNS-induced contractions (Figure 2). A similar result was observed when protein synthesis was prevented by cycloheximide (Figure 2).

As can be observed in figure 2, addition of L-arginine (300 μM) after L-NMMA administration returned the TNS-induced contractions to control levels in intact arteries. However, in vessels without endothelium, responses to TNS after addition of L-

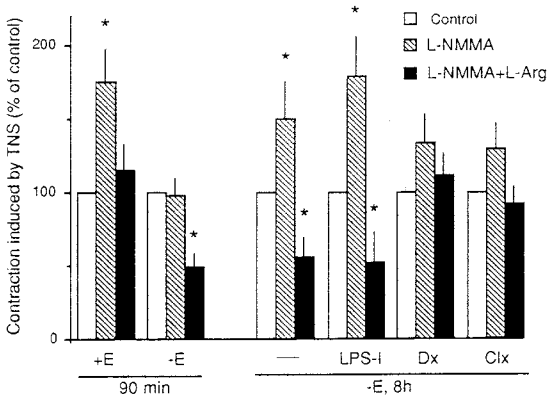

Figure 2. Effect of L-NMMA (100 μM), or L-NMMA plus L-arginine (300 μM) on TNS-induced contractions (8 Hz) in vessels with (+E) or without (-E) endothelium. Experiments were performed either 90 min or 8 hours after mounting the tissues in the organ bath and were carried out in the absence or presence of endotoxin and γ-interferon (LPS-I), dexamethasone (Dx) or cycloheximide (Clx). The data represent the mean ± SE of the values obtained in 9-13 arteries isolated from groups of 6-8 animals, and are expressed as percentage of the control response in each vessel.
* $p<0.05$ as compared to control values.

arginine were significantly decreased as compared with controls, except when vessels were treated with dexamethasone or cycloheximide. Such an effect was apparent in vessels maintained throughout the experiment either in regular PSS, or in PSS supplemented with 50 μM arginine (data not shown).

DISCUSSION

The present results show that NO produced within the vessel wall inhibits arterial contractions induced by transmural stimulation of perivascular noradrenergic nerves. Among vessels studied immediately after an equilibration period of 90 minutes (i.e. the whole experiment was performed between 2 and 4 hours after the animal's death), intact, but not endothelium-denuded, rings showed an increased response to TNS in the presence of L-NMMA. This finding suggests that NO originated in the endothelial cells, and agrees with previous observations in perfused isolated arteries showing an enhancement in the TNS response after removal of the endothelium (6-8).

We cannot distinguish from the present experiments whether endothelial NO is released as a consequence of the noradrenergic stimulation, or if there is a basal NO production, which would become manifest only after a constrictor stimulus is applied. Whatever the releasing stimulus, the inhibitory effect of endothelial NO on TNS-induced contractions is probably mediated by its direct effect on smooth muscle

cells. A modulatory action of NO on the nerve ending function is unlikely, according to previous studies in pulmonary arteries, in which L-NMMA did not modify TNS-induced noradrenaline release (9).

When arteries without endothelium were studied 8 hours after setting up the rings, a non-endothelial production of NO was revealed. Such NO production can be attributed to an induction of NO synthase in smooth muscle cells, as has been described in other blood vessels (10,11). This possibility is supported by the observation that L-NMMA had a greater effect in vessels incubated with LPS and γ-IFN. Accordingly, in the presence of dexamethasone or after prevention of protein synthesis, the enhancing effect of L-NMMA on TNS-induced contractions disappeared. Experiments performed with isolated arteries have also shown a NO-mediated decrease of the constrictor response to exogenous noradrenaline in vessels maintained in physiological solutions for 6-8 hours; this effect is probably due to the presence of small concentrations of LPS in the laboratory water (10).

In some vessels (13,15), as well as in other smooth muscle-containing organs such as the intestine (16,17), trachea (18), etc. NO has been shown to mediate NANC relaxations, and its release from intramural nerves has been postulated. However, this was not the case in the rat iliac artery, since vessels pretreated with adrenergic receptor blockers and precontracted with $PGF_{2\alpha}$ did not relax when stimulated.

Addition of L-arginine to intact vessels reversed the effect of L-NMMA and allowed TNS-induced responses to return to control levels. However, in endothelium-denuded vessels, L-arginine produced a significant decrease in TNS-induced contractions as compared with controls. This observation suggests that the activity of the smooth muscle NO synthase was limited by the availability of substrate. When NO synthase expression was prevented by dexamethasone or cycloheximide, neither L-NMMA nor L-arginine modified the TNS-induced response. The rate-limiting role of L-arginine does not seem to be a consequence of arginine depletion during the experimental period, since it was observed as soon as 90 minutes after setting up the tissue, and also in experiments performed in the presence of physiological concentrations of L-arginine.

Taken together, these results indicate that, a) in rat iliac arteries, the vascular response to noradrenergic nerve stimulation is modulated by NO, and b) NO originates in endothelial cells, and when NO synthase induction occurs, also in smooth muscle cells. The inhibitor role of smooth muscle cell-derived NO may be relevant in pathological conditions such as local inflammatory processes or endotoxic shock, in

which LPS and cytokines are present, and persistent vasodilation occurs. The substrate dependence of the muscle NO synthase activity suggests that in these pathological states, plasma L-arginine concentrations may have an influence on the vascular response to the sympathetic tone and, hence, on the local or systemic vascular resistance.

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