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Differential effects of L-arginine on the inhibition by N^G-nitro-L-arginine methyl ester of basal and agonist-stimulated EDRF activity

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1 An isolated, buffer-perfused rabbit ear preparation was used to investigate the influence of N^G-nitro-L-arginine methyl ester (L-NAME) on endothelium-dependent vasodilatation and modulation of vasoconstrictor responses and vascular conductance.

2 Acetylcholine (0.55 pmol–1.6 nmol) caused dose-related vasodilatation of preparations constricted by the combination of 5-hydroxytryptamine and histamine (both 1 μM), with an $ED_{50} = 31.1 \pm 7.8$ pmol and a maximum dilatation of $69.9 \pm 4.3\%$. In the presence of 10 μM L-NAME the dose-response for vasodilator effects was shifted significantly ($P < 0.001$) to the right ($ED_{50} = 3.07 \pm 1.18$ nmol) and there was a significant ($P < 0.01$) depression of the maximum response ($R_{max} = 44.3 \pm 4.0\%$). The higher concentration of 100 μM L-NAME completely abolished vasodilatation to acetylcholine. L-Arginine (10 mM) did not reverse the inhibitory actions of L-NAME at either concentration.

3 L-NAME 100 μM, augmented vascular tone induced by 1 μM 5-hydroxytryptamine and 1 μM histamine, thus altering the characteristics of both pressure/flow and conductance/flow relationships such that conductance was reduced at all flow rates. The augmentation of constrictor tone was reversed in a concentration-dependent manner by L-arginine (10 μM–10 mM) and the effect of L-NAME on the conductance/flow relationships was similarly reversed by 10 mM L-arginine. The augmentation of tone was endothelium-dependent as it did not occur following functional destruction of the endothelium by perfusion of the vascular bed with the detergent CHAPS (0.3%) for 150 s.

4 In conclusion, L-NAME is a potent inhibitor of agonist-induced endothelium-dependent vasodilatation. L-NAME reduces vascular conductance in pharmacologically constricted preparations and this emphasizes the important role of EDRF in vascular regulation. The ability of L-arginine to reverse L-NAME-induced inhibition of basal EDRF activity but not L-NAME-induced inhibition of agonist-induced endothelium-dependent relaxations suggests that there is pharmacological heterogeneity in the mechanisms responsible for the conversion of L-arginine to EDRF.

Keywords: Rabbit ear; EDRF; N^G-nitro-L-arginine methyl ester; pressure/flow relationships; vascular conductance; L-arginine

Introduction

For some time it has been known that the vascular endothelium releases a potent vasorelaxant, endothelium-derived relaxing factor (EDRF), in response to various agonists (Furchgott & Zawadzki, 1980) and also basally (Griffith *et al.*, 1984a,b). It has recently been demonstrated that EDRF is either nitric oxide (Palmer *et al.*, 1987) or a nitric oxide containing moiety (Myers *et al.*, 1990) with the nitrogen atom being derived from the terminal guanidino group of L-arginine (Palmer *et al.*, 1988).

The elucidation of the identity of EDRF and its precursor has led to the use of analogues of L-arginine to inhibit EDRF synthesis (Rees *et al.*, 1989a; Moore *et al.*, 1990). The first analogue used, N^G-monomethyl-L-arginine (L-NMMA), inhibited endothelium-dependent relaxations of the rabbit aorta without affecting endothelium-independent relaxations to nitrovasodilators (Rees *et al.*, 1989a). More recently N^G-nitro-L-arginine and its methyl ester (L-NAME) have emerged as more potent inhibitors of endothelium-dependent relaxations in both conduit and resistance vessels (Moore *et al.*, 1990; Rees *et al.*, 1990). In addition to inhibiting endothelium-dependent relaxations such compounds augment pharmacologically induced tone in an endothelium-dependent manner (Rees *et al.*, 1989a) and cause hypertension in whole animal preparations (Rees *et al.*, 1989b; 1990). The former action demonstrates the modulatory effects of EDRF on vascular tone and the hypertensive effect is indicative of the important role of EDRF in blood pressure regulation.

The inhibitory actions of L-arginine analogues have been shown to be reversible by L-arginine but the extent varies between inhibitors and preparations. In rat aortic rings and isolated mesentery Moore *et al.* (1990) found that the partial inhibition of endothelium-dependent relaxations to acetylcholine by 30 μM N^G-nitro-L-arginine was reversed by < 150 μM L-arginine. In contrast, Unmans (1990) reported that the inhibitory effects of 100 μM N^G-nitro-L-arginine on endothelium-dependent relaxations to acetylcholine in the rat aorta were irreversible even at high concentrations of L-arginine. Similarly the hypertensive effects of N^G-nitro-L-arginine in the anaesthetized guinea-pig are only slightly attenuated by administration of L-arginine, in contrast to the pressor effects of L-NMMA which were fully reversed by L-arginine (Steinberg *et al.*, 1990).

The biological activity of nitric oxide or a nitric oxide-like substance is not confined to vascular systems. It has recently been shown that non-adrenergic, non-cholinergic (NANC) transmission in the rat anococcygeus muscle is specifically inhibited by 200 μM L-NAME while the partial inhibitory effects of 20 μM L-NAME are almost completely reversed by a 5 fold molar excess of L-arginine (Hobbs & Gibson, 1990). Similarly 5 μM N^G-nitro-L-arginine inhibits NANC relaxations of the guinea-pig trachea where the effect was partially reversed by 500 μM L-arginine (Tucker *et al.*, 1990). EDRF is also known to prevent platelet aggregation (Azuma *et al.*, 1986), thus inhibitors of nitric oxide synthesis enhance platelet aggregation but the maximum effect of L-NAME is not reversed by high concentrations of L-arginine (Radomski *et al.*, 1990a). It would thus appear that the effects of nitric oxide synthase inhibitors and their sensitivity to reversal by L-arginine are dependent on species and the physiological system under consideration.

In the present study we have investigated the effects of L-NAME on both agonist and basally released EDRF in a

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resistance bed. It has previously been shown that in the blood-perfused rat mesentery, destruction of the endothelium and also administration of methylene blue alter the characteristics of pressure/flow relationships indicating a substantial increase in vascular resistance presumably as a consequence of loss of basally released EDRF (Randall & Hiley, 1988b). We have previously reported similar effects on diameter/flow, pressure/flow and conductance/flow relationships for haemoglobin (Griffith *et al.*, 1987a), which avidly scavenges EDRF (Kelm *et al.*, 1988), and L-NMMA in the rabbit isolated ear (Griffith & Edwards, 1990). In the present study we have investigated whether L-NAME similarly affects the pressure/flow and conductance/flow relationships in the rabbit ear and have examined the characteristics of L-NAME-induced blockade of both basal and agonist-dependent EDRF activity in this preparation.

Methods

Preparation of the rabbit ear vascular bed

Male New Zealand White rabbits (2–2.5 kg) were killed by cervical dislocation. An ear was removed and the central artery cannulated (Portex PP50 tubing). The preparation was perfused, by means of a peristaltic pump, at 2 ml min^{-1} with Holman's solution (mM): NaCl 120, KCl 5, $CaCl_2$ 2.5, NaH_2PO_4 1.3, $NaHCO_3$ 25, sucrose 10 and D-glucose at 11. The physiological buffer also contained 5% dextran (mol. wt. 80,000) and 10 μM indomethacin. The buffer was gassed with 95% O_2/5% CO_2 (Po_2 ca. 600 mmHg; pH 7.4) and maintained at 35°C.

Experimental protocol

In order to study the effects of L-NAME on the endothelium-dependent vasodilatation to acetylcholine in the isolated ear preparation, tone was established by addition of 5-hydroxytryptamine and histamine to the perfusate to achieve individual concentrations of 1 μM. We have previously shown that this combination of vasoconstrictor agents produces consistent and well maintained tone and does not release EDRF in this preparation (Griffith *et al.*, 1988). The vasodilator properties of acetylcholine were assessed by bolus injections of this agent, in volumes less than 30 μl, into the perfusion system. The effects of either 10 μM or 100 μM L-NAME on responses to acetylcholine were examined by addition of the agent after tone had been established but 30 min before experimental determinations. After constructing dose-response curves to acetylcholine the effect of 10 mM L-arginine on inhibition of endothelium-dependent responses by L-NAME was investigated by addition to the perfusate 30 min before further doses of acetylcholine.

The influence of L-NAME on the pressor effects of 5-hydroxytryptamine and histamine was investigated by constructing concentration-response curves to equimolar concentrations of these agonists in the absence of L-NAME and also in preparations which had been pretreated with 100 μM L-NAME 30 min before experimental determinations. To investigate further the effects of L-NAME on constriction induced by the combination of 5-hydroxytryptamine and histamine (both 1 μM), tone was established and allowed to stabilize for 30 min, at which time L-NAME was added to the perfusion fluid at a concentration of 100 μM. The ability of L-arginine and its enantiomer D-arginine to oppose the increase in perfusion pressure in response to L-NAME was then examined. Vascular responses to L-arginine were also examined in the absence of L-NAME. In some preparations the concentrations of 5-hydroxytryptamine and histamine were increased to 3 μM which gave a level of tone comparable to that induced by 1 μM of these agents in the presence of L-NAME. The endothelium-dependent nature of the augmented constrictor responses by L-NAME was examined by addition of L-NAME to preparations denuded of endothelium. Destruction of the endothelium was achieved by perfusion of the bed with 0.3% CHAPS in distilled water for 150 s, a technique which has been used in other resistance beds, both *in vitro* and *in vivo*, to cause selective destruction of the endothelium (Randall & Hiley, 1988a,b). After an equilibration period of 30 min functional destruction of the endothelium was confirmed by the inability of 550 pmol of acetylcholine to cause relaxation of established tone while constrictor responses to 5-hyroxytryptamine and histamine and vasodilatation to sodium nitroprusside (1 nmol) remained intact. Once this had been confirmed, L-NAME (100 μM) was added to the perfusion fluid.

The effects of L-NAME on vascular conductance in pharmacologically constricted (1 μM 5-hydroxytryptamine and 1 μM histamine) preparations were also considered. In these experiments pressure/flow relationships were established at 8 flow rates, from 0.959 to 5.5 ml min^{-1}. Each flow was used twice and changed every 2 min according to a Latin square design while the pressure was measured by a transducer placed close to the inflow cannula. Correction for the resistance of the inflow cannula was made by subtracting the pressure drop across the cannula alone at each of the flow rates employed. In these experiments pressure/flow relationships were determined for constricted preparations and then in the presence of 100 μM L-NAME alone followed by the addition of 10 mM L-arginine or in the presence of L-arginine followed by L-NAME. Conductance was determined from the pressure/flow data by the following relationship:

$$\text{conductance (G)} = \frac{\text{flow rate (Q)}}{\text{pressure drop across the ear (P)}}$$

Data and statistical analysis

All data are given as the mean ± s.e.mean. The dose-response curve for the vasodilator response to acetylcholine responses was analysed by fitting the logistic equation:

$$R = \frac{R_{max} \times A^{n_H}}{ED_{50}^{n_H} + A^{n_H}}$$

where R is the percentage relaxation of tone, A the dose of acetylcholine, R_{max} the maximum relaxation, n_H the slope function and ED_{50} the dose of acetylcholine giving the half maximal relaxation.

A modified Marquardt procedure, as implemented in the Harwell routine VB01A on a mainframe computer, was used to carry out the curve fitting (Aceves *et al.*, 1985).

The variation of conductance with flow rate was analysed by linear regression or construction of a polynomial function depending on the best fit. This was carried out by means of Cricket Graph 1.2 (Cricket Software, Malvern PA, U.S.A.) running on an Apple Macintosh IIcx.

Comparison between experimental groups was carried out either by paired or unpaired Student's *t* tests or by one-way analysis of variance.

Drugs

All solutions were prepared on the day of the experiment. N^G-nitro-L-arginine methyl ester, L-arginine hydrochloride, D-arginine hydrochloride, histamine dihydrochloride, sodium nitroprusside, and 5-hydroxytryptamine as creatinine sulphate complex were dissolved in saline and then diluted to the required concentrations in the Holman's solution. Indomethacin was made up as a 10 mM stock solution in 5% (w/v) sodium bicarbonate and was dissolved in the Holman's solution at a concentration of 10 μM. Acetylcholine chloride was dissolved in 0.9% (w/v) saline. CHAPS (3-[(3-cholamidopropyl)-dimethyl ammonio]-1-propane sulphonate) was dissolved at a concentration of 0.3% (w/v) in distilled water. All drugs were obtained from Sigma Chemical Company, Poole, Dorset.

Results

Effects of N^G-nitro-L-arginine methyl ester on endothelium-dependent vasodilatation to acetylcholine

The basal perfusion pressure was 31.5 ± 3.8 mmHg and the combination of 1 μM 5-hydroxytryptamine and 1 μM histamine increased perfusion pressure to 147 ± 14 mmHg (n = 13). Acetylcholine (0.55 pmol–1.6 nmol) caused dose-related reductions in the established pressure. The dose-response curve (Figure 1a) for the dilatations was described by an ED_{50} = 31.1 ± 7.8 pmol, an R_{max} = 69.9 ± 4.3% and a slope function of 0.98 ± 0.13 (n = 5–7).

Addition of 10 μM L-NAME significantly ($P < 0.05$) increased perfusion pressure to 190 ± 12 mmHg (n = 9). In the presence of this concentration of L-NAME there was a significant ($P < 0.001$) rightward shift of the dose-response curve (ED_{50} = 3.07 ± 1.18 nmol; n_H = 0.93 ± 0.11) for the relaxation of tone by acetylcholine (0.55 pmol–160 nmol) with a significant ($P < 0.01$) depression of the maximum relaxation (R_{max} = 44.3 ± 4.0%) (Figure 1a). In the same 9 preparations the reductions in potency and maximum relaxation to acetylcholine were unaffected by addition of 10 mM L-arginine to the perfusate (ED_{50} = 5.39 ± 2.53 nmol; R_{max} = 48.4 ± 6.0%; n_H = 0.82 ± 0.10) (Figure 1a). Following addition of this amino acid, perfusion pressure was 154 ± 17 mmHg.

In another 6 preparations basal perfusion pressure was 37.6 ± 4.1 mmHg and in the presence of 5-hydroxytryptamine, histamine and 100 μM L-NAME perfusion pressure was 180 ± 23 mmHg while acetylcholine (0.55 pmol–1.6 nmol) did not cause any significant relaxation of tone (Figure 1b; n = 5–6). In the presence of both 100 μM L-NAME and 10 mM L-arginine perfusion pressure was 147 ± 24 mmHg and acetylcholine (0.55 pmol–1.6 nmol) did not cause significant relaxation of tone (Figure 1b; n = 5).

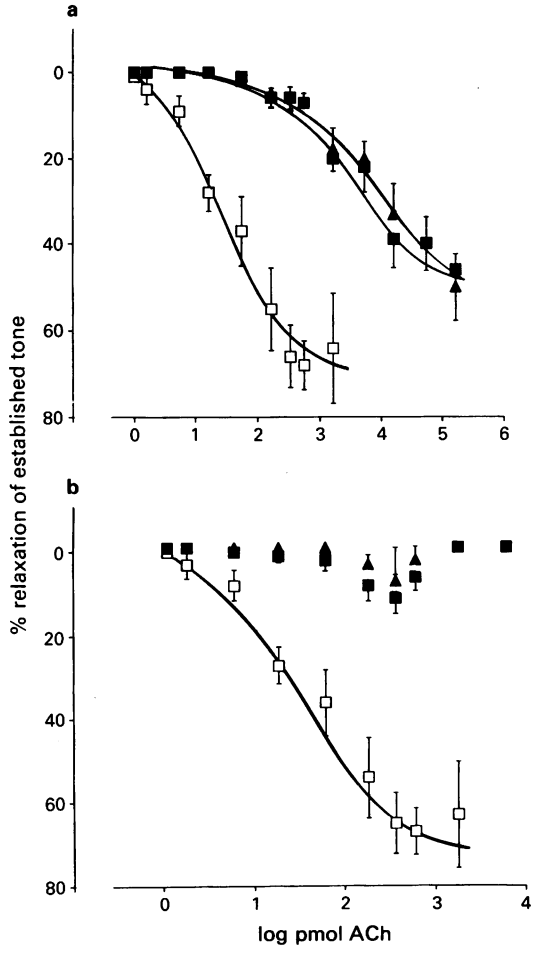

Figure 1 The relaxation of established tone by acetylcholine (ACh) in the isolated perfused ear of the rabbit; (a) shows the relaxations to acetylcholine in the absence of N^G-nitro-L-arginine methyl ester (L-NAME) (□, n = 7), in the presence of 10 μM L-NAME (■, n = 9) and in the presence of 10 μM L-NAME plus 10 mM L-arginine (▲, n = 9); (b) shows the relaxations to acetylcholine in the presence of 100 μM L-NAME (■, n = 5–6) and in the presence of 100 μM L-NAME and 10 mM L-arginine (▲, n = 5) with the control data from (a) represented by (□). The dose-response curves were computer fitted as described in the Methods. The points show the mean and the vertical bars the s.e.mean.

Effects of N^G-nitro-L-arginine methyl ester on the constrictor activity of the combination of 5-hydroxytryptamine and histamine

In seven preparations resting perfusion pressure was 29.0 ± 4.3 mmHg and the equimolar mixture of 5-hydroxytryptamine and histamine brought about concentration-related increases in perfusion pressure with a maximum increase of 174 ± 9 mmHg (to a perfusion pressure of 203 ± 10 mmHg) and the EC_{50} for the agonist mixture was 790 ± 230 nM (Figure 2). In another five preparations resting perfusion pressure in the presence of 100 μM L-NAME was 41.8 ± 7.6 mmHg and this value did not differ significantly from that obtained in its absence. In these preparations the mixture of 5-hydroxytryptamine and histamine similarly gave rise to concentrated-related increases in perfusion pressure with a maximum increase of 166 ± 12 mmHg (to a perfusion pressure of 207 ± 9 mmHg) and these values did not differ from those obtained in the absence of L-NAME. However, in the presence of L-NAME there was a significant ($P < 0.01$) 30 fold leftward shift in the concentration-response curve (Figure 2) indicating that the mixture of constrictor agents was significantly ($P < 0.01$) more potent (EC_{50} = 29 ± 9 nM).

Effects of L-arginine on the augmentation of vascular tone by 100 μM N^G-nitro-L-arginine methyl ester

In resting preparations (n = 11) perfused at 2 ml min^{-1}, perfusion pressure was 30.5 ± 4.4 mmHg. Inclusion of 1 μM 5-hydroxytryptamine and 1 μM histamine increased perfusion pressure to 133 ± 9 mmHg (n = 11). Addition of 100 μM L-NAME at the plateau of the response increased perfusion

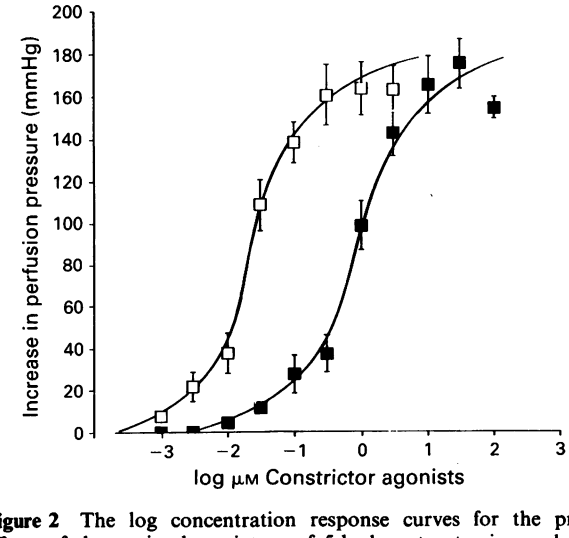

Figure 2 The log concentration response curves for the pressor effects of the equimolar mixture of 5-hydroxytryptamine and histamine in the absence (■) and presence (□) of 100 μM N^G-nitro-L-arginine methyl ester in the isolated perfused ear preparation of the rabbit. The points show mean with s.e.mean indicated by vertical bars.

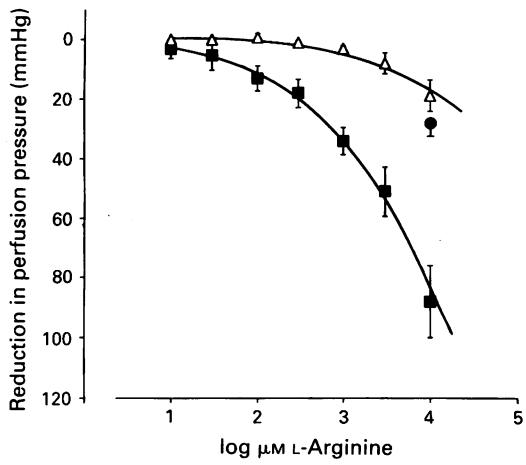

Figure 3 Shows the sustained relaxations of tone in the preconstricted rabbit ear preparation caused by L-arginine in the absence (△, $n = 7$) and presence (■, $n = 6$–11) of 100 μM N^G-nitro-L-arginine methyl ester (L-NAME). The (●) indicates the relaxation caused by 10 mM D-arginine in the presence of 100 μM L-NAME ($n = 3$). The points show the mean and the vertical bars the s.e.mean.

pressure significantly ($P < 0.001$) to 206 ± 9 mmHg ($n = 11$). Figure 3 shows that this augmentation of pharmacologically induced tone was reversed by L-arginine (10 μM–10 mM) in a concentration-dependent manner such that at the highest concentration used there was a significant ($P < 0.001$) reduction in perfusion pressure to 114 ± 12 mmHg (or a 50.0 ± 6.7% relaxation of established tone) ($n = 10$). The IC_{50} for the reversal of the augmentation of vasoconstriction by L-NAME was greater than 1 mM. Addition of L-arginine (10 μM–10 mM) did not significantly alter the pH of the physiological salt solution.

In a further three preparations the relaxant properties of the enantiomer D-arginine were assessed against constrictor tone in the presence of L-NAME. In these experiments basal perfusion pressure was 46.1 ± 7.2 mmHg and in the presence of 1 μM 5-hydroxytryptamine, 1 μM histamine and 100 μM L-NAME perfusion pressure was increased to 197 ± 9 mmHg and the depressor response induced by 10 mM D-arginine was 25 ± 7 mmHg (16.6 ± 4.7% reduction of tone) and this is significantly ($P < 0.01$) less than that evoked by 10 mM L-arginine under similar conditions (Figure 3).

In another seven resting preparations the perfusion pressure was 29.7 ± 4.1 mmHg and was increased to 155 ± 14 mmHg by the combination of 5-hydroxytryptamine and histamine (each 1 μM). In the absence of L-NAME additions of L-arginine brought about modest relaxations of tone which only became significant ($P < 0.05$) at 10 mM when there was a relaxation of 18.6 ± 5.3 mmHg (which represents a 14.9 ± 4.2% reduction of overall tone) and this was significantly ($P < 0.001$) less than the relaxation seen in the presence of both constrictors and L-NAME (Figure 3). Subsequent addition of 100 μM L-NAME brought about an increase in perfusion pressure to 196 ± 20 mmHg and this was significantly ($P < 0.01$) less than seen in the absence of L-arginine. In another 4 preparations the activity of L-arginine was examined at a level of tone comparable to that induced by the constrictor agents in the presence of L-NAME. In these preparations basal perfusion pressure was 32.4 ± 6.2 mmHg and this was increased to 177 ± 11 mmHg by increasing the individual concentrations of 5-hydroxytryptamine and histamine to 3 μM. Under these conditions 10 mM L-arginine reduced perfusion pressure by 18.8 ± 7.4 mmHg and this represents a 10.6% reduction in perfusion pressure. This reduction in perfusion pressure was not significantly different from that observed at the lower perfusion pressures described above but was significantly ($P < 0.001$) less than observed in the presence of L-NAME.

Effects of perfusion of the ear with CHAPS on the augmentation of constrictor responses

In six preparations the basal perfusion pressure was 41.3 ± 8.0 mmHg and the inclusion of 1 μM 5-hydroxytryptamine and 1 μM histamine increased perfusion pressure to 179 ± 18 mmHg. Perfusion of the bed with 0.3% CHAPS completely abolished endothelium-dependent relaxations to the test dose of acetylcholine (550 pmol) and the perfusion pressure of the constricted bed was 148 ± 16 mmHg and this did not differ significantly from that before perfusion with the detergent. Following perfusion of the bed with CHAPS, addition of 100 μM L-NAME did not alter the level of tone as the perfusion pressure was 150 ± 17 mmHg.

Effects of 100 μM N^G-nitro-L-arginine methyl ester and 10 mM L-arginine on vascular conductance at high constrictor tone

Submaximal (ca. 60%) pharmacological tone was induced by addition of 1 μM 5-hydroxytryptamine and 1 μM histamine to the perfusion fluid. In the presence of constrictor agents alone, pressure increased with flow (Figure 4a) and addition of 100 μM L-NAME caused an upward shift in the plot such that perfusion pressure was generally higher in the presence than in the absence of L-NAME at most flow rates. This effect of L-NAME was totally reversed by addition of L-arginine (10 mM).

In the 16 control preparations the conductance/flow relationships were determined and found to be linear with a gradient of 0.0039 ± 0.0002 $mmHg^{-1}$ and the mean elevation was 0.0173 ± 0.0007 $ml\,min^{-1}\,mmHg^{-1}$ ($r = 0.92$). It can be seen from Figure 4b that inclusion of L-NAME (100 μM) in the perfusion fluid altered the characteristics of the plot which became less linear and was best fitted to a 3rd order polynomial function ($y = 0.0042 + 0.0046x + 0.0012x^2 + 0.0004x^3$; $n = 10$). The deviation from the control conductance/flow relationships was particularly pronounced at the higher flow rates used. This effect of L-NAME was reversed by addition of 10 mM L-arginine to the buffer and the conductance/flow plot again approached linearity with a gradient of 0.0041 ± 0.005 $mmHg^{-1}$ and a mean elevation of 0.0196 ± 0.0013 $ml\,min^{-1}\,mmHg^{-1}$ ($r = 0.890$) (Figure 4b; $n = 8$). The slope and mean elevation point of this plot does not differ significantly from that of the control plot.

When L-arginine alone was added to constricted preparations there was no change in the pressure/flow relationships compared to the controls but when L-NAME was added there was a slight but non-significant upward shift in the regression line (Figure 5a). From the conductance/flow relationships it can be seen that addition of 10 mM L-arginine to the perfusion fluid did not alter either the slope (0.0037 ± 0.0003 $mmHg^{-1}$) or the mean elevation (0.0186 ± 0.0018 $ml\,min^{-1}\,mmHg^{-1}$) and r was 0.903 (Figure 5b; $n = 9$). Subsequent addition of 100 μM L-NAME did not alter the linear nature of the relationship between conductance and flow (Figure 5b). Both the gradient (0.0032 ± 0.0003 $mmHg^{-1}$) and the mean elevation (0.0144 ± 0.0009 $ml\,min^{-1}\,mmHg^{-1}$) ($r = 0.915$ and $n = 8$) did not differ significantly from the control values or those in the presence of L-arginine.

Discussion

The results presented in this paper clearly demonstrate that L-NAME is an inhibitor of endothelium-dependent relaxations in the isolated ear. Furthermore the effects of L-NAME on vascular conductance emphasize the important role of EDRF in modulating vascular tone. Interestingly, the inhibition of basal EDRF activity by L-NAME was sensitive to L-arginine while the inhibition of agonist-dependent EDRF activity was not.

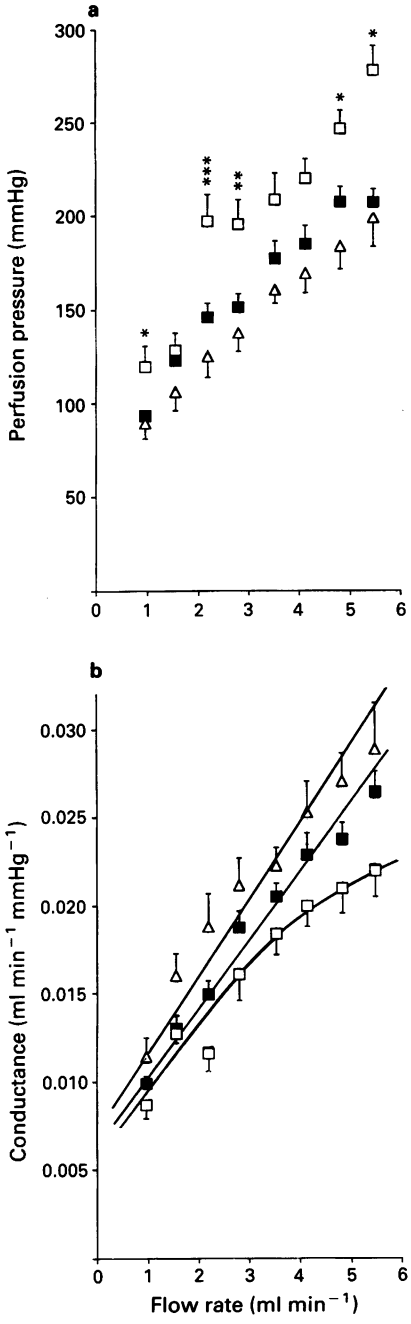

Figure 4 Effects of N^G-nitro-L-arginine methyl ester (L-NAME) and L-arginine on pressure/flow and conductance/flow relationships in the isolated rabbit ear preparation constricted with 5-hydroxytryptamine and histamine: (a) shows the pressure/flow relationships and (b) shows the conductance/flow relationships. In each case (■) shows the control data ($n = 16$), (□) shows the data in the presence of 100 μM L-NAME alone ($n = 10$) and (△) indicates the data ($n = 8$) following subsequent addition of L-arginine (10 mM). Statistically significant differences between the pressure/flow data obtained in the absence and presence of L-NAME are indicated: * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$.

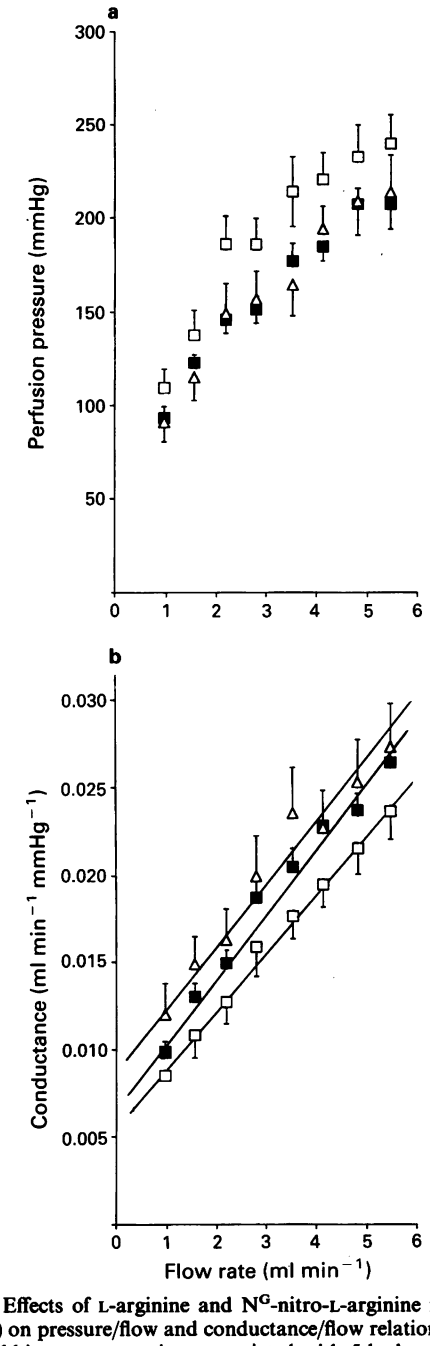

Figure 5 Effects of L-arginine and N^G-nitro-L-arginine methyl ester (L-NAME) on pressure/flow and conductance/flow relationships in the isolated rabbit ear preparation constricted with 5-hydroxytryptamine and histamine: (a) shows the pressure/flow relationships and (b) shows the conductance/flow relationships. In each case (■) shows the control data taken from Figure 4 ($n = 16$), (△) shows the regressions in the presence of 10 mM L-arginine alone ($n = 9$) and (□, $n = 8$) indicates the data following subsequent addition of L-NAME (100 μM).

The inhibition of the endothelium-dependent vasodilatation by L-NAME confirms the activity of this agent which has been described in other preparations (Rees *et al.*, 1990). The ability of 100 μM L-NAME to abolish vasodilatation to acetylcholine completely suggests that an L-arginine-derived substance is the sole mediator of endothelium-dependent vasodilatation in this preparation and rules out the participation of any other factors such as endothelium-derived hyperpolarizing factor (Taylor *et al.*, 1988). Low concentrations of L-NAME brought about non-competitive inhibition of acetylcholine-induced vasodilatation as shown by depression of the maximum response and the rightward shift of the dose-response curve. Complete inhibition was observed at the higher concentration of the inhibitor. At both concentrations of L-NAME the inhibition was insensitive to reversal by L-arginine. This finding contrasts with findings of Moore *et al.* (1990) and Kobayashi *et al.* (1990) but accords with those of Unmans (1990). Since we found that L-arginine did not influence the inhibitory effects of L-NAME in the rabbit ear, even at low concentrations of the inhibitor, then it is possible that the mechanisms responsible for the conversion of L-arginine to nitric oxide in the rabbit ear differ from those in some of the other vascular preparations studied (e.g. rat mesentery). Possible differences could exist in the mode of action of L-NAME at the level of

the enzymes responsible for the synthesis of nitric oxide or at the level of L-arginine uptake. The possibility of heterogeneity in the synthetic mechanisms for EDRF is also suggested by the *in vivo* findings of Gardiner *et al.* (1990) where administration of L-arginine reversed the haemodynamic changes induced by L-NAME in the mesenteric and renal beds but not apparently in the hindquarters of the rat.

It was observed that pretreatment of preparations with L-NAME increased the potency of the mixture of constrictor agonists while addition of this agent to preconstricted ears caused an increase in perfusion pressure. These actions are likely to reflect augmentation of tone since in the unconstricted preparation, L-NAME does not cause direct vasoconstriction as shown by the lack of significant effect on perfusion pressure in the resting preparations and previous findings (Randall *et al.*, 1990). Such augmentation of tone is thought to be due to loss of modulation by basally released EDRF as it has been shown to occur both on administration of inhibitors of EDRF synthesis/activity and on loss of the endothelium (Griffith *et al.*, 1984a,b; Martin *et al.*, 1986). In the present context the constriction caused by L-NAME was probably endothelium-dependent as it did not occur following functional destruction of the endothelium produced by perfusion with CHAPS. The augmentation of tone by L-NAME is probably due to the loss of modulation of constrictor responses by basally released EDRF, rather than that released by agonist stimulation, as neither 5-hydroxytryptamine nor histamine directly release EDRF in this preparation (Griffith *et al.*, 1988). Similar augmentations of tone have also been found to occur in the phenylephrine-constricted rat aorta in response to L-NAME, L-NMMA and N-iminoethyl ornithine (Rees *et al.*, 1990) and in response to L-NMMA and N^G-nitro-L-arginine in the noradrenaline-constricted rat isolated mesentery (Moore *et al.*, 1990). In the endothelium-intact ear preparation we found that the increases in perfusion pressure caused by L-NAME were completely reversed in a concentration-dependent manner by L-arginine. This action appeared to be selective and stereospecific as under identical conditions the enantiomer D-arginine (10 mM) only caused a modest relaxation. The action of D-arginine was comparable to the relaxation caused by L-arginine against established tone in the absence of L-NAME. Thomas *et al.* (1989) have also found that millimolar concentrations of both enantiomers cause modest endothelium-independent relaxations of phenylephrine-induced tone in rat aortic rings and this would appear due to non-specific interactions with vascular smooth muscle. It would thus appear that, while there might be a slight non-specific effect, as shown by the action of L-arginine against tone *per se*, L-arginine appears to cause appreciable reversal of the augmentation of tone caused by L-NAME.

In view of the augmentation of vascular tone discussed above, pressure/flow relationships were determined in the constricted preparation. In the control preparations perfusion pressure increased with increasing flow rate. Addition of L-NAME caused an upward shift in the regression of pressure on flow such that perfusion pressure was higher at most flow rates in the presence compared to the absence of L-NAME. These changes in pressure/flow characteristics are similar to those observed following endothelial destruction in the blood perfused mesentery (Randall & Hiley, 1988b) and in the presence of L-NMMA or haemoglobin in the rabbit ear (Griffith *et al.*, 1989; Griffith & Edwards, 1990). From the pressure/flow data, vascular conductance was determined at each flow rate used. We found a linear relationship between conductance and flow in the presence of EDRF, addition of L-NAME, however, caused a depression of the conductance/flow relationship with a departure from linearity which was particularly pronounced at the high flow rates, so that at these flow rates conductance showed smaller changes with increases in flow. This phenomenon has been observed in the ear for other inhibitors of EDRF activity/synthesis (Griffith & Edwards, 1990) and implies that in the absence of EDRF activity the vessels in the bed tend towards 'rigid tube' behaviour. The effects of L-NAME on pressure/flow relationships and hence conductance were completely reversed by addition of excess L-arginine. This effect is likely to be due to L-arginine reversing the blockade of nitric oxide synthesis by L-NAME as addition of L-arginine in the absence of L-NAME did not affect the pressure/flow relations. It should be noted also that prior addition of L-arginine to the perfusion fluid prevented L-NAME from altering significantly the pressure/flow characteristics. Thus it would appear that inhibitory effects of L-NAME on endothelium-dependent vascular regulation are sensitive to reversal by L-arginine.

The effects of L-NAME on vascular conductance may be accounted for by an inhibition of EDRF synthesis coupled to basal release. This release of EDRF may be spontaneous (Griffith *et al.*, 1984a,b) or in response to fluid flow (Rubanyi *et al.*, 1986) which is sensed through changes in shear stress (Pöhl *et al.*, 1986). Thus it would appear that, in common with agonist-dependent EDRF activity, basal EDRF activity is also inhibited by L-NAME. However, the results presented in this paper also demonstrate that the blockade by L-NAME of basal, but not agonist-stimulated, EDRF activity is reversed by L-arginine. This important observation may suggest that the enzymes coupled to EDRF release in response to agonist stimulation have different characteristics from those coupled to basal release. Other differences between agonist and basally released EDRF have previously been reported in respect to their differential sensitivity towards metabolic inhibitors (Griffith *et al.*, 1987b). In the present context one possible explanation for the differences in blockade by L-NAME could be that there are isoenzymes for nitric oxide synthesis. This is supported by the identification in N1E-115 neuroblastoma cells of cytosolic enzymes linked to humoral release and particulate enzymes accounting for tonic release of nitric oxide (Förstermann *et al.*, 1990). Other biochemical differences for the nitric oxide synthase have been reported, for example, the enzyme from rat cerebellum requires calmodulin (Bredt & Snyder, 1990) while that in rat peritoneal polymorphonuclear neutrophils is calmodulin-independent (Kosuga *et al.*, 1990). Heterogeneity in the synthetic mechanisms for nitric oxide has also been reported for porcine aortic endothelial cells (Mülsch *et al.*, 1989). In this respect Mülsch *et al.* (1989) identified a synthetic pathway which was calcium-dependent and one which was not. Both of the pathways were inhibited by 1 mM N^G-nitro-L-arginine and the calcium-independent pathway appeared to account for basal EDRF activity. More recently Radomski *et al.* (1990b) have found that vascular endothelial cells contain an inducible and a constitutive nitric oxide synthase. The former enzyme is calcium-independent and is induced by lipopolysaccharide and this may be prevented by glucocorticoids and cycloheximide. It would therefore appear that there is more than one enzyme system responsible for the production of EDRF.

In conclusion, we have found that L-NAME is a potent inhibitor of agonist-induced endothelium-dependent vasorelaxation. In this respect the action of L-NAME is not reversed by excess L-arginine and this contrasts with the action of this inhibitor in some other resistance beds. L-NAME would also appear to oppose endothelium-dependent modulation of tone in constricted preparations. In the latter situation L-arginine reverses the actions L-NAME. Taken together these results may point to differences between the mechanisms responsible for the synthesis of basal and those for agonist-dependent EDRF activity.

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