

Endogenous nitric oxide modulates adrenergic neural vasoconstriction in guinea-pig pulmonary artery

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1 Electrical field stimulation (EFS) of guinea-pig isolated pulmonary artery induced a frequency-dependent contraction. This was abolished by tetrodotoxin (1 μM) and prevented by phentolamine and prazosin (both 1 μM), indicating a role for α_1 -adrenoceptors activated by noradrenaline (NA) released from perivascular adrenergic nerves.

2 L-N^G-monomethyl arginine (L-NMMA, 0.3–100 μM) caused a concentration-dependent enhancement of the EFS-induced contraction with a 3.4 ± 0.5 fold increase at 100 μM ($n = 6$). The augmenting effect of 30 μM L-NMMA on the contraction to EFS was completely reversed by 100–300 μM L-arginine, but not by an identical concentration of D-arginine.

3 The contractile response to exogenous NA was similarly enhanced by 30 μM L-NMMA (2.9 ± 0.6 fold increase, $n = 5$).

4 The contractile responses to exogenous phenylephrine and prostaglandin F_{2 α} which matched the contraction to EFS (4 Hz) were equally augmented by 30 μM L-NMMA.

5 In vessel rings submaximally contracted with the thromboxane analogue U44069 (2 μM), the selective α_2 -adrenoceptor agonist UK14304 induced concentration-dependent relaxation, which was abolished by removal of endothelium. NA had little relaxant effect on these precontracted vessel rings unless in the presence of prazosin (1 μM).

6 Indomethacin had no significant effect on the contractile response to EFS or NA, indicating that vasodilator cyclo-oxygenase products such as prostacyclin are not involved in modulating these responses.

7 Our results suggest that endogenous nitric oxide inhibits the contractile response to adrenergic nerve stimulation in the guinea-pig pulmonary artery by a postjunctional mechanism, but release of prostacyclin does not modulate these responses. Basal release of nitric oxide from endothelial cells may account for this inhibition.

Keywords: Pulmonary artery; innervation; adrenoceptor; nitric oxide; prostacyclin; endothelium; EDRF

Introduction

Endothelium plays an important modulatory role in the response of vascular smooth muscle to a variety of stimuli (De Mey *et al.*, 1982; Furchgott, 1984; Bullock *et al.*, 1986). Endothelium has an inhibitory effect on the contractile responses to α -adrenoceptor stimulation either by exogenous administration of noradrenaline (Cocks & Angus, 1983) and other α -adrenoceptor agonists (Egleme *et al.*, 1984; Lues & Schumann, 1984; Martin *et al.*, 1986) or by adrenergic nerve stimulation in rabbit carotid artery (Tsfamariam *et al.*, 1987; Cohen & Weisbrod, 1988) and rat caudal artery (Hynes *et al.*, 1988). This inhibitory effect of endothelium on the contractile response to adrenergic nerve stimulation is believed to be due to the release of relaxing factor(s) from endothelial cells (Tsfamariam *et al.*, 1987; Tsfamariam & Halpern, 1987; Cohen & Weisbrod, 1988). The factor(s) responsible for the inhibition of neurogenic adrenergic vasoconstriction has not yet been identified.

The pulmonary circulation differs markedly from the systemic circulation in several aspects; the different response to acute hypoxia, which causes contraction in pulmonary, but relaxation in systemic vessels (Staub, 1985) is well known. There are also differences in other control mechanisms (Fishman, 1990), the distribution (Hyman *et al.*, 1989) and affinities (Shaul *et al.*, 1990) of adrenoceptors, and in the response to some peptides, such as vasoactive intestinal polypeptide (Sata *et al.*, 1986). Although the presence of endothelial α_2 -adrenoceptors has been demonstrated in the systemic vessels of several regions (Angus *et al.*, 1986; Bullock *et al.*, 1986; Vanhoutte & Miller, 1989), the presence of these

receptors on pulmonary artery is still uncertain (Vanhoutte & Miller, 1989).

The two major relaxing factors released from vascular endothelium are prostacyclin (PGI₂) and endothelium-derived relaxing factor (EDRF). EDRF has been characterized as nitric oxide (NO) or a related compound (Palmer *et al.*, 1987; Ignarro *et al.*, 1987; Moncada *et al.*, 1988; 1989), which is synthesized from L-arginine (Palmer *et al.*, 1988a,b; Schmidt *et al.*, 1988; Moncada *et al.*, 1989) in vascular endothelial cells. The L-arginine analogue L-N^G-monomethyl arginine (L-NMMA) is a specific inhibitor of NO production (Palmer *et al.*, 1988b; Moncada *et al.*, 1989; Johns *et al.*, 1990). Using the inhibitor and precursor of NO formation and the PGI₂ synthesis inhibitor indomethacin, we have explored the modulatory role of endogenous NO and PGI₂ on the contractile response to adrenergic nerve stimulation in guinea-pig pulmonary artery. We also investigated the possible presence of endothelial α_2 -adrenoceptors.

Methods

Tissue preparation

Male Dunkin-Hartley guinea-pigs (300–350 g) were killed by cervical dislocation and pulmonary arteries were rapidly removed. The two branches of the vessels were carefully dissected, cut into rings 2 mm in length, mounted over a pair of rigid wires and suspended in an organ bath containing 2 ml Krebs-Henseleit (KH) solution of the following composition (mM): NaCl 118, KCl 5.9, MgSO₄ 1.2, CaCl₂ 2.5, NaH₂PO₄ 1.2, glucose 5.6, NaHCO₃ 25.5, EDTA 0.027 and ascorbic acid 0.03. One wire was fixed and the other attached to a force transducer (FT.03 Grass Instruments, Quincy, U.S.A.).

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Changes in isometric force were recorded on a polygraph (Grass Model 7). The KH solution was maintained at 37°C and bubbled with a mixture of 95% O₂ and 5% CO₂. Initially, the vessel rings were repeatedly stimulated by EFS (50 V, 0.2 ms duration, 16 Hz, 15 s) starting from zero resting tension, until the maximum response to EFS was achieved at an increment of 100 mg, and the optimal resting tension determined. Rings were then allowed to equilibrate at this tension (700 mg) in the bath for at least 60 min and washed with fresh KH solution every 20 min during the equilibration period.

Nerve stimulation

Electrical field stimulation (EFS) was applied by 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.). To activate the intramural nerves without inducing a myogenic response, voltage-duration curves were performed in the presence and absence of 0.3 μM tetrodotoxin by recording the smallest detectable response (<2% of maximal response) and optimal parameters (50 V, 0.2 ms duration) determined in a preliminary study. Frequency-response relationships were constructed in a frequency range of 1–16 Hz, each stimulation being applied for 15 s every 4 min. To assess the nature of the contractile response to EFS, the vessel rings were incubated with tetrodotoxin (1 μM), phentolamine (1 μM) or prazosin (1 μM) for 15 min and stimulated at 1–16 Hz or 8–24 Hz in separate experiments. To assess the effects of endogenous NO and PGI₂ on the adrenergic contraction, vessel rings were stimulated with fixed electrical stimuli (50 V, 0.2 ms, 4 Hz) at 4 min intervals. When responses were constant, the vessel rings were incubated with inhibitory agents for 10 min and a further 3–4 stimulations performed. In the reversibility study, L- or D-arginine were added to the organ bath after the maximal effect of a dose of L-NMMA was achieved. To clarify the relative pre- or postjunctional action of endogenous NO and PGI₂, the effect of 30 μM L-NMMA and 1 μM indomethacin on the matched contractions induced by EFS (4 Hz) and exogenous noradrenaline (NA) were compared in paired rings from the same vessel. Comparisons were also made among the matched contractions induced by EFS, NA, phenylephrine (PE) and prostaglandin F_{2α} (PGF_{2α}) in paired vessel rings. To match the EFS-induced contraction, variable concentrations of NA (0.1–0.3 μM), PE (0.08–0.1 μM) and PGF_{2α} (0.3–0.6 μM) were used.

Concentration-response curves

To determine the effect of L-NMMA on contraction to exogenous NA, concentration-response curves to NA were constructed in the presence and absence of 30 μM L-NMMA. In the relaxation study, paired vessel rings were precontracted with 2 μM U44069 and concentration-relaxation curves to NA in the absence and presence of prazosin (1 μM), prazosin plus propranolol (both 1 μM) and prazosin plus yohimbine (both 1 μM), and to UK14304 in the presence and absence of an intact endothelium or 30 μM L-NMMA were obtained.

Drugs

The following drugs were used: noradrenaline hydrochloride, prazosin hydrochloride, yohimbine hydrochloride, indomethacin, U44069 (9,11-dideoxy-11α,9α-epoxymethano-prostaglandin F_{2α}), tetrodotoxin, phentolamine hydrochloride, propranolol hydrochloride, L-arginine hydrochloride, D-arginine hydrochloride, sodium nitroprusside (Sigma, Poole, Dorset), prostaglandin F_{2α} solution (Upjohn, Crawley, Sussex), UK14304 (Pfizer, Sandwich, Kent) and L-N^G-monomethyl arginine (a generous gift from Dr S. Moncada, Wellcome Research Laboratory, Beckenham, Kent).

Analysis of results

Contraction is presented in absolute tension or expressed as a percentage of its maximum. An individual concentration-response curve to NA was fitted and the EC₅₀ value estimated by use of a computer programme (Graph PAD InPlot, Graph PAD Software, San Diego, CA, U.S.A.). Relaxation was expressed as a percentage of the U44069-induced contraction or the maximum relaxation to nitroprusside (in the endothelial denudation study). The contractile responses to EFS and other vasoconstrictors in the presence of the inhibitors or their vehicles were compared with the responses before adding these inhibitors or vehicles, and expressed as percentage augmentation. Values were presented as mean ± s.e.mean and *n* indicates the number of animals in each group. Statistical analysis of results was performed by use of Student's *t* test or one way analysis of variance following by *t* test with Bonferroni correction, when multiple comparisons were made. For data which were abnormally distributed or with unequal variance, a Mann-Whitney U test was used. A *P* value < 0.05 was considered to be significant.

Results

Adrenergic response to electrical field stimulation

EFS (50 V, 0.2 ms duration, 1–16 Hz, 15 s) induced a frequency-dependent contraction of the guinea-pig pulmonary artery rings at resting tension. This contraction was abolished by 1 μM tetrodotoxin and antagonized by phentolamine and prazosin (both 1 μM), indicating that it was due to the activation of α₁-adrenoceptors by neurally released NA from perivascular sympathetic nerves. The EFS-elicited contraction was 25.0 ± 5.3, 44.2 ± 6.9, 78.6 ± 8.4, 112.1 ± 9.2 and 161.2 ± 13.2 mg at 1, 2, 4, 8 and 16 Hz respectively (*n* = 6).

Effects of L-N^G-monomethyl arginine and L-arginine on electrical field stimulation-induced contraction

L-NMMA (0.3–100 μM) enhanced the EFS-induced contractile responses in a concentration-dependent manner (Figure 1) and increased basal tone, especially at high concentrations. The increase in basal tone caused by L-NMMA was 59.4 ± 3.0, 98.3 ± 15.0 and 126.3 ± 28.9 mg at concentrations of 10, 30 and 100 μM respectively (*P* < 0.02, compared with control, *n* = 6). The augmentation of EFS-induced contraction

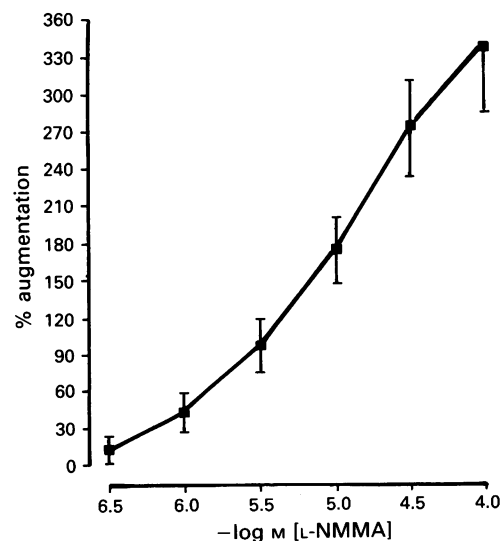


Figure 1 Effect of L-N^G-monomethyl arginine (L-NMMA) on the contractile response to adrenergic nerve stimulation (50 V, 0.2 ms duration, 4 Hz for 15 s) in guinea-pig pulmonary artery rings, showing concentration-dependent augmentation of the adrenergic neurogenic vasoconstriction. Mean of 6 animals with s.e.mean shown by vertical bars.

by $30\ \mu\text{M}$ L-NMMA was completely reversed by $100\text{--}300\ \mu\text{M}$ L-arginine, although an identical concentration of D-arginine was ineffective (Figure 2).

Effects of L-N^G-monomethyl arginine on noradrenaline-induced contraction

NA caused a concentration-dependent contraction of pulmonary vessel rings at resting tension. Rings pretreated with $30\ \mu\text{M}$ L-NMMA had a lower threshold concentration of NA than the control rings (15 ± 6 vs 162 ± 59 nM, $P < 0.05$, $n = 5$). The pD₂ values for control and L-NMMA-treated rings was 6.39 ± 0.18 and 7.08 ± 0.05 , respectively ($P < 0.03$, $n = 5$), representing a 4.9 fold increase in sensitivity to NA in L-NMMA pretreated rings. The maximal tension generation was 717 ± 123 and 860 ± 53 mg for control and L-NMMA groups, which was not significantly different ($P > 0.05$, $n = 5$).

Comparison of the effects of L-N^G-monomethyl arginine on electrical field stimulation- and noradrenaline-induced contractions

To investigate the relative pre- and postjunctional effect of L-NMMA, we compared the augmenting effects of $30\ \mu\text{M}$ L-NMMA on the contractile response to EFS (4 Hz) and a matched contractile response to NA ($0.1\text{--}0.3\ \mu\text{M}$), which was 61.2 ± 8.9 and 61.2 ± 10.3 mg respectively ($P > 0.05$, $n = 6$) before L-NMMA treatment. L-NMMA augmented EFS- and NA-induced contraction equally (Figure 3), suggesting that L-NMMA enhanced adrenergic responses via a postjunctional mechanism.

Vasorelaxant response to UK14304 and noradrenaline

To determine the presence and the possible role of α_2 -adrenoceptors in this vessel, vasorelaxant responses of U44069-precontracted vessel rings with and without endothelium to the selective α_2 -adrenoceptor agonist UK14304 and endothelium intact rings to NA in the absence and presence of various blockers, were determined. U44069 raised the vascular tone to 405 ± 46 , 378 ± 53 and 548 ± 52 mg in the rings with and without endothelium, and in the presence of $30\ \mu\text{M}$ L-NMMA respectively ($n = 5$). UK14304 induced a

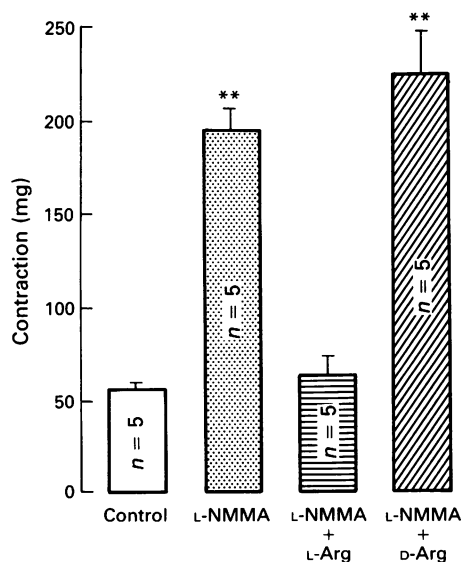


Figure 2 Reversibility of $30\ \mu\text{M}$ L-N^G-monomethyl arginine (L-NMMA)-induced augmentations of adrenergic contraction to electrical field stimulation (EFS, 50 V, 0.2 ms, 4 Hz for 15 s) by L- and D-arginine. L-arginine (L-Arg) $100\text{--}300\ \mu\text{M}$, but not identical concentrations of D-arginine (D-Arg) completely reversed the L-NMMA effect. **: $P < 0.01$, compared with control.

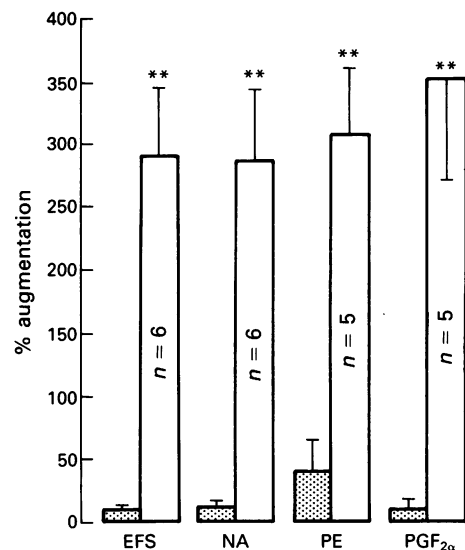


Figure 3 Comparison of the effect of L-N^G-monomethyl arginine (L-NMMA) on matched contractile responses to adrenergic nerve stimulation (EFS, 50 V, 0.2 ms, 4 Hz for 15 s), exogenous noradrenaline (NA), phenylephrine (PE) and prostaglandin F_{2α} (PGF_{2α}). Controls are shown in stippled columns and L-NMMA ($30\ \mu\text{M}$) in open columns. **: $P < 0.01$, compared with control.

concentration-dependent relaxation of the U44069-precontracted rings (Figure 4). This relaxation was abolished by endothelial denudation. Pretreatment of the vessel rings with $30\ \mu\text{M}$ L-NMMA mimicked the effect of endothelium denudation (Figure 4), indicating that α_2 -adrenoceptors located on endothelial cells mediate vasorelaxation by stimulation of NO release. The U44069-produced contraction was 715 ± 69 , 727 ± 29 , 623 ± 69 and 718 ± 118 mg in control, prazosin, prazosin plus propranolol and prazosin plus yohimbine groups, respectively ($P > 0.05$, $n = 5$). NA had little relaxant effect even on the precontracted rings. The maximal relaxation achieved at the concentration of $3\ \mu\text{M}$ was a $4.0 \pm 3.1\%$ reduction of the U44069-elicited tension. In the presence of prazosin ($1\ \mu\text{M}$), NA induced a concentration-

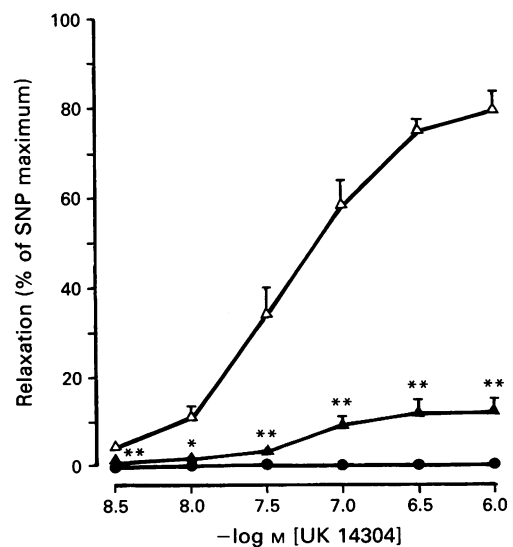


Figure 4 Effects of endothelium removal and L-N^G-monomethyl arginine (L-NMMA) on the relaxant response of the U44069-precontracted vessel rings to UK14304. Relaxation was expressed as percentage of nitroprusside (SNP) maximum relaxation: (Δ) with control, (●) without intact endothelium and (▲) in the presence of $30\ \mu\text{M}$ L-NMMA. * $P < 0.05$ and ** $P < 0.01$, compared with control rings. Mean \pm s.e.mean of 5 animals with s.e.mean shown by vertical bars.

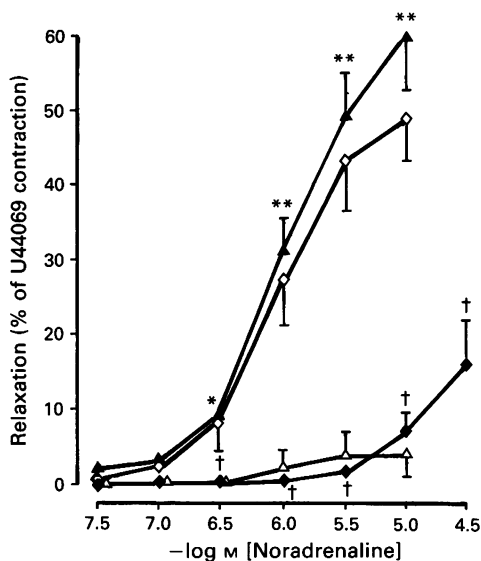


Figure 5 Relaxation of U44069 precontracted vessel rings to noradrenaline (NA) in the absence (Δ , control) and presence of prazosin (\blacktriangle , $1 \mu\text{M}$), prazosin plus propranolol (\diamond , both $1 \mu\text{M}$) and prazosin plus yohimbine (\blacklozenge , both $1 \mu\text{M}$). Relaxation was expressed as percentage of U44069 ($2 \mu\text{M}$)-induced contraction. NA caused little relaxant effect (Δ) except in the presence of prazosin (\blacktriangle). * $P < 0.05$ and ** $P < 0.01$, compared with control. † $P < 0.01$, compared with prazosin pre-treated rings, $n = 5$ in all groups.

dependent reduction of the U44069-induced vascular tone. This relaxation was significantly antagonized by yohimbine ($1 \mu\text{M}$), but unaffected by propranolol ($1 \mu\text{M}$, Figure 5), suggesting that although α_2 -adrenoceptors are present on these vessels, they play little role in mediating the response to NA.

Effects of L-N^G-monomethyl arginine on the contractions to other vasoconstrictors

The contraction induced by a single concentration of PE and PGF_{2 α} was respectively 62.5 ± 12.0 and 68.0 ± 16.3 mg before treatment with L-NMMA ($P > 0.05$, $n = 5$). These contractions, which were similar to the contractile response to EFS, were enhanced to the same extent by $30 \mu\text{M}$ L-NMMA (Figure 3).

Effects of indomethacin

Indomethacin slightly, but not significantly enhanced the contractile responses to EFS at concentrations of 1 and $3 \mu\text{M}$ but had no effect at $10 \mu\text{M}$. At a concentration of $30 \mu\text{M}$, indomethacin slightly enhanced EFS-induced contraction in 4 of the 6 tested vessel rings from 6 guinea-pigs, and slightly inhibited the EFS-induced contraction in 2 rings. Indomethacin ($1 \mu\text{M}$) slightly enhanced the contraction to NA. These results suggest that endogenous cyclo-oxygenase products are not important in modulating response to adrenergic nerve stimulation.

Discussion

Removal of endothelium increases vasoconstrictor responses to α -adrenoceptor agonists (Cocks & Angus, 1983; Egleme *et al.*, 1984; Lues & Schumann, 1984; Carrier & White, 1985; Martin *et al.*, 1986) and to adrenergic nerve stimulation (Tsfamariam *et al.*, 1987; Cohen & Weisbrod, 1988; Hynes *et al.*, 1988). However, it has not yet been established which factor is responsible for this inhibitory action of endothelium. We now report that the NO synthase inhibitor L-NMMA enhances the contractile response to adrenergic nerve stimulation concentration-dependently in guinea-pig pulmonary

artery. This augmentation is completely reversed by L-arginine but not D-arginine. By contrast, this neurogenic contraction is unaffected by indomethacin. These results indicate that endogenous NO but not PGI₂ modulates the adrenergic neurogenic vasoconstriction.

It has been reported that NO can be released from inhibitory non-adrenergic, non-cholinergic nerve (i-NANC) endings of several tissues (Gillespie *et al.*, 1989; Tucker *et al.*, 1990; Li & Rand, 1991), including cerebral arteries (Toda & Okamura, 1990). It is possible that stimulation by EFS of i-NANC nerves, which have been demonstrated in this vessel (Liu *et al.*, 1991b), induces NO release, or alternatively, stimulation of i-NANC nerves releases mediator(s), which act on endothelial cells to stimulate NO release (Liu *et al.*, 1991b). However, release of NO from i-NANC is unlikely. Firstly, we found, in our previous study, that NO mediating the i-NANC relaxation is released from endothelium (Liu *et al.*, 1991b). Secondly, we demonstrated in the present study that EFS- and exogenous NA-induced contractions were augmented by L-NMMA to an equal extent. It is possible that different mechanisms in uptake and access to receptors of endogenous and exogenous NA and the presence of a negative feedback mechanism in endogenous NA release in response to EFS may make a strict comparison of EFS- and exogenous NA-induced contraction difficult. However, the equal augmentation of EFS- and NA-induced contractions by L-NMMA at least indicates that prejunctional action of NO is not a major mechanism. Taken together with the equal augmentation of matched contractions to PE and PGF_{2 α} , our results suggest L-NMMA augments adrenergic responses via a common mechanism at a postjunctional level. The possibility that a mediator from i-NANC nerves stimulates NO release cannot be totally excluded in the present study, but it would exert a postjunctional effect. Thus, our results suggest that L-NMMA augments the contractile response to adrenergic nerve stimulation by a postjunctional mechanism.

The presence of endothelial α_2 -adrenoceptors in guinea-pig pulmonary artery, as in systemic vessels (Vanhoutte & Miller, 1989), is indicated by the demonstration that the selective α_2 -adrenoceptor agonist UK14304 and nonselective agonist NA, in the presence of an α_1 -adrenoceptor antagonist, induced a concentration-dependent relaxation of precontracted vessel rings. This relaxation was unaffected by the β -adrenoceptor antagonist, propranolol, but was significantly antagonized by the α_2 -adrenoceptor antagonist yohimbine. We also provide evidence to indicate that activation of these receptors induce vasorelaxation by stimulating endothelium-derived NO release. Release of EDRF can offset NA-induced contraction (Cocks & Angus, 1983; Angus *et al.*, 1986; Vanhoutte & Miller, 1989). It is possible that NA from adrenergic nerves stimulates α_2 -adrenoceptors to induce NO release, which then inhibits the contractile response to EFS. However, there is evidence against this possibility. Firstly, NA relaxed the precontracted vessel rings only in the presence of α_1 -adrenoceptor blockade and NA had little relaxant effect even when the vascular tone was raised, whereas EFS was applied when the vessel rings were at resting tension. This implies that the weak α_2 -adrenoceptor action of NA is profoundly masked by its α_1 -adrenoceptor action. Secondly, a similar magnitude of contractile response to PE and PGF_{2 α} was augmented by L-NMMA to the same extent as in vessels contracted with NA, although neither had any relaxant effect on the precontracted vessel rings. It is more likely that there is a continuous basal release of NO from vascular endothelium which gives a tonic inhibition of the contractile responses to NA and adrenergic nerve stimulation. Inhibition of this NO release by L-NMMA enhances contraction to adrenergic stimulation. This explanation is supported by our observation that L-NMMA increased basal smooth muscle tone in a concentration-related manner. Basal release of NO has previously been reported both in systemic (Rees *et al.*, 1989) and pulmonary (Wiklund *et al.*, 1990; Liu *et al.*, 1991a) circulations.

An increase in endothelial shear stress by changing the perfusate velocity and viscosity has been demonstrated to inhibit adrenergic contraction to electrical field stimulation in perfused carotid artery (Teschfamiar & Cohen, 1988). Whether smooth muscle contraction also imposes shear stress on endothelial cells and increases NO release remains to be determined.

In summary, our results demonstrate that endogenous nitric oxide inhibits adrenergic neurogenic vasoconstriction via a

postjunctional mechanism. Basal release of nitric oxide from endothelial cells may account for this inhibition. Stimulation of α_2 -adrenoceptors on endothelium is unlikely to contribute to this effect, although these receptors are present on endothelial cells of these vessels.

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