Role of Nitric Oxide and Cyclic GMP as Mediators of Endothelium-Independent Neurogenic Relaxation in Bovine Mesenteric Artery

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Electrical field stimulation (EFS) of phenylephrine-contracted bovine mesenteric arteries pretreated with guanethidine elicited a relaxation that amounted to roughly 40%. This relaxation was sensitive to tetrodotoxin pretreatment, suggesting a neurogenic origin. The EFS-induced relaxation was correlated to an increase in cGMP level, from 14.2±2.5 pmol/g wet wt in nonstimulated arteries to 31.6±3.4 pmol/g wet wt after 1 minute of EFS. cAMP values were not affected by EFS. Methylene blue (5 μM) and the compound LY 83583 (10 μM), inhibitors of soluble guanylate cyclase, inhibited the EFS-induced relaxation by 60% and 50%, respectively. Zaprinast (1 μM), a selective inhibitor of cGMP degradation, significantly (p=0.005) potentiated the EFS-induced relaxation. The relaxation induced by EFS in bovine mesenteric arteries exhibits characteristics similar to the relaxations evoked by organic nitroesters and endothelium-dependent vasodilators, both of which are suggested to be mediated by cGMP and probably with nitric oxide as the common activator of the cGMP system. The possible involvement of nitric oxide as a mediator of EFS-induced relaxations was investigated with the use of known modulators of endogenous nitric oxide production. Preincubation of the arteries with 1 mM arginine or 1 mM N-α-benzoyl-L-arginine, both reported to potentiate endogenous nitric oxide production, or 5 mM L-canavanine, 0.25 mM N⁶-monomethyl-L-arginine, or 0.1 mM N⁶-nitro-L-arginine, alleged inhibitors of endogenous nitric oxide production, were without effect on the relaxation induced by EFS. However, pyrogallol, a generator of superoxide anions, was a potent inhibitor of relaxations induced by EFS in bovine mesenteric arteries. These results suggest the existence of a neurogenic and cGMP-mediated relaxation in bovine mesenteric arteries, and also suggest that nitric oxide may participate in this signal transduction, although no evidence for an involvement of the L-arginine pathway in the generation of nitric oxide could be found. (Circulation Research 1991;68:756–762)

Vascular tone is regulated by various neurogenic mechanisms, including adrenergic, cholinergic, and peptidergic neurons. Circulating factors may also affect vascular dynamics, either through direct action on the vascular smooth muscle cells or through interaction with endothelial cells. This latter mechanism is thought to include liberation of diffusible factors from the endothelial cells. Depending on the type, these factors may cause either vascular relaxation or vascular constriction. Regarding endothelium-dependent relaxation of vascular smooth muscle, it has recently been claimed that at least two different factors may be involved, namely, endothelium-derived relaxing factor (EDRF) and endothelium-derived hyperpolarizing factor. Evidence suggesting that EDRF is identical to nitric oxide has also been presented. Nitric oxide has previously been claimed to be the molecular species liberated from organic nitroesters and responsible for the potent vasodilator action of these drugs. EDRF and organic nitroesters both activate the cGMP system and thus share the same physiological pathway in mediating their vascular smooth muscle relaxant actions.

In a recent publication we described a novel neurogenic vasodilator mechanism in isolated bo-
vine mesenteric arteries. In the present study we have further characterized this vasodilator system, and we now report a possible role of the cGMP-nitric oxide system as mediator of this endothelium-independent relaxation.

**Materials and Methods**

**Tension Recording**

Bovine mesenteric arteries were obtained from a local slaughterhouse. The arteries were transported to the laboratory in warm (37°C) Krebs-bicarbonate solution of the following millimolar composition: Na+ 137, K+ 6, Mg2+ 1.3, Ca2+ 2.2, Cl− 134, HCO3− 15.4, H2PO4− 1.2, and glucose 5.6. The solution was equilibrated with 95% O2-5% CO2. The arteries were carefully cleaned of adventitia and opened longitudinally. Unless the arteries were to be used to test for endothelium-dependent relaxation, the endothelium was removed by gentle rubbing of the arteries with a scalpel. This procedure abolished the relaxant response to ionomycin. Tissue specimens, weighing 150–200 mg, were tied to sewing cotton and mounted in Plexiglas chambers of 5 ml capacity. The chambers were fitted with platinum electrodes to allow electrical field stimulation (EFS) of the tissue. The isometric tension of the circular muscle layer was recorded with an isometric strain gauge transducer and a polygraph (both from Grass Instrument Co., Quincy, Mass.). To avoid release of norepinephrine during EFS, the arteries were treated with 5 μM guanethidine for 10 minutes before addition of 2.5 μM phenylephrine. The contraction was allowed to stabilize, whereupon the arteries were stimulated with trains of biphasic rectangular pulses (1 msec, 10 Hz, 100 V nominal output; representing supramaximal stimulation) with a Grass SD9 stimulator.

**Cyclic Nucleotide Determinations**

Arteries were mounted for tension recording, and EFS was applied for 60 seconds, whereupon the tissue was quickly frozen. Cyclic nucleotides were measured by radioimmunoassay.

**Statistical Methods**

Values are presented as mean±SEM. The level of statistical significance has been tested with Student’s *t* test on paired observations.

**Drugs**

Guanethidine was a gift from CIBA-GEIGY, Västra Frölunda, Sweden. N6-Monomethyl-L-arginine was from Calbiochem Corp., La Jolla, Calif. 6-Anilino-5,8-quinolinedione (LY 83583) was a gift from Eli Lilly and Company, Indianapolis, Ind., and Zaprinast was a gift from Rhone-Poulenc, Dagenham, England. All other chemicals were from Sigma Chemical Co., St. Louis.

**Results**

EFS of phenylephrine-contracted bovine mesenteric arteries elicited a tetrodotoxin-sensitive relaxation of 40% (Figure 1). Methylene blue (5 μM) inhibited this relaxation by 59%, and LY 83583 (10 μM) inhibited the relaxation by 49% (Figure 1). Methylene blue and LY 83583 in concentrations up to 10 μM had no inhibitory effect on other nerve-mediated responses, such as noncholinergic, nonadrenergic inhibitory responses in bovine trachea, phasic and tonic contractions in rat vas deferens, and melanosome aggregation in fish melanophores (data not shown).

EFS for 1 minute elicited near-maximal relaxation obtainable by this route and increased the tissue cGMP level from a control value of 14.2±2.5 pmol/g wt to 31.6±3.4 pmol/g wt (Figure 2). cAMP values were 60.7±5.4 pmol/g wt in control specimens and 51.7±4.9 pmol/g wt in field-stimulated artery specimens (*n*=8).

The selective cGMP-phosphodiesterase inhibitor Zaprinast (1 μM) significantly potentiated the EFS-induced relaxation (Figure 3), and the rate of relaxation was also increased. Zaprinast alone at concentrations up to 1 μM had no or slight inhibitory effect on the vascular smooth muscle tension, whereas higher concentrations caused a marked loss of tension.

Arginine and different arginine derivatives have been shown to modulate EDRF-induced vascular smooth muscle relaxation. Preincubation with 1 mM arginine for 15 minutes or 1 mM N-α-benzoyl-L-arginine ethyl ester for 5 minutes, both reported to
potentiate endothelium-dependent relaxations, had no effect on the EFS-induced relaxation (Figure 4). Preincubation with inhibitors of endothelium-dependent relaxations such as L-canavanine (5 mM for 60 minutes), or \( N^\circ \)-monomethyl-L-arginine (0.25 mM for 5 minutes) was also without effect on the EFS-induced relaxation (Figure 4). \( N^\circ \)-Nitro-L-arginine (30–100 \( \mu \)M), which is another inhibitor of EDRF relaxations, was also ineffective on EFS-induced relaxation, but rapidly reversed endothelium-dependent relaxations induced by ionomycin (0.25 \( \mu \)M) (Figure 5). Furthermore, incubation of bovine mesenteric arteries for 24 hours at 37°C had no effect on the EFS-induced relaxations (Figure 5). This procedure has previously been shown to deplete vascular tissue of L-arginine and to decrease endothelium-dependent relaxations.\(^{21}\)

Superoxide dismutase (100 or 200 units/ml), which has been reported to potentiate vascular smooth muscle relaxations induced by EDRF and nitric oxide,\(^{12,22,23}\) was without effect on EFS relaxations but potentiated EDRF relaxations induced by 0.25 \( \mu \)M ionomycin (Figures 6 and 7). The superoxide anion–generator pyrogallol (0.1–0.5 mM) inhibited and rapidly reversed relaxations induced by both ionomycin and EFS (Figure 7).

**Discussion**

In a previous study we reported the existence of a neurogenic endothelium-independent vasodilator mechanism in bovine mesenteric arteries.\(^{19}\) In the present study we show that this relaxation is correlated to an increased cGMP level and is probably mediated through endogenous nitric oxide produc-

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**Figure 2.** Electrical field stimulation (EFS) elicited a two-fold increase in cGMP level as measured after 1 minute of continuous stimulation. Mean±SEM (n=8); \(^*\)p<0.01. cAMP values were 60.7±5.4 pmol/g wet wt in control specimens and 51.7±4.9 pmol/g wet wt in specimens subjected to EFS.

**Figure 3.** Effect of the selective cGMP-phosphodiesterase inhibitor Zaprinast on relaxation induced by electrical field stimulation in bovine mesenteric artery. Representative polygraph recording (top panel), and mean±SEM (n=6) (bottom panel). Horizontal bars indicate field stimulation.

**Figure 4.** Effect of preincubation with 1 mM L-arginine (15 minutes), 5 mM L-canavanine (60 minutes), 0.25 mM \( N^\circ \)-monomethyl L-arginine (NMMA) (5 minutes), and 1 mM \( N^\circ \)-\( \alpha \)-benzoyl-L-arginine ethyl ester (BAEE) (5 minutes) on relaxation induced by electrical field stimulation (stimulation parameters as in Figure 1). Mean±SEM (n=4–8). NS, not statistically significant (p>0.1).

**Figure 5.** Representative original tracings showing the effect of \( N^\circ \)-nitro-L-arginine (N-ARG) on endothelium-dependent relaxations induced by 0.25 \( \mu \)M ionomycin (top) and electrical field stimulation (middle) in bovine mesenteric artery. Bottom tracing shows the relaxations induced by electrical field stimulation in bovine mesenteric arteries incubated for 24 hours at 37°C and the effect of N-ARG in these arteries. Horizontal bars indicate electrical field stimulation.
cGMP has previously been suggested as the mediator of vascular smooth muscle relaxation induced by three different types of vasodilators: organic nitroesters (e.g., nitroglycerin), EDRF, and atrial natriuretic peptide. In addition to this, ultraviolet light has been shown to induce cGMP-mediated vascular smooth muscle relaxation. The present report thus adds yet another function of cGMP in the regulation of vascular smooth muscle dynamics.

Organic nitroesters and EDRF act through activation of the soluble form of guanylate cyclase, while atrial natriuretic peptide activates the particulate form of guanylate cyclase. The relaxation induced by organic nitroesters and EDRF has been shown to be antagonized by methylene blue and the compound LY 83583. Both these compounds seem to interfere with the activation of soluble guanylate cyclase by nitric oxide, which is suggested to be the common chemical species liberated from organic nitroesters and from an unknown precursor by endothelium-dependent vasodilators. LY 83583 and methylene blue have not been found to have any significant effects on vascular smooth muscle relaxation and cGMP elevations induced by atrial natriuretic peptide. The finding that preincubation of bovine mesenteric arteries with either methylene blue or LY 83583 inhibited the relaxation induced by EFS to a similar extent (50–60%) could therefore indicate that activation of soluble guanylate cyclase is involved in the mediation of this response. It seems unlikely that the inhibitory action of LY 83583 and methylene blue on EFS-induced relaxations is due to an inhibition of the neuronal transmission process since nerve-mediated responses in other tissues were unaffected by pretreatment with these drugs.

The EFS-induced relaxation was completely and promptly inhibited by tetrodotoxin. It is therefore possible that some neurotransmitter is released from nerve terminals located within the vascular wall and that this neurotransmitter subsequently interacts with the vascular smooth muscle cells to elicit relaxation through an elevation of the cGMP level. It was recently reported that excitatory amino acids induced nitric oxide production and elevations of cGMP in cerebellar tissue. It might be speculated that EFS of bovine mesenteric arteries causes release of some unknown neurotransmitter that stimulates nitric oxide production/release in the vascular tissue. However, the possibility also exists that a compound (e.g., nitric oxide), which directly activates guanylate cyclase, is liberated directly from the neurons during EFS.

It has previously been shown that L-arginine and various derivatives of L-arginine can modulate the vascular response to endothelium-dependent vasodilators, suggesting that L-arginine or some L-arginine-containing peptide could constitute the source for nitric oxide. In the present study, however, we were unable to demonstrate an effect of either L-arginine or N-α-benzoyl-L-arginine, which have been reported to potentiate endothelium-dependent relaxations. Incubation of bovine mesenteric arteries at 37°C for 24 hours did not have any effect on the EFS-induced relaxation. This procedure has previously been shown to deplete vascular smooth muscle of endogenous L-arginine. Furthermore, L-canavanine, N⁶-monomethyl-L-arginine, and N⁶-nitro-L-arginine, which inhibit endogenous nitric oxide production and inhibit endothelium-dependent relaxations, were without effect on EFS-induced relaxations. These findings may seem puzzling, although it is known that different cell types show varying substrate/inhibitor profiles for L-arginine and its analogues, possibly because of the existence of different isoenzymes catalyzing nitric oxide synthesis. Another possibility is that the different L-arginine compounds do not penetrate the vascular tissue to reach their site of action. The bovine mesenteric...
artery is a rather thick preparation, probably representing a substantial diffusional barrier, which is in marked contrast to the situation with EDRF-induced relaxations where the site of nitric oxide production is in a superficial cell layer in direct contact with the bathing solution.

Pyrogallol, which is a potent inhibitor of endothelium-dependent relaxations by its capacity to inactivate nitric oxide through generation of superoxide anions in oxygenated solutions, inhibited EFS-induced relaxations. Conversely, superoxide dismutase, which protects nitric oxide from degradation by superoxide anions, was without effect on the EFS-induced relaxations. Superoxide dismutase potentiated EDRF relaxations, as previously reported by other authors.12,22,23 Superoxide dismutase has also been found to potentiate nitric oxide–mediated vascular smooth muscle relaxation by ultraviolet light exposure.46,47 The reason for the discrepancy regarding the effects of pyrogallol and superoxide dismutase is unclear, although it may be that superoxide dismutase, representing a big molecule, does not gain access to the site of production of nitric oxide (possibly nerve terminals and synapses) within the vascular tissue. Similar findings have recently been reported in dog cerebral artery.

Endothelium-independent neurogenic relaxations induced by transmural nerve stimulation have previously been identified in cat cerebral artery, where both vasoactive intestinal peptide and calcitonin gene–related peptide have been suggested as mediators of the vasodilation. Calcitonin gene–related peptide has also been suggested to act as the transmitter responsible for the vasodilation induced by perivascular nerve stimulation in rat mesenteric resistance vessels, and the release of calcitonin gene–related peptide on nerve stimulation has also been demonstrated in this vascular bed. Furthermore, the existence of a neurogenic muscarinic and endothelium-independent vasodilation has been shown in the feline caudal femoral artery and in feline posterior auricular arteries. However, calcitonin gene–related peptide or acetylcholine seem to be unlikely candidates as the neurotransmitters mediating the EFS-induced relaxations in bovine mesenteric arteries because exogenously added calcitonin gene–related peptide had only slight relaxatory potency in this tissue and because both capsaicin and atropine were ineffective.

Neurogenic mechanisms mediating smooth muscle relaxation via an activation of the cGMP system have previously been demonstrated in bovine retractor penis muscle, opossum lower esophageal sphincter, and rat anococcygeus muscle. Evidence has been presented that nitric oxide may function as a mediator of these relaxations. During the final preparation of this manuscript, several additional articles have appeared that report the existence of smooth muscle relaxations induced by activation of noncholinergic, nonadrenergic nerves and release of endogenous nitric oxide. The tissues where such mechanisms have been demonstrated include rabbit corpus cavernosum, canine ileocolonic junction, guinea pig trachea, and canine cerebral artery.

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