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Endothelium-dependent modulation of resistance vessel contraction: studies with N^G-nitro-L-arginine methyl ester and N^G-nitro-L-arginine

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1 The effect of N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-nitro-L-arginine (L-NOARG) on noradrenaline (NA)-induced contractility and acetylcholine (ACh)-induced endothelium-dependent relaxation was studied in rat mesenteric resistance arteries.

2 Third order branches of mesenteric arteries were dissected and mounted on two forty micron wires in a Mulvany myograph.

3 Incubation with L-NAME and L-NOARG (10 μM) caused a time-dependent shift in the 50% response to NA (ED_{50}) (0.01 μM–10 μM) but was not associated with an increase in the maximum contractile response.

4 L-NAME and L-NOARG (10 μM) caused a time-dependent inhibition of ACh (1 μM)-induced relaxation with a maximum effect after 120 min.

5 Following endothelium removal, incubation with either L-NAME or L-NOARG caused no significant shift in the ED_{50}, although the residual relaxation response to ACh (1 μM) was further attenuated.

6 Incubation with the cylco-oxygenase inhibitor, indomethacin, enhanced the relaxation to ACh and reduced the inhibitory effects of L-NAME and L-NOARG.

7 In conclusion, L-NAME and L-NOARG are potent inhibitors of acetylcholine-induced endothelium-dependent relaxation in mesenteric resistance arteries. The shift in ED_{50} associated wtih these inhibitors suggests a probable role for the endothelium in modulating the contractility of the resistance vasculature.

Keywords: Mesenteric resistance vessel; endothelium-derived relaxing factor; Wistar Kyoto rats; N^G-nitro-L-arginine methyl ester (L-NAME); N^G-nitro-L-arginine (L-NOARG); cyclo-oxygenase; indomethacin

Introduction

It is known now that many vasodilators act by causing the endothelium to release a vasorelaxant, endothelium-derived relaxing factor (EDRF) that relaxes vascular smooth muscle (Furchgott & Zawadski, 1980). The relaxing properties of EDRF have been well documented in large and medium sized arteries (Yang *et al.*, 1991) but few studies have been performed on resistance arteries. These have revealed that in normotensive rats, maximally contracted resistance vessels relax almost completely when exposed to acetylcholine (De Mey & Gray, 1985). Similar responses have been demonstrated in vessels from normotensive rabbits (Owen & Bevan, 1985) and man (Aalkjaer *et al.*, 1987). In addition, endothelium-dependent relaxation has been shown to increase as the internal diameter of the vessel decreases (Owen & Bevan, 1985). A variety of vasoconstrictor agents also induce EDRF release (Griffith *et al.*, 1985) but any vasorelaxant effect is submerged in the overriding contraction. However, there is evidence that the endothelium can reduce the contractile response to vasoconstrictors and may play an important role in the modulation of smooth muscle contraction of the small arteries concerned with the regulation of peripheral vascular resistance.

Studies of the modulating function of the endothelium in larger arteries have involved the use of physical methods (rubbing, perfusion with collagenase or detergent) to remove the endothelium (Harder, 1987; Osol *et al.*, 1989; Wiest *et al.*, 1989). Unfortunately, these techniques produce inconsistent results because of incomplete removal of the endothelium and varying degrees of damage to the underlying vascular smooth muscle. Recently it has been suggested that EDRF is nitric oxide (Palmer *et al.*, 1987) or a substance containing nitric oxide (Myers *et al.*, 1989) formed by the conversion of L-arginine to L-citrulline by the enzyme nitric oxide synthase (Mulsch & Busse, 1990). Furthermore, this led to the use of analogues of L-arginine which inhibit EDRF synthesis (Palmer *et al.*, 1988), attenuating endothelial dependent relaxation in the rabbit aorta (Rees *et al.*, 1989). Such analogues have also been used *in vivo* where the infusion of N^G-monomethyl-L-arginine (L-NMMA) into the human forearm caused inhibition of acetylcholine induced vasodilatation (Vallance *et al.*, 1989a) and arterial dilatation (Vallance *et al.*, 1989b) showing that EDRF has a major influence on basal blood flow. *In vitro* studies of rat aorta have shown that two other L-arginine analogues N^G-nitro-L-arginine (L-NOARG) and N^G-nitro-L-arginine methyl ester (L-NAME) are potent inhibitors of acetylcholine-induced endothelium-dependent relaxation (Moore *et al.*, 1990; Rees *et al.*, 1990) and similar results have been obtained in human subcutaneous resistance arteries with L-NOARG (Woolfson & Poston, 1990). The actions of the L-arginine analogues are reversible following the exposure to high concentrations of L-arginine (Levic *et al.*, 1990). The L-arginine analogues provide the opportunity to study endothelium modulation of resistance vessel contractility. Accordingly, we have studied the effects of two inhibitors of EDRF synthesis, L-NOARG and L-NAME alone or in the presence of the cyclo-oxygenase inhibitor indomethacin on the noradrenaline contraction and

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acetylcholine-induced endothelium-dependent relaxation of mesenteric resistance arteries from 12 week old Wistar Kyoto rats.

Methods

Preparation of vessels

The experiments were performed on 12 week old female normotensive Wistar Kyoto rats (WKY) bred in our own colony at Leicester University. Rats were killed by stunning followed by cervical dislocation. Arterial resistance vessels were taken from the superior mesenteric bed which supplies the jejunum at a point 8–10 cm from the pylorus. Two segments 2 mm in length of the third generation branch resistance vessels, with a mean internal diameter of less than 300 μm, were dissected and mounted on two 40 μm wires in a myograph. One of the wires was attached to a force transducer and the other to a micrometer. This arrangement enables wall tension to be measured at a pre-determined internal diameter. Both dissection and mounting of the vessels were carried out in cold (4°C) physiological salt solution (PSS).

Experimental protocol and solutions

The resistance vessels were allowed to equilibrate for 1 h in PSS at 37°C. The PSS was made up of (mM): NaCl 118, $NaHCO_3$ 25, KCl 4.5, KH_2PO_4 1.0, $CaCl_2$ 2.5, $MgSO_4$ 1.0 and glucose 6. A high potassium PSS was made by replacing NaCl with KCl. All solutions were gassed with 95% O_2:5% CO_2 to achieve a pH of 7.4 at 37°C. After this equilibration period the length tension characteristic for each vessel was determined and then the internal circumference was set to $0.9 \times L_{100}$, where L_{100} is the internal circumference the artery would have had *in vivo* when relaxed and under a transmural pressure of 100 mmHg (Mulvany & Halpern, 1976). Following this normalization procedure, the vessels were incubated in PSS for a further 60 min before starting the contraction studies. Two stimulations with a high-potassium PSS were followed by one contraction with 10 μM noradrenaline. Each stimulation was maintained for 2 min before being rinsed in PSS and allowed to return to baseline. The vessels were then rinsed three times with PSS and left to recover for a further 15 min.

Cumulative dose-contraction curves to noradrenaline over the range 0.01 to 30 μM were obtained in the presence of 1 μM cocaine which was added 20 min before the start of the contraction curve. Maximally contracted vessels were then relaxed with a single dose of acetylcholine (1 μM). Following this initial contraction/relaxation curve, the vessels were selected arbitrarily for studies with either L-NAME or L-NOARG before and after incubation with indomethacin.

(1) Nine vessels with a mean internal diameter of 280 ± 17 μm served as time controls. Noradrenaline dose contraction curves in the presence of 1 μM cocaine were performed 30, 60 and 120 min after the preliminary study.

(2) Sixteen vessels with a mean internal diameter of 252 ± 11 μm were incubated with L-NOARG (10 μM) and dose-contraction curves to noradrenaline performed in the presence of 1 μM cocaine after 12, 60 and 120 min.

(3) Fifteen vessels with a mean internal diameter of 280 ± 17 μm were incubated with L-NAME (10 μM) and dose-contraction curves performed as for protocol (2). The remaining two groups of vessels were subjected to endothelial removal after the preliminary noradrenaline dose-contraction curve had been obtained. Following endothelial removal a second dose-contraction was obtained and the vessels arbitrarily selected for either protocol (4) or (5).

(4) Ten vessels with a mean internal diameter of 260 ± 9 μm were incubated with L-NAME (10 μM) and dose-contraction curves obtained in the presence of 1 μM cocaine after 12 and 60 min.

(5) Fourteen vessels with a mean internal diameter of 282 ± 10 μm were incubated with L-NOARG (10 μM) and dose-contraction curves obtained as for protocol (4).

Protocols (1), (2) and (3) were repeated in the presence of 10 μM indomethacin which was added 30 min before the start of the contraction/relaxation curve.

Endothelium removal

The endothelium was removed by the technique described by Osol *et al.* (1989). A human hair was washed in ethanol and rinsed in PSS before being inserted into the lumen of the vessel mounted under tension in the myograph. The endothelium was mechanically removed by repeatedly drawing the hair through the lumen of the vessel. Following removal of the endothelium the vessel was left for 45 min. Endothelial function was assessed by observing the relaxation produced by exposing a maximally contracted vessel to 10 μM ACh.

Drugs

All solutions were freshly prepared on the day of study. Cocaine hydrochloride, noradrenaline, acetylcholine, L-NAME, L-NOARG and indomethacin were obtained from Sigma Chemical Company, St Louis, Missouri, U.S.A. All drugs were dissolved in distilled water except for indomethacin which was dissolved in absolute ethanol. Ethanol was found to have no effect on responses induced by acetylcholine. Drugs were diluted to the final bath concentration with PSS.

Data and statistical analysis

Results are expressed as the mean ± standard error of the mean (s.e.mean) and the statistical significance determined by Student's paired *t* test with Dunnett's correction for multiple comparisons. The noradrenaline contractile responses are expressed as active tension (milli Newtons/mm) which is calculated from the measured force (milli Newtons) divided by twice the vessel length (millimetres). Noradrenaline sensitivity is expressed in terms of the ED_{50}, that is the concentration required to produce a half-maximal contraction. ACh-induced relaxations are expressed as a percentage decline of the maximum contraction.

Results

Contraction studies

The controls showed that there was no difference in either the noradrenaline sensitivity and maximum contraction between the dose-response curves performed at the baseline and after incubation in PSS for 30, 60 and 120 min (Figure 1). Incubation with L-NAME for the same time period produced a non-significant rise in the maximum contraction with an enhanced noradrenaline sensitivity (Figure 2, Table 1); the ED_{50} fell from 3.61 ± 0.72 μM at the baseline to 1.21 ± 0.27 μM after 60 min ($P<0.05$). A similar response was observed after a 2 h incubation with L-NOARG but the sensitivity was significantly increased at 12, 60 and 120 min (Figure 3, Table 1). Mechanical removal of the endothelium reduced the maximum contractile response to noradrenaline from 3.63 ± 0.21 mN mm^{-1} to 2.91 ± 0.32 mN mm^{-1} ($P<0.05$) in the group subsequently treated with L-NAME. There was no associated change in the sensitivity, the ED_{50} changed from 5.20 ± 1.03 μM to 3.26 ± 0.72 μM. This pattern was also observed in the endothelium-denuded arteries treated with L-NOARG where the maximum contraction fell from 3.06 ± 0.30 mN mm^{-1} to 2.56 ± 0.36 mN mm^{-1} ($P<0.05$) without a significant change in the ED_{50} of 3.80 ±

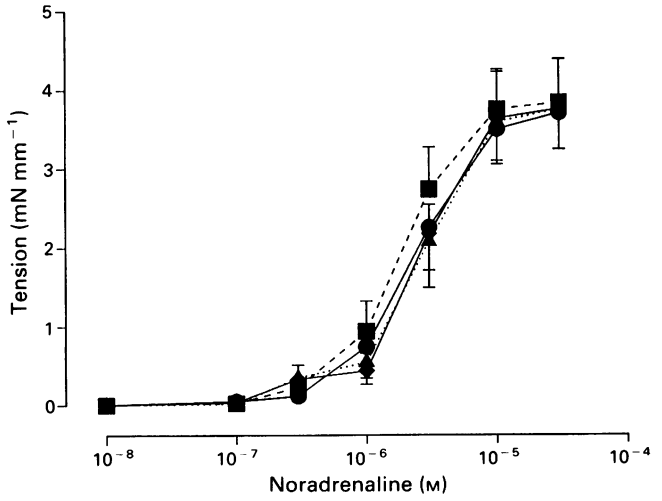

Figure 1 Noradrenaline-induced contractile responses in the control rat mesenteric resistance arteries at 0 (●), 30 (■), 60 (▲) and 120 min (◆).

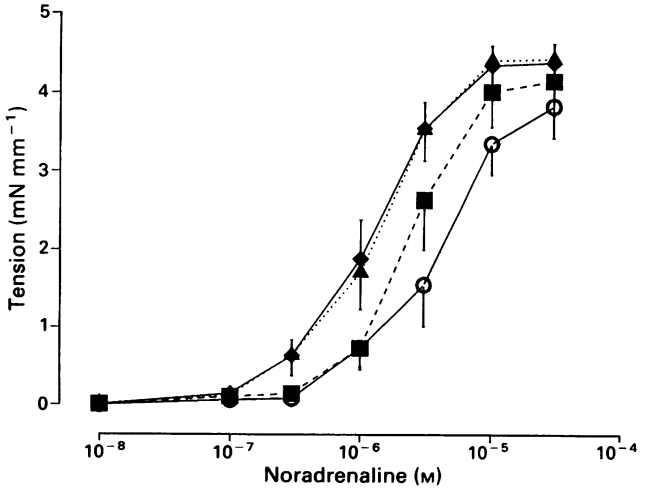

Figure 3 The contractile response to noradrenaline in intact rat mesenteric resistance vessels before (○) and after incubation with N^G-nitro-L-arginine (L-NOARG) for 12 (■), 60 (▲) and 120 min (◆).

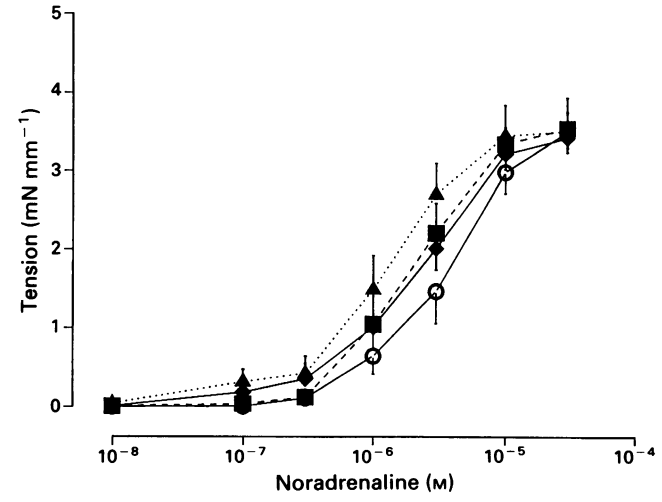

Figure 2 The contractile response to noradrenaline in intact rat mesenteric resistance vessels before (○) and after incubation with N^G-nitro-L-arginine methyl ester (L-NAME) for 12 (■), 60 (▲) and 120 min (◆).

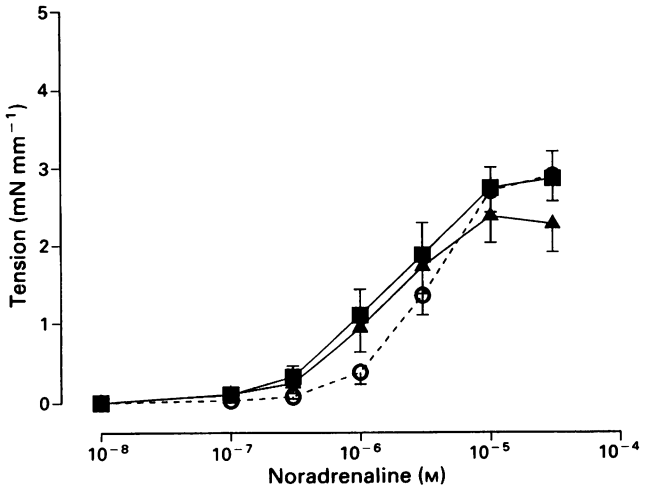

Figure 4 The contractile response to noradrenaline in endothelium-denuded rat mesenteric resistance vessels before (○) and after incubation with N^G-nitro-L-arginine methyl ester (L-NAME) for 12 (■) and 60 (▲) min.

0.65 μM in the intact arteries and 3.17 ± 0.78 μM after removing the endothelium. The effects of L-NAME (Figure 4 and Table 1) and L-NOARG (Figure 5 and Table 1) on the noradrenaline contractile response were abolished in the endothelium-denuded arteries.

Relaxation

The controls showed that there was no difference in the relaxation to ACh between curves performed at the baseline (83 ± 4%) and after incubation in PSS for 30 min (78 ± 3%), 60 min (83 ± 5%) and 120 min (74 ± 7%) (Table 3). The relaxation of maximally contracted resistance arteries decreased after incubation with L-NAME and L-NOARG in a time-dependent manner (Table 2). A bolus dose of ACh (to give a final concentration of 1 μM) produced 68 ± 4% relaxation in the untreated arteries in the group subsequently treated with L-NAME and 79 ± 4% in the L-NOARG group. Incubation with L-NAME reduced the relaxation response to 64 ± 6% at 12 min, 46 ± 8% ($P<0.05$) at 60 min and 32 ± 10% ($P<0.05$) at 120 min. Incubation with L-NOARG produced similar changes with 63 ± 4% relaxation at 12 min, 59 ± 3% ($P<0.05$) at 60 min and 44 ± 9% ($P<0.05$) after 120 min (Table 2). Endothelium removal produced a marked fall in ACh-induced relaxation. In the L-NAME group, ACh-induced relaxation was 66 ± 5% before and 10 ± 3% ($P<0.05$) after denuding the endothelium. Incubation with L-NAME further reduced the acetylcholine relaxation to 5 ± 3% at 12 min and 1 ± 1% at 60 min. In the L-NOARG group of arteries, ACh relaxation was 73 ± 4% before and 21 ± 8% ($P<0.05$) after removing the endothelium. Incubation with L-NOARG caused a reduction to 5 ± 3% at 12 min ($P<0.05$) and 2 ± 2% at 60 min (Table 2).

Indomethacin enhanced ACh-induced endothelium-dependent relaxation in mesenteric resistance arteries from the WKY. In addition the effect of L-NAME and L-NOARG was significantly reduced ($P<0.05$) in arteries which had been pre-incubated with 10 μM indomethacin (Table 3).

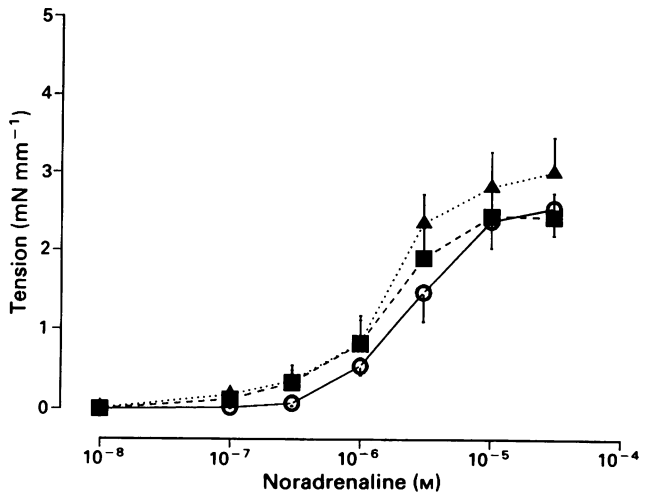

Figure 5 The contractile response to noradrenaline in endothelium-denuded rat mesenteric resistance vessels before (○) and after incubation with N^G-nitro-L-arginine (L-NOARG) for 12 (■) and 60 (▲) min.

Discussion

The present experiments show that the L-arginine analogues L-NAME and L-NOARG, are potent inhibitors of endothelium-dependent relaxation in the rat isolated mesenteric resistance artery. In addition, the enhanced vasoconstrictor response to noradrenaline after inhibition of EDRF synthesis supports an important role for the endothelium in the regulation of the contractile response of these arteries. There was a time-dependent decrease in the ED_{50} for a period up to 60 min of incubation but this was not associated with a significant increase in the maximum contraction. Moreover, the effects of both inhibitors were attenuated after mechanical removal of the endothelium by passing a human hair back and forward through the vessel lumen. Similar augmentation of vascular tone has been documented using L-NAME in the phenylephrine-contracted rat aorta (Rees *et al.*, 1990) and with L-NOARG in both the rat aorta and the rat isolated perfused mesentery (Moore *et al.*, 1990). However, by contrast incubation with another L-arginine analogue L-N^G-monomethyl-arginine (L-NMMA), did not affect the noradrenaline contractility of human subcutaneous arteries (Woolfson & Poston, 1990). This discrepancy may depend on differences between the vascular beds and also the potency of the EDRF inhibitor used. In the perfused rat mesenteric bed L-NOARG is approximately four times more active in inhibiting acetylcholine-induced endothelial-dependent relaxation than is L-NMMA. Recent evidence suggests that the difference in potency of EDRF inhibitors could be due to differences in the mechanisms by which they enter the endothelial cell (McCall *et al.*, 1991). It appears that in porcine cultured endothelial cells, L-NMMA acts by inhibiting the transport system Y^+ which is responsible for the entry of L-arginine into the endothelial cell. In contrast L-NAME and L-NOARG do not affect this system and therefore enter the cell by a different mechanism (Bogle *et al.*, 1992).

The change in noradrenaline sensitivity probably results from a decrease in basal EDRF release since there was no significant increase in the maximum contraction. However, a reduction in stimulated EDRF release cannot be excluded and it is known that noradrenaline, along with other vasoconstrictors, can stimulate the release of EDRF (Peach *et al.*, 1985). Acetylcholine evoked potent endothelium-

Table 1 The maximum contraction and the ED_{50} values for noradrenaline before and after incubation with N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-nitro-L-arginine (L-NOARG) in vessels with and without an intact endothelium

		Endothelium intact			
		Control	*12 min*	*60 min*	*120 min*
L-NAME	NA_{max} (mN mm^{-1})	3.51 ± 0.21	3.64 ± 0.29	3.57 ± 0.42	3.50 ± 0.32
	ED_{50} (μM)	3.61 ± 0.72	2.37 ± 0.77	1.21 ± 0.27*	2.17 ± 0.59
L-NOARG	NA_{max} (mN mm^{-1})	4.01 ± 0.38	4.20 ± 0.40	4.53 ± 0.32	4.44 ± 0.25
	ED_{50} (μM)	3.88 ± 0.65	2.16 ± 0.42*	1.75 ± 0.32*	1.84 ± 0.42*
		Endothelium denuded			
		Before	*After* (0 min)	*12 min*	*60 min*
L-NAME	NA_{max} (mN mm^{-1})	3.63 ± 0.21	2.91 ± 0.32†	2.89 ± 0.34	2.38 ± 0.35
	ED_{50} (μM)	5.20 ± 1.03	3.26 ± 0.72	2.40 ± 0.99	1.62 ± 0.40
L-NOARG	NA_{max} (mN mm^{-1})	3.06 ± 0.30	2.56 ± 0.36†	2.49 ± 0.30	3.06 ± 0.46
	ED_{50} (μM)	3.80 ± 0.65	3.17 ± 0.78	1.76 ± 0.54	1.43 ± 0.31

*$P<0.05$ as compared to control (incubation in PSS).
†$P<0.05$ as compared to before endothelial removal.

Table 2 The maximum relaxation to acetylcholine before and after incubation with N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-nitro-L-arginine (L-NOARG) in vessels with and without an intact endothelium

		Endothelium intact		
Inhibitor	*Control*	*12 min*	*60 min*	*120 min*
L-NAME	68 ± 4	64 ± 6	46 ± 8*	32 ± 10*
L-NOARG	79 ± 4	63 ± 4	59 ± 3*	44 ± 9*
		Endothelium denuded		
Inhibitor	*Before*	*After*	*12 min*	*60 min*
L-NAME	66 ± 5	10 ± 3†	5 ± 3	1 ± 1
L-NOARG	73 ± 7	21 ± 8†	5 ± 3	2 ± 2

*$P<0.05$ as compared to control (incubation in PSS).
†$P<0.05$ as compared to before endothelial removal.

Table 3 The maximum relaxation to acetylcholine in vessels incubated with N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-nitro-L-arginine (L-NOARG) alone and after incubation with indomethacin (Indom)

	Control	*30 min*	*60 min*	*120 min*
PSS	83 ± 4	78 ± 3	83 ± 5	74 ± 7
Indom	74 ± 11	90 ± 4	92 ± 5	92 ± 4
	Control	*12 min*	*60 min*	*120 min*
L-NAME	68 ± 4	64 ± 6	46 ± 8*	32 ± 10*
L-NAME + Indom	88 ± 4 †	79 ± 6	73 ± 8†	71 ± 7†
L-NOARG	79 ± 4	63 ± 4	59 ± 3*	44 ± 9*
L-NOARG + Indom	93 ± 1 †	90 ± 3†	88 ± 5†	80 ± 11†

*$P<0.05$ (compared to control)
†$P<0.05$ (inhibitor vs inhibitor plus indomethacin)

dependent relaxation in the mesenteric resistance arteries which were comparable to previous studies (Watt & Thurston, 1989). Incubation with either L-NAME or L-NOARG caused a time-dependent decrease in acetylcholine-induced relaxation with maximum inhibition after 120 min. However, even after incubation with the inhibitors for 2 h, the initial burst of relaxation caused by acetylcholine was unchanged but the sustained relaxation seen in the control curve was markedly reduced. This is in keeping with the findings of Furchgott *et al.* (1990) and suggests that there are two stores of EDRF. The first is a limited and readily available store which is released almost immediately upon exposure to an endothelium-dependent agonist such as acetylcholine but becomes exhausted. The exposure to this agonist then initiates the synthesis of EDRF from L-arginine and it is this store which is responsible for the sustained relaxation seen in the control curves. It is possible that incubation with the EDRF inhibitor blocks the synthesis of this second store of EDRF and hence the relaxation seen in the treated vessels is only of a transient nature.

Neither L-NAME nor L-NOARG completely inhibited acetylcholine-induced endothelium-dependent relaxation. Thus, even after incubation with the inhibitors for 120 min there was approximately 40% relaxation (L-NAME, 32 ± 10% and L-NOARG, 44 ± 9%). It is possible that the concentration of inhibitor used in this study may not have been sufficient to inhibit completely the endothelium-dependent relaxation produced by the L-arginine pathway. However, other studies using higher concentrations of L-NAME and L-NOARG (30 μM) have achieved comparable levels of inhibition of relaxation in larger arteries (Moore *et al.*, 1990). On the other hand the residual relaxation may depend on the release of other vasodilator substances such as prostaglandin E_2 and prostacyclin. It is apparent that acetylcholine can release another EDRF which is generated from a source other than L-arginine (Ray *et al.*, 1988). This substance has been named endothelium-derived hyperpolarizing factor (EDHF) and relaxes vascular smooth muscle cells through hyperpolarization as a result of opening of potassium channels (Van de Voorde *et al.*, 1992).

We have investigated the likelihood of a cyclo-oxygenase-derived vasodilator being responsible for the residual relaxation seen after incubation with the EDRF inhibitors. The results however indicate that this is not the case since incubation with indomethacin enhanced the relaxation to a bolus dose of ACh. Furthermore, incubation with indomethacin reduced the effectiveness of both L-NAME and L-NOARG such that there was no significant inhibition of ACh-induced endothelium-dependent relaxation. These results suggest that the residual relaxation in the presence of L-NAME and L-NOARG is not due to ACh stimulated release of a cyclo-oxygenase-derived relaxing factor such as prostacyclin but may represent partial inhibition of EDRF synthesis. Moreover, the attenuation of the inhibitory effects of the EDRF inhibitors in the presence of indomethacin suggests that a contracting factor is released from the mesenteric resistance arteries taken from normotensive WKY rats. Interestingly, pre-incubation with the thromboxane A_2 antagonist, SQ 29548, did not affect the action of the EDRF inhibitors on ACh-induced endothelium-dependent relaxation. Thus it would appear that this weak contracting factor is not thromboxane A_2 but may be another vasoconstrictor prostaglandin such as prostaglandin H_2. There is evidence that the reduced endothelium-dependent relaxation seen in the spontaneously hypertensive rat (SHR) is due to a cyclo-oxygenase contracting factor (Luscher *et al.*, 1990) but as yet there is no evidence for the release of such a factor in the WKY. It appears that in the SHR the reduced relaxation arises because of a shift in the balance of relaxing and contracting factors. The discovery of a contracting factor in the WKY supports such a claim, suggesting that in the WKY the balance is in favour of relaxing factors. Furthermore since the contracting factor is only apparent after inhibition of EDRF it suggests that it is fairly weak in nature and is easily overridden by EDRF.

In conclusion we have demonstrated that both L-NAME and L-NOARG are potent inhibitors of acetylcholine-induced endothelium-dependent relaxation. Both of these inhibitors blocked endothelium-dependent regulation of vascular contraction probably by inhibiting the basal rather than the stimulated release of EDRF. These results show that the endothelium plays an important role in the modulation of the contraction of the resistance vasculature and therefore in the maintenance of vascular resistance.

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