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Role of nitric oxide and guanosine 3′,5′-cyclic monophosphate in mediating nonadrenergic, noncholinergic relaxation in guinea-pig pulmonary arteries

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1 Nonadrenergic, noncholinergic (NANC) nerves mediate vasodilatation in guinea-pig pulmonary artery (PA) by both endothelium-dependent and endothelium-independent mechanisms. The transmitter(s) involved in the endothelium-independent pathway have not yet been identified. We have therefore investigated the possibility that nitric oxide (NO) and guanosine 3′,5′-cyclic monophosphate (cyclic GMP) may mediate this neural vasodilator response in guinea-pig branch PA rings denuded of endothelium.

2 Electric field stimulation (EFS, 50 V, 0.2 ms) induced a frequency-dependent (1–24 Hz), tetrodotoxin-sensitive relaxation of the U44069-precontracted PA rings in the presence of adrenergic and cholinergic blockade.

3 The NO synthase inhibitors N^G-monomethyl L-arginine (L-NMMA, 100 μM) and N^G-nitro L-arginine methyl ester (L-NAME, 30 μM), and the guanylyl cyclase inhibitor methylene blue (5 μM) inhibited the EFS (16 Hz)-induced relaxation by 53 ± 5, 74 ± 9 and 82 ± 9% respectively ($n = 5–7$, $P < 0.01$, compared with control rings).

4 Excess concentrations of L-, but not D-arginine (300 μM) completely reversed the inhibitory effect of L-NMMA.

5 The EFS-elicited relaxation (4 Hz) was potentiated by 1 μM zaprinast, a type V phosphodiesterase inhibitor which inhibits guanosine 3′:5′-cyclic monophosphate (cyclic GMP) degradation, but was unaffected by 0.1 μM zardaverine, a type III/IV phosphodiesterase inhibitor which inhibits cyclic AMP degradation.

6 EFS (50 V, 0.2 ms, 16 Hz) induced a 3 fold increase in tissue cyclic GMP content, an action which was inhibited by L-NMMA (100 μM).

7 Pyrogallol (100 μM), a superoxide anion generator, also inhibited the EFS-induced relaxation by 53 ± 9%, and this effect was prevented by superoxide dismutase.

8 Chemical sympathetic denervation with 6-hydroxydopamine had no effect on the relaxant response to EFS in the endothelium-denuded PA rings.

9 In endothelium-denuded branch PA rings at resting tone, L-NMMA (100 μM) significantly augmented the adrenergic contractile response, an effect which was completely reversed by L-arginine, but not by D-arginine. In the same groups of vessel rings, L-NMMA had no significant effect on the matched contractile response to exogenous noradrenaline.

10 These results suggest that NO may be released from intramural nerve endings other than adrenergic nerves (probably NANC nerves), and this leads to vasodilatation via activation of guanylyl cyclase.

Keywords: Innervation; NANC nerves; pulmonary artery; nitric oxide; electrical field stimulation; 6-hydroxydopamine; guanylyl cyclase; cyclic GMP; vasodilatation

Introduction

In a previous study we have shown that electrical field stimulation (EFS) of precontracted guinea-pig branch pulmonary artery (PA) rings causes a nonadrenergic, noncholinergic (NANC) relaxation, which is mediated by two pathways, one of which is endothelium-dependent and one of which is endothelium-independent (Liu *et al.*, 1992). The endothelium-dependent component appears to be mediated by adenosine 5′-triphosphate (ATP), but the transmitters responsible for the endothelium-independent component of this relaxation remain unknown.

Recently, evidence has accumulated for nitric oxide (NO) as a NANC transmitter in both vascular and nonvascular tissues (for review see Gillespie *et al.*, 1990; Moncada *et al.*, 1991; Rand, 1992). NO causes vasorelaxation by activating soluble guanylyl cyclase, resulting in an increase in intracellular guanosine 3′,5′-cyclic monophosphate (cyclic GMP) (Ignarro *et al.*, 1989; Moncada *et al.*, 1991). Endogenous NO also modulates pulmonary adrenergic vasoconstriction (Liu *et al.*, 1991b) and hypoxic pulmonary vasoconstriction (Liu *et al.*, 1991a). However, the NO could be released from NANC nerves as a transmitter, from adrenergic nerve endings as a cotransmitter, or from both.

In the present study, we tested the possibility that NO and cyclic GMP may mediate the endothelium-independent component of this neural relaxation. We studied the effect of EFS on tissue cyclic GMP content. Since cyclic GMP is broken down by phosphodiesterase (PDE), we also investigated the role of cyclic GMP in this neural relaxation using a type V PDE inhibitor which inhibits cyclic GMP degradation and a type III/IV PDE inhibitor which inhibits cyclic AMP breakdown (Nicholson *et al.*, 1991). Additionally, we studied the effect of chemical sympathetic denervation on the neural relaxant response to EFS in order to ascertain whether adrenergic nerves also release NO.

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Methods

Tissue preparation

Branch PA rings from male Dunkin-Hartley guinea-pigs (300–350 g) were prepared and mounted in organ baths for tension recording as described previously (Liu *et al.*, 1992). All the vessel rings were allowed to equilibrate at their optimal resting tension (700 mg) in the baths for at least 60 min and washed with fresh Krebs-Henseleit (K-H) solution every 20 min during the equilibration period. The K-H solution is composed of (mM): NaCl 118, KCl 5.9, $MgSO_4.7H_2O$ 1.2, $CaCl_2.6H_2O$ 2.5, NaH_2PO_4 1.2, glucose 5.6 and $NaHCO_3$ 25.5. Endothelium was removed from all vessel rings by gently rubbing their intimal surfaces with a piece of fine abrasive paper as previously described (Liu *et al.*, 1992). Removal of endothelium was confirmed by loss of the relaxant response to substance P prior to the experiment in all vessel rings (Bolton & Clapp, 1986; Maggi *et al.*, 1990) and by histological examination at the end of the experiment in 12 of these rings.

Nerve stimulation

Electrical field stimulation (EFS, 50 V, 0.2 ms, for 15 s at 4 min interval) was applied via two platinum wire electrodes positioned at each end of the vessel ring and connected to a Grass S88 stimulator (Grass Instruments, Quincy, U.S.A.). The vessel rings were preincubated with phentolamine, atropine and propranolol (all 1 μM) for 20 min and precontracted with 3 μM U44069 (9,11-dideoxy-9α, 11α-epoxymethano-prostaglandin $F_{2\alpha}$). After a stable contraction was obtained, frequency-response relationships (1–24 Hz) were constructed in the chemical adrenergic denervation study. In other studies, the vessel rings were stimulated with fixed EFS stimuli (50 V, 0.2 ms, 16 Hz, for 15 s). After 3–4 reproducible responses had been obtained, the vessel rings were incubated with N^G-monomethyl L-arginine (L-NMMA), N^G-nitro L-arginine methyl ester (L-NAME), methylene blue or pyrogallol or their respective vehicles for 10 min and a further 4–5 EFS stimulations performed. EFS was continued until the effect of these inhibitory agents was maximal. The effect of an increase in vascular tone on the NANC relaxation was evaluated by comparing the EFS (16 Hz)-induced relaxation before and after the vascular tone had been raised further by 5-hydroxytryptamine (5-HT) (10 μM) in a group of U44069-precontracted PA rings. In the reversibility studies, L-arginine or superoxide dismutase were added to the organ baths at the peak of L-NMMA or pyrogallol effects. The effects of two PDE inhibitors, zaprinast (1 μM) and zardaverine (0.1 μM) on EFS-induced relaxation were evaluated in 1 μM U44069-precontracted PA rings. Similarly, 3–4 reproducible relaxant responses to EFS were recorded before and after the addition of the two inhibitors. In two groups of non-precontracted PA rings, 3–4 reproducible contractile responses to exogenous noradrenaline (NA) were recorded before and after treatment of the vessel rings with 100 μM L-NMMA. To match the NA-induced contraction with EFS-induced contraction, variable concentrations (20–30 nM) of NA were used.

Concentration-response curve

Paired PA rings were precontracted with 3 μM U44069 and concentration-response curves to zaprinast in the absence and presence of 100 μM pyrogallol were obtained.

6-Hydroxydopamine pretreatment

To achieve chemical sympathetic denervation, a group of guinea-pigs (300 g) were injected with 6-hyroxydopamine (6-OHDA 20 mg kg^{-1}), intraperitoneally, every 12 h, on 4 occasions. A batch matched control group received 4 intraperitoneal injections of the same volume of vehicle for 6-OHDA (ascorbic acid, 1 mg ml^{-1}) at the same intervals. On the third day after the first 6-OHDA (or vehicle) injection, the animals were killed and pulmonary arteries dissected for use. The effectiveness of this 6-OHDA treatment protocol in destroying the adrenergic nerve endings has been proved previously by Tranzer & Thoenen (1968) using ultrastructural examination.

Cyclic GMP determination

Tissue cyclic GMP levels were determined in endothelium-denuded branch PA rings that had been equilibrated under optimal resting tension and subjected to precontraction (3 μM U44069) as performed in other rings. Tension was monitored and EFS (50 V, 0.2 ms, 16 Hz, for 15 s) applied, whereupon the tissue was quickly frozen in liquid nitrogen. Samples were extracted and assayed for cyclic GMP by radioimmunoassay as previously described (Crawley *et al.*, 1992).

Histological examination

At the end of the functional studies, 12 paired branch PA rings (6 rubbed and 6 unrubbed) from 6 animals were chosen in a randomized manner and fixed in 10% formal saline for histological examination. Three μM sections were cut and stained with haematoxylin and eosin. Sections were then examined under a light microscope from which it was possible to confirm the presence or absence of an endothelial lining.

Drugs

The following drugs were used: noradrenaline hydrochloride, substance P, tetrodotoxin, phentolamine hydrochloride, pyrogallol, atropine sulphate, propranolol hydrochloride, 5-hydroxytryptamine, methylene blue, U44069 (9,11-dideoxy-9α,11α-epoxymethano-prostaglandin $F_{2\alpha}$), L-arginine hydrochloride, D-arginine hydrochloride, superoxide dismutase, N^G-monomethyl L-arginine, N^G-nitro L-arginine methyl ester, 6-hydroxydopamine (all from Sigma, Poole, U.K.), zaprinast (Rhone-Poulenc, Dagenham, U.K.) and zardaverine (Byk-Gulden Pharmaceutics, Koustanz, Germany).

Analysis of results

Contraction is presented as absolute tension (mg), and relaxation as a percentage of the U44069-induced contraction. The contractions induced by EFS and NA after L-NMMA or its vehicle treatment were compared with those before treatment and calculated as percentage augmentations. Likewise, vasorelaxant responses to EFS in the presence of various inhibitors or their vehicles were compared with EFS responses before adding these inhibitors or vehicles, and expressed as percentage inhibition. Mean values at each concentration or frequency point were used to compare the difference between two frequency-response or concentration-response curves. Values are presented as mean ± s.e. and n indicates the number of animals from which the tissues were dissected. Statistical analysis of results was performed by use of paired and unpaired Student's t test or by one way analysis of variance followed by Bonferroni corrected t test, when multiple comparisons were made. A P value < 0.05 was considered to be significant.

Results

Histological examination

Light microscopic examination revealed that an intact endothelial lining was consistently present in the unrubbed

and absent in the rubbed rings, confirming the successful removal of vascular endothelium.

Effects of L-NMMA and L-NAME

In the presence of adrenergic and cholinergic blockade, EFS induced a transient, frequency-dependent relaxation of the U44069-precontracted, endothelium-denuded branch PA rings, which was abolished by 1 μM tetrodotoxin. EFS induced a reduction of the U44069-induced vascular tone of 3 ± 1, 10 ± 2.5, 18.6 ± 2.7, 26.9 ± 3.9, 31 ± 3.5 and 29.7 ± 3.8% at 1, 2, 4, 8, 16 and 24 Hz respectively. Treatment with L-NMMA (100 μM) or L-NAME (30 μM) had no effect on baseline or U44069-generated tension, but markedly inhibited EFS-elicited relaxation (Figure 1). The inhibitory effect of L-NMMA was completely reversed by 300 μM L-arginine but was unaffected by an identical concentration of D-arginine (Figure 2).

Effects of methylene blue and zaprinast

U44069-induced contraction was 725 ± 72 and 693 ± 48 mg for control and methylene blue-treated rings respectively ($P > 0.05$, $n = 7$). In the presence of phentolamine, atropine and propranolol (all 1 μM), EFS (16 Hz) induced a reproducible relaxation of U44069-precontracted vessel rings. Methylene blue (5 μM) treatment caused a further increase in vascular tension of 192 ± 50 mg ($n = 7$) and a significant ($P < 0.001$) inhibition of the NANC relaxation (Figure 1). To determine whether the tension increase by methylene blue was contributory to the inhibition of the NANC relaxation, the vascular tone of precontracted rings was elevated further with 5-HT (10 μM). Increase of the vascular tone by 5-HT (225 ± 48 mg) had no significant effect on the relaxant response to EFS (Figure 1). We also evaluated the effects of zaprinast (1 μM) and zardaverine (0.1 μM), which are type V and type III/IV specific PDE inhibitors respectively, on the relaxant response to EFS. To minimize the effect of functional antagonism on the possible potentiation of the EFS-induced relaxation by these inhibitors, vessel rings were precontracted with 1 μM U44069 and stimulated at 4 Hz. U44069-generated vascular tone was 623 ± 123, 589 ± 88 and 551 ± 104 mg for control, zaprinast and zardaverine-treated rings respectively ($P > 0.05$, $n = 5$). Zaprinast (1 μM) significantly potentiated the EFS-induced relaxation, but zardaverine had no effect (Figure 3).

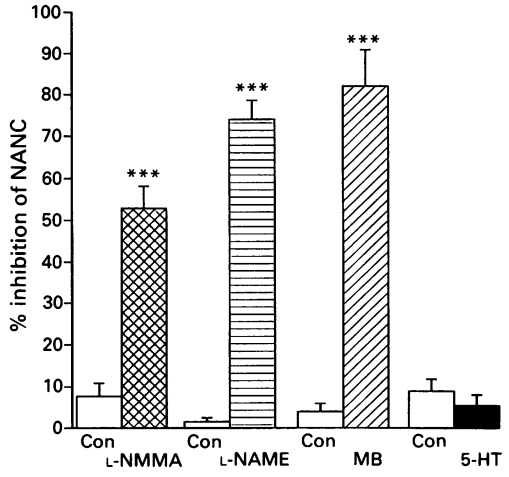

Figure 1 Inhibition of the nonadrenergic noncholinergic (NANC) relaxant response to electrical field stimulation (EFS, 50 V, 0.2 ms, 16 Hz, for 15 s) in U44069 (3 μM)-precontracted endothelium-denuded branch pulmonary artery (PA) rings by N^G-monomethyl L-arginine (L-NMMA, 100 μM), N^G-nitro L-arginine methyl ester (L-NAME, 30 μM) and methylene blue (MB, 5 μM), and the effect of increase in vascular tension by 5-hydroxytryptamine (5-HT, 10 μM) on NANC relaxation. MB further increased the vascular tension by 192 ± 50 mg and greatly inhibited the NANC relaxation. 5-HT further increased the vascular tone by 225 ± 48 mg, but had no effect on the NANC relaxation. Con: vehicle controls. ***$P < 0.001$, compared with vehicle control rings, $n = 5$–7.

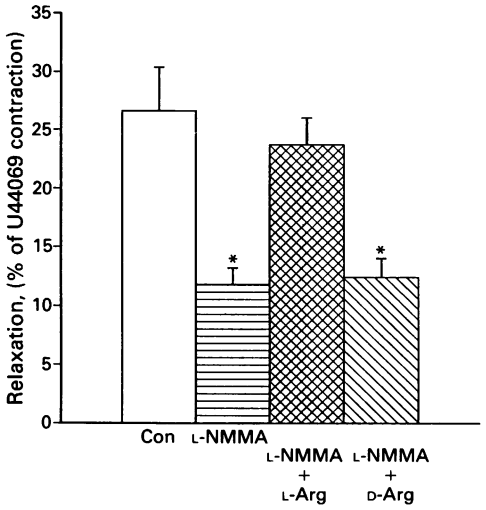

Figure 2 Reversibility of N^G-monomethyl L-arginine (L-NMMA) induced inhibition of nonadrenergic, noncholinergic (50 V, 0.2 ms, 16 Hz, for 15 s) vasodilator response in endothelium-denuded branch PA rings precontracted with U44069 (3 μM) by L-arginine (L-Arg) and D-arginine (D-Arg). 300 μM L-Arg, but not 300 μM D-Arg completely reversed the inhibitory effect of 100 μM L-NMMA on NANC relaxation. Relaxation was expressed as a percentage of the U44069-generated contraction. *$P < 0.05$, compared with vehicle control (Con) and L-NMMA plus L-Arg-treated rings, $n = 5$.

EFS-induced cyclic GMP formation

Tissue cyclic GMP content was determined in 3 groups ($n = 6$, in each group) of endothelium-denuded branch PA rings precontracted with 3 μM U44069. EFS (50 V, 0.2 ms, 16 Hz, for 15 s) caused a 3 fold increase in tissue cyclic GMP level. The EFS-induced cyclic GMP accumulation was significantly inhibited by pretreatment with 100 μM L-NMMA (Figure 4).

Effects of pyrogallol

U44069-generated contractions were 562 ± 42 and 512 ± 47 mg in control and pyrogallol-treated rings, respectively ($P > 0.05$, $n = 6$). EFS (16 Hz) induced a reduction of 18.7 ± 3.7% of the U44069-induced tone. Pyrogallol (100 μM) markedly inhibited the relaxant response to EFS (Figure 5), but had no effect on zaprinast-induced relaxation. Zaprinast-evoked relaxations were 11 ± 4, 21 ± 6, 35 ± 9 and 64 ± 8% in control rings and 12 ± 3, 21 ± 5, 36 ± 7 and 78 ± 2% in pyrogallol (100 μM)-treated rings at concentrations of 3, 10, 30 and 100 μM respectively ($n = 5$, $P > 0.05$). The relaxation to EFS was fully restored by adding superoxide dismutase (100 u ml^{-1}) at the peak of pyrogallol-induced inhibition (Figure 5).

Effect of L-NMMA on the contractile responses to EFS and noradrenaline

In endothelium-denuded PA rings at resting tension, EFS (50 V, 0.2 ms, 4 Hz) caused a concentration of 46 ± 5 mg, which was blocked by phentolamine (1 μM) and tetrodotoxin (1 μM). Treatment of the vessel rings with L-NMMA (100 μM) significantly enhanced the contractile response to

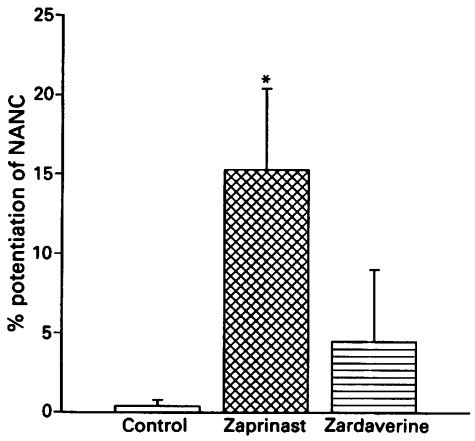

Figure 3 Potentiation of the nonadrenergic, noncholinergic (NANC) vasodilator response to electrical field stimulation (50 V, 0.2 ms, 4 Hz for 15 s) in the U44069 (1 μM) precontracted endothelium-denuded branch pulmonary artery (PA) rings by the cyclic GMP specific phosphodiesterase (PDE) inhibitor, zaprinast (1 μM), but not by the cyclic AMP specific PDE inhibitor, zardaverine (0.1 μM). $*P<0.05$, compared with vehicle control rings, $n=5$.

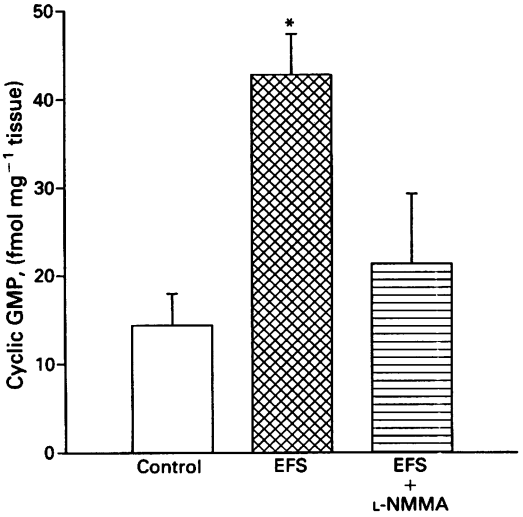

Figure 4 Stimulation of cyclic GMP formation by electrical field stimulation (EFS, 50 V, 0.2 ms, 16 Hz, for 15 s) in the endothelium-denuded branch pulmonary artery rings precontracted with 3 μM U44069. Control rings were subjected to precontraction and quick frozen. EFS stimulated rings, and rings treated with 100 μM N^G-monomethyl L-arginine (L-NMMA), were precontracted and quick frozen at 15 s after the onset of EFS. $*P<0.05$, compared with both control and L-NMMA-treated rings, $n=6$.

EFS ($n=9$, $P<0.01$, compared with control rings), but had no significant effect on the matched contractile response to NA (Figure 6). This effect of L-NMMA on the adrenergic contractions was completely reversed by 300 μM L-arginine, whereas an identical concentration of D-arginine was ineffective (Figure 7).

Effect of sympathetic denervation on NANC relaxation

In the presence of atropine (1 μM), U44069 (3 μM) contracted the endothelium-denuded PA rings from both vehicle and 6-OHDA pretreated animals by 1162 ± 152 and 1256 ± 153 mg respectively ($n=5$, $P>0.05$). EFS reduced U44069-generated vascular tensions by 2 ± 1, 12 ± 2, 25 ± 3, 34 ± 4, 41 ± 5 and 41 ± 5% in PA rings from vehicle-treated animals, and by 6 ± 2, 14 ± 4, 26 ± 5, 30 ± 5, 32 ± 4 and

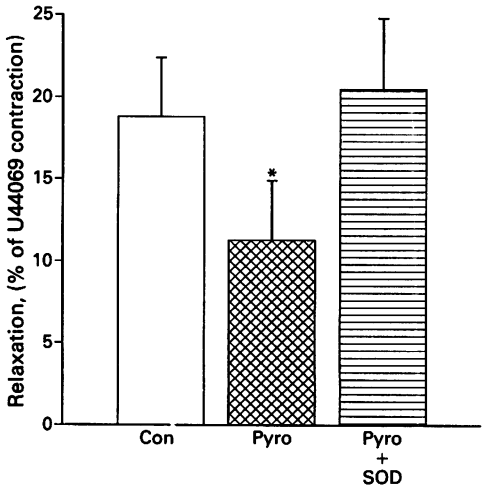

Figure 5 Inhibition of the nonadrenergic, noncholinergic vasodilator response to electrical field stimulation (50 V, 0.2 ms, 16 Hz, for 15 s) by pyrogallol (Pyro, 100 μM) and reversal of this inhibition by superoxide dismutase (SOD, 100 u ml^{-1}) in the endothelium-denuded branch pulmonary artery (PA) rings. Relaxation was expressed as a percentage of U44069-induced contraction. Con: control rings. $*P<0.05$, $n=6$.

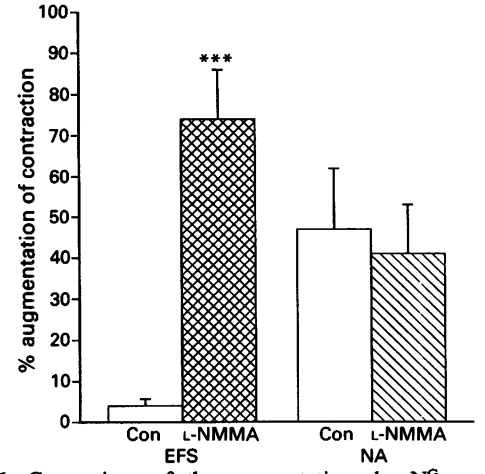

Figure 6 Comparison of the augmentations by N^G-monomethyl L-arginine (L-NMMA, 100 μM) of the matched contractile responses to electrical field stimulation (EFS, 50 V, 0.2 ms, 4 Hz, for 15 s) and exogenous noradrenaline (NA, 20–30 nM). Con: vehicle control rings. $***P<0.001$, compared with control rings, $n=6$.

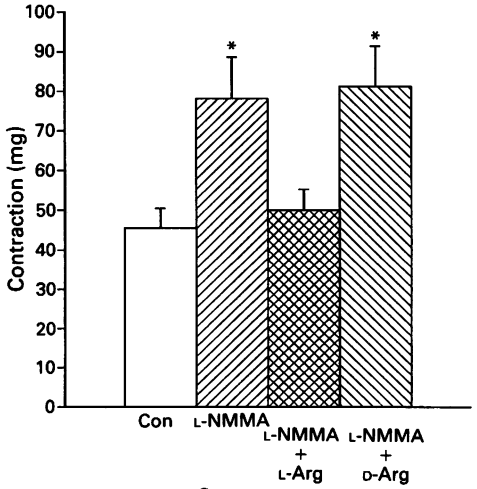

Figure 7 Reversibility of N^G-monomethyl L-arginine (L-NMMA)-induced augmentation of the contractile response to electrical field stimulation (EFS, 50 V, 0.2 ms, 4 Hz, for 15 s). L-Arginine (L-Arg, 300 μM), but not D-arginine (D-Arg, 300 μM) reversed the augmenting effect of L-NMMA (100 μM). $*P<0.05$, compared with control (Con) and, L-NMMA plus L-Arg group rings, $n=6$.

28 ± 4% in the PA rings from 6-OHDA-treated animals, at a frequency of 1, 2, 4, 8, 16 and 24 Hz respectively ($P > 0.05$, $n = 5$), indicating that chemical adrenergic denervation by 6-OHDA did not affect the NO-mediated neural relaxation. The effectiveness of adrenergic denervation was confirmed by the abolition of the adrenergic contractile response to EFS in the PA rings from 6-OHDA-treated animals, whereas EFS caused a frequency-dependent adrenergic contraction in the rings from vehicle-treated animals (data not shown).

Discussion

In previous studies we demonstrated that, in the presence of adrenergic and cholinergic blockade, stimulation of the intramural nerves of the guinea-pig branch PA by EFS induced a tetrodotoxin-sensitive relaxation. This relaxation was reduced but not abolished after endothelial denudation, indicating that both endothelium-dependent and endothelium-independent mechanisms were involved (Liu *et al.*, 1992). We also demonstrated that the endothelium-dependent component may be mediated by ATP. Here, we expand our previous study by showing that the endothelium-independent component of NANC relaxation is mediated predominantly through NO and cyclic GMP formation. We have also provided evidence against NO as a cotransmitter of sympathetic nerves.

NO is synthesized from the semi-essential amino acid L-arginine (Palmer *et al.*, 1988a,b; Schmidt *et al.*, 1988; Moncada *et al.*, 1991) by NO synthase. L-NMMA and L-NAME are specific inhibitors of NO synthesis (Palmer *et al.*, 1988b; Johns *et al.*, 1990; Moore *et al.*, 1990; Moncada *et al.*, 1991). NO exerts its effect via the activation of guanylyl cyclase and elevation of intracellular cyclic GMP concentration (Katsuki *et al.*, 1977; Ignarro, 1989). Methylene blue is a specific inhibitor of this enzyme (Martin *et al.*, 1985). Pyrogallol also inhibits NO action by generating superoxide anions that inactivate NO (Moncada *et al.*, 1986). Our results showed that L-NMMA and L-NAME markedly inhibited the endothelium-independent component of the NANC relaxation. The inhibitory effects of L-NMMA on the NANC relaxation were completely reversed by the NO precursor L-arginine, whereas D-arginine was ineffective. Additionally, pyrogallol also significantly reduced the NANC relaxation, whereas it had no effect on zaprinast-induced relaxation, indicating that its effect is not due to nonspecific inhibition of smooth muscle relaxation. Furthermore, the inhibitory action of pyrogallol was completely reversed by adding superoxide dismutase. All these results are consistent with the hypothesis that NO mediates the NANC relaxation. This NANC relaxation is highly sensitive to methylene blue (82% inhibition at 5 μM) and is significantly potentiated by zaprinast, a type V PDE inhibitor which prevents cyclic GMP degradation. By contrast, zardaverine, a type III/IV PDE inhibitor which prevents cyclic AMP degradation, had no significant effect on the NANC relaxation. Moreover, EFS induced a 3 fold increase in tissue cyclic GMP concentration. The EFS-induced elevation in tissue cyclic GMP concentration was markedly inhibited by L-NMMA. These results indicate that cyclic GMP is a key component in the mechanism underlying this neural relaxation in the guinea-pig branch PA. Thus, the endothelium-independent component of the NANC relaxation in this vessel is mediated through NO and cyclic GMP generation.

Although a large body of evidence supports a role for NO in the mediation of NANC relaxation, the possibility that other nerves may also release NO has not been explored. Moreover, NO may be released as a sympathetic nerve cotransmitter rather than as a primary transmitter from separate NANC nerves. In most reported studies, either adrenoceptor blockade or catecholamine depletion was employed to exclude the adrenergic response during EFS. Because catecholamine depletion does not necessarily mean the destruction of adrenergic nerve endings, such studies cannot preclude the possibility that adrenergic nerves may release NO. 6-Hydroxydopamine is neurotoxic agent that selectively destroys sympathetic nerves (Tranzer & Thoenen, 1968; Bennett *et al.*, 1970). The failure of 6-OHDA treatment to affect the relaxant response to EFS in precontracted branch PA rings indicates that the NO mediating this neural relaxation in this vessel is not released by, or related to, adrenergic nerve activation. We could not rule out the possibility that NO is released from parasympathetic nerves as a cotransmitter with acetylcholine, but NO may also be released by separate NANC nerves. The possibility that NO may be released from cholinergic nerves cannot be further investigated until it is possible to deplete these nerves selectively.

If the hypothesis that NO is a NANC transmitter is correct, we reasoned that inhibition of NO synthesis or release should augment adrenergic contraction. Application of EFS activates both adrenergic and NANC nerves, and NO released from NANC nerves will exert a functional antagonism on adrenergic contraction, which is mediated by noradrenaline acting on α-adrenoceptors (Liu *et al.*, 1991b). Our demonstration that L-NMMA induced an L-arginine-reversible augmentation of adrenergic contraction in the endothelium-denuded PA rings provides further evidence for NO as a NANC neurotransmitter.

In the endothelium-denuded guinea-pig PA rings, both L-NMMA and L-NAME had no effects on either the basal or U44069-generated tension, suggesting that there may be no smooth muscle-derived NO production in this tissue, which is in contrast to the endothelium-denuded bovine pulmonary arteries where both basal and stimulated release of smooth muscle-derived NO was demonstrated (Wood *et al.*, 1990). The ability of methylene blue to elevate further U44069-generated tension indicates that there is an intrinsic NO-independent generation of cyclic GMP, which may also modulate the intrinsic smooth tone. Similar findings have been made in bovine pulmonary arteries (Ignarro *et al.*, 1987).

Based on the equal enhancement of the contractile response to both EFS and the matched contraction to exogenous NA, we concluded that L-NMMA augments adrenergic contraction by a postjunctional mechanism in the endothelium intact guinea-pig branch PA rings (Liu *et al.*, 1991b). In the present study, L-NMMA potentiated the contractile response to adrenergic nerve stimulation but had not effect on the matched contraction induced by exogenous NA, suggesting that L-NMMA augments adrenergic contraction via a prejunctional mechanism in the endothelium-denuded PA rings. The presence or absence of endothelium could explain this difference. In intact rings, 100 μM L-NMMA enhanced the adrenergic contraction by 341%, whereas it caused only 74% augmentation of the contraction in endothelium-denuded rings. These results suggest that L-NMMA enhances adrenergic contraction mainly through the inhibition of endothelially-derived NO, although inhibition of neurally-released NO is also contributory.

In summary, together with our previous studies, our results indicate that in the guinea-pig branch pulmonary artery, EFS induces a NANC vasodilator response. This neural relaxation is mediated via two pathways, an endothelium-dependent, and an endothelium-independent pathway. The former pathway is at least partially mediated by ATP, which causes vasodilatation through the activation of endothelial P_{2y}-purinoceptors and NO release. The endothelium-independent pathway is mediated predominantly by NO and cyclic GMP.

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References

BENNETT, T.G., BURNSTOCK, G., COBB, J.L.C. & MALMFORS, T. (1970). An ultrastructural and histochemical study of the short-term effect of 6-hydroxydopamine on adrenergic nerves in the domestic fowl. *Br. J. Pharmacol.*, **38**, 802–809.

BOLTON, T.B. & CLAPP, L.H. (1986). Endothelium-dependent relaxant actions of carbachol and substance P in arterial smooth muscle. *Br. J. Pharmacol.*, **87**, 713–723.

CRAWLEY, D.E., ZHAO, L., GIEMBYCZ, M.A., LIU, S.F., BARNES, P.J., WINTER, R.J.D. & EVANS, T.W. (1992). Chronic hypoxia selectively impairs soluble guanylyl cyclase-mediated pulmonary arterial relaxation in the rat. *Am. J. Physiol.*, (in press).

GILLESPIE, J.S., LIU, X.R. & MARTIN, W. (1990). The transmitter of the non-adrenergic, non-cholinergic inhibitory nerves to smooth muscle of the genital system. In *Nitric Oxide from L-Arginine: a Bioregulatory System*. ed. Moncada, S. & Higgs, E.A., pp. 147–164. Amsterdam: Elsevier.

IGNARRO, I.J. (1989). Endothelium-derived nitric oxide: actions and properties. *FASEB J.*, **3**, 31–36.

IGNARRO, L.J., BYRNS, R.E. & WOOD, K.S. (1987). Endothelium-dependent modulation of cGMP levels and intrinsic smooth muscle tone in isolated bovine intrapulmonary artery and vein. *Circ. Res.*, **60**, 82–92.

JOHNS, R.A., PEACH, M.J., LINDEN, J. & TICHOTSKY, A. (1990). N^G-monomethyl L-arginine inhibits endothelium-derived relaxing factor-stimulated cyclic GMP accumulation on co-cultures of endothelial and vascular smooth muscle cells by an action specific to the endothelial cell. *Circ. Res.*, **67**, 979–985.

KATSUKI, S., ARNOLD W.P., MITTAL, C. & MURAD, F. (1977). Stimulation of guanylate cyclase by sodium nitroprusside, nitroglycerin and nitric oxide in various preparations and comparison to the effects of sodium azide and hydroxylamine. *J. Cycl. Nucleotide Res.*, **3**, 23–25.

LIU, S.F., CRAWLEY, D.E., BARNES, P.J. & EVANS, T.W. (1991a). Endothelium-derived relaxing factor inhibits hypoxic pulmonary vasoconstriction in rats. *Am. Rev. Respir. Dis.*, **143**, 32–37.

LIU, S.F., CRAWLEY, D.E., EVANS, T.W. & BARNES, P.J. (1991b). Endogenous nitric oxide modulates adrenergic neural vasoconstriction in guinea-pig pulmonary artery. *Br. J. Pharmacol.*, **104**, 565–569.

LIU, S.F., CRAWLEY, D.E., EVANS, T.W. & BARNES, P.J. (1992). Endothelium-dependent nonadrenergic, noncholinergic neural relaxation in guinea pig pulmonary artery. *J. Pharmacol. Exp. Ther.*, **260**, 541–548.

MAGGI, C.A., PATACCHINI, R., PERRETTI, F., TRAMONTANA, M., MANZINI, S., GEPPETTI, P. & SANTICIOLI, I. (1990). Sensory nerves, vascular endothelium and neurogenic relaxation of the guinea-pig isolated pulmonary artery. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **342**, 78–84.

MARTIN, W., VILLANI, G.M., JOTHIANANDAN, D. & FURCHGOTT, R.F. (1985). Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by haemoglobin and methylene blue in the rabbit aorta. *J. Pharmacol. Exp. Ther.*, **232**, 708–716.

MONCADA, S., PALMER, R.M.J. & GRYGLEWSKI, R.J. (1986). Mechanism of action of some inhibitors of endothelium-derived relaxing factor. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 9164–9168.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.*, **43**, 109–142.

MOORE, P.K., AL-SWAYEH, A.O., CHONG, N.W.S., EVANS, R.A. & GIBSON, A. (1990). N^G-nitro-L-arginine (L-NOARG), a novel L-arginine-reversible inhibitor of endothelium-dependent vasodilatation *in vitro*. *Br. J. Pharmacol.*, **99**, 408–412.

NICHOLSON, C.D., CHALLISS, R.A.J. & SHAHID, M. (1991). Differential modulation of tissue function and therapeutic potential of selective inhibitors of cyclic nucleotide phosphodiesterase isoenzymes. *Trends Pharmacol. Sci.*, **12**, 20–27.

PALMER, R.M.J., ASHTON, D.S. & MONCADA, S. (1988a). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature*, **333**, 664–666.

PALMER, R.M.J., REES, D.D., ASHTON, D.S. & MONCADA, S. (1988b). L-arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. *Biochem. Biophys. Res. Commun.*, **153**, 1251–1256.

RAND, M.J. (1992). Nitrergic transmission: nitric oxide as a mediator of non-adrenergic, non-cholinergic neuro-effector transmission. *Clin. Exp. Pharmacol. Physiol.*, **19**, 147–169.

SCHMIDT, H.H.H.W., NAU, H., WITTFOHT, W., GERLACH, J., PRESCHER, K.E., KLEIN, M.M., NIROOMAND, F. & BOHME, E. (1988). Arginine is physiological precursor of endothelium-derived nitric oxide. *Eur. J. Pharmacol.*, **154**, 213–216.

TRANZER, J.P. & THOENEN, H. (1968). An electron microscopic study of selective, acute degeneration of sympathetic nerve terminals after acute administration of 6-hydroxydopamine. *Experientia*, **24**, 155–156.

WOOD, K.S., BUGA, G.M., BYRNS, R.E. & IGNARRO, L.J. (1990). Vascular smooth muscle-derived relaxing factor (EDRF) and its similarity to nitric oxide. *Biochem. Biophys. Res. Commun.*, **170**, 80–88.

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