Br. J. Pharmacol. (1990), **101**, 166–170

Inhibitory role of endothelium-derived relaxing factor in rat and human pulmonary arteries

D.E. Crawley, S.F. Liu, T.W. Evans & [1]P.J. Barnes

Department of Thoracic Medicine, National Heart and Lung Institute, Dovehouse Street, London SW3 6LY

1 The inhibitory role of endothelium-derived relaxing factor was studied in both rat and human pulmonary arteries *in vitro* by inhibiting its synthesis with the L-arginine analogue N^G-monomethyl-L-arginine (L-NMMA).

2 In rat pulmonary arteries, L-NMMA pretreatment (10–300 μM) dose-dependently inhibited acetylcholine-induced relaxation (which is endothelium-dependent). N^G-monomethyl-D-arginine (D-NMMA, 100 μM) was without effect. L-Arginine, but not D-arginine, dose-dependently reversed this inhibition. L-NMMA had no effect on relaxation induced by sodium nitroprusside.

3 In human small pulmonary arteries L-NMMA (100 μM) pretreatment similarly inhibited the acetylcholine-induced relaxation but had no effect on the sodium nitroprusside-induced relaxation.

4 In both rat and human pulmonary arteries, L-NMMA, but not D-NMMA, always caused contraction of preconstricted tissues whereas it had no effect on baseline tone. In the rat this contraction was completely prevented by prior treatment with L-arginine.

5 L-NMMA (100 μM) pretreatment mimicked the effect of endothelium removal on phenylephrine-induced vasoconstriction, both resulting in an increase in tension development at each concentration of phenylephrine. This enhancement was greatest at low concentrations of phenylephrine but was still present even at the highest concentrations. Pretreatment with L-NMMA (100 μM) also significantly increased the responses to single doses of phenylephrine.

6 These results suggest that endothelium-derived relaxing factor from endothelial cells both mediates the relaxation response to acetylcholine and also acts as a physiological brake against vasoconstriction in pulmonary vessels.

Introduction

Since Furchgott & Zawadzki (1980) first demonstrated that vascular relaxation by acetylcholine (ACh) results from the release of relaxant factor from the endothelium, the identity and physiology of this endothelium-derived relaxing factor (EDRF) has been the target for much research. EDRF has now been identified as nitric oxide (NO) (Palmer *et al.*, 1987), and the source of NO has recently been elucidated. Infusions of L-arginine through a column of porcine aortic endothelial cells result in an increased bradykinin-induced release of NO only in those cells deprived of L-arginine for 24 h. This effect is stereospecific as the D-isomer is without effect (Palmer *et al.*, 1988a). In bovine aortic endothelial cells incubation with L-[guanidino-$^{15}N_2$] arginine results in the formation of ^{15}NO, $^{15}NO_2$, and $^{15}NO_3$, indicating that the terminal guanidino nitrogens of L-arginine are the physiological precursors of NO (Schmidt *et al.*, 1988).

The L-arginine analogue N^G-monomethyl-L-arginine (L-NMMA) inhibits NO release and also inhibits endothelium-dependent relaxation *in vitro* (Rees *et al.*, 1989a). *In vivo* it causes marked hypertension in anaesthetized rabbits (Rees *et al.*, 1988b); an effect reversed by L-arginine, indicating that L-NMMA restricts the availability of L-arginine for the synthesis of NO. In rabbit aortic rings *in vitro*, L-NMMA inhibits endothelium-dependent relaxation and this effect can be reversed by L-arginine but not D-arginine, indicating stereospecificity of NO formation from L-arginine (Palmer *et al.*, 1988b). However, it has been demonstrated that L-arginine, N-α-benzoyl-L-arginine, D-arginine and L-histidine produce endothelium-independent relaxation, and that L-arginine *per se* cannot be the immediate precursor of NO (Thomas & Ramwell, 1988; Thomas *et al.*, 1989b).

Endothelial-dependent relaxation has been demonstrated in pulmonary arteries of several species, including dogs (Chand & Altura, 1981) and man (Greenberg *et al.*, 1987). We have studied the effect of L-NMMA on the endothelium-dependent vasodilatation induced by ACh in rat and human pulmonary arteries *in vitro*. We have also used L-NMMA to determine whether the release of EDRF has an effect on vasoconstriction in these vessels.

[1] Author for correspondence.

Methods

Tissue preparation

The heart and major blood vessels of 250 g male Wistar rats were placed into Krebs-Henseleit (Krebs) solution containing the following (in mM): NaCl 118, KCl 5.9, $MgSO_4 \cdot 7H_2O$ 1.2, $CaCl_2 \cdot 6H_2O$ 2.5, $NaH_2PO_4 \cdot H_2O$ 1.2, glucose 5.6, $NaHCO_3$ 25.5. The right and left pulmonary arteries were then dissected free of surrounding tissue and each cut into two rings, approximately 2 mm in length. Care was taken not to touch or damage the intimal surface.

Human small pulmonary arteries (0.7–1.5 mm internal diameter) were obtained from surgical lobectomy specimens from patients with lung carcinoma. Tissues were always used within 12 h of surgery. Pulmonary arteries were identified at the cut surface of the lung and then traced peripherally. The specimens were placed in Krebs solution at 4°C and oxygenated with 95% O_2 and 5% CO_2. Following removal of the surrounding connective tissue, the vessels were cut into rings (2–3 mm in length).

Experimental procedure

The rings were mounted over two parallel wires, one being fixed and the other attached to a force-displacement transducer (FT.03 Grass, Quincy, U.S.A.). Measurements of tension were recorded on a polygraph (Grass model 7). Resting tensions of 0.5 g (rat) and 0.5–0.7 g (human) were applied to the vessels, in order to give optimal response characteristics. After the vessels had been mounted, the wires were lowered into a 2 ml organ bath filled with Krebs solution (37°C) and bubbled

with 95% O_2 and 5% CO_2. The vessels were then left to stabilize for one hour and were washed with fresh Krebs every 15 min. Endothelium-dependent and endothelium-independent relaxation were studied by preconstriction with phenylephrine (PE, 1 μM) in rat or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 30 μM) in human vessels, and then a cumulative concentration-response curve to ACh (1 nM–10 μM), which acts via the endothelium, or to sodium nitroprusside (1 nM–10 μM), which acts directly on vascular smooth muscle, constructed. Contractile responses were studied in rat vessels either by the construction of a cumulative concentration-response curve to PE (1 nM–10 μM) or by exposing the vessels to a single concentration of PE. In experiments involving single concentrations of PE, a single concentration of PE was applied to the vessels until a consistent constrictor response was obtained. Following a washout/recovery period, the tissues were then exposed to a similar concentration of PE, some tissues having been pretreated with L-NMMA. In experiments involving endothelium-denuded vessels, the endothelium was removed by lightly rubbing the vessel lumen with a small steel probe and removal confirmed by the loss of the relaxation response to ACh in preconstricted tissues. In all experiments involving pretreatment, the drug(s) was added 10 min before the experiment.

The effects of L-NMMA, D-NMMA, L-arginine and D-arginine on both relaxant and constrictor responses were investigated. The interactions of L-histidine, N-α-benzoyl-L-arginine and N-α-L-histidine with L-NMMA were also investigated.

Drugs and chemicals

Drugs were obtained from the following sources: phenylephrine, acetylcholine, sodium nitroprusside, L-histidine, L-arginine, D-arginine, N-α-benzoyl-L-arginine, and N-α-benzoyl-L-histidine (Sigma, Poole, U.K.). Propranolol hydrochloride (ICI, Cheshire, U.K.). $PGF_{2\alpha}$ Upjohn Ltd, Crawley, U.K.). N^G-monomethyl-L-arginine and N^G-monomethyl-D-arginine were kindly supplied by Dr S. Moncada (Wellcome, Beckenham, U.K.).

Statistics

The results were statistically analysed by either Student's paired t test (probability values <0.05 being considered significant), or a one-way analysis of variance followed by modified t-statistics using the Bonferroni correction for multiple comparisons.

Results

Relaxation studies

Rat pulmonary artery ACh (1 nM–30 μM) caused a dose-dependent relaxation of the rat isolated pulmonary artery rings (mean $EC_{50} = 97 \pm 12$ nM). This relaxation was unaffected by pretreatment with L-arginine (100 μM), but was inhibited dose-dependently by pretreatment with L-NMMA. The tension generated by 1 μM PE together with 3 μM L-NMMA was 400 ± 30 mg. ACh reduced this tension to 43 ± 7 mg at the highest concentration of ACh tested (10 μM). This is compared to control values of 375 ± 48 mg (1 μM PE alone) versus 62 ± 20 mg (10 μM ACh). By increasing the concentration of L-NMMA, the lowering of tension by 10 μM ACh was reduced such that at 10 μM L-NMMA, ACh reduced the tension in the tissues from 342 ± 30 mg to 120 ± 10 mg; at 30 μM L-NMMA, ACh reduced the tension from 366 ± 11 mg to 125 ± 21 mg; at 100 μM L-NMMA, from 380 ± 40 mg to 178 ± 9 mg and at 300 μM L-NMMA from 430 ± 35 mg to 215 ± 11 mg (Figure 1). Concentrations of L-NMMA up to 1 mM did not increase the inhibition of the ACh-induced relaxation beyond that seen

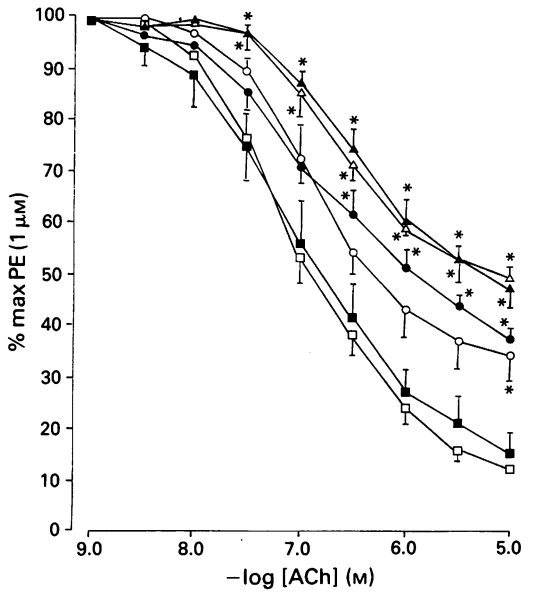

Figure 1 Concentration-response curves for the relaxation (expressed as a % of the contraction induced by 1 μM phenylephrine, PE) in rat pulmonary artery induced by acetylcholine (ACh, 1 nM–30 μM) in the absence (■) and presence of 3 μM (□), 10 μM (●), 30 μM (○), 100 μM (▲) and 300 μM (△) N^G-monomethyl-L-arginine. Mean observations are shown ($n = 6$) and vertical lines indicate s.e.mean. * $P < 0.01$ compared to control values ($n = 6$).

with 100 μM and 300 μM. By contrast, D-NMMA had no effect. This inhibition was not overcome by ACh, but was dose-dependently reversed by L-arginine pretreatment. L-Arginine 10 μM had no significant effect on the reduction in tension by 10 μM ACh (final tension 193 ± 10 mg). However, both 100 μM and 1 mM L-arginine significantly reduced the effect of 100 μM L-NMMA, final tensions in the tissues being 138 ± 21 mg and 73 ± 8 mg respectively (Figure 2). D-Arginine was without effect. L-NMMA did not significantly affect the sodium nitroprusside-induced relaxation (mean EC_{50} for untreated vessels $= 44 \pm 5$ nM compared to mean EC_{50} for treated

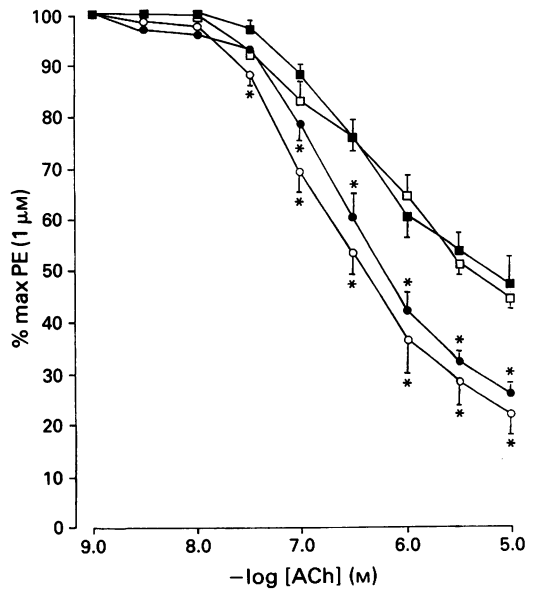

Figure 2 Reversal by L-arginine of the inhibition by 100 μM N^G-monomethyl-L-arginine (L-NMMA) of the acetylcholine (ACh)-dependent relaxation (expressed as a % of the contraction induced by 1 μM phenylephrine, PE) in rat pulmonary artery at 10 μM (□), 100 μM (●), and 1 mM (○). Relaxation to ACh in the presence of 100 μM L-NMMA alone (■). * $P < 0.05$ as compared to control values. Mean observations are shown ($n = 6$) and vertical lines indicate s.e.mean.

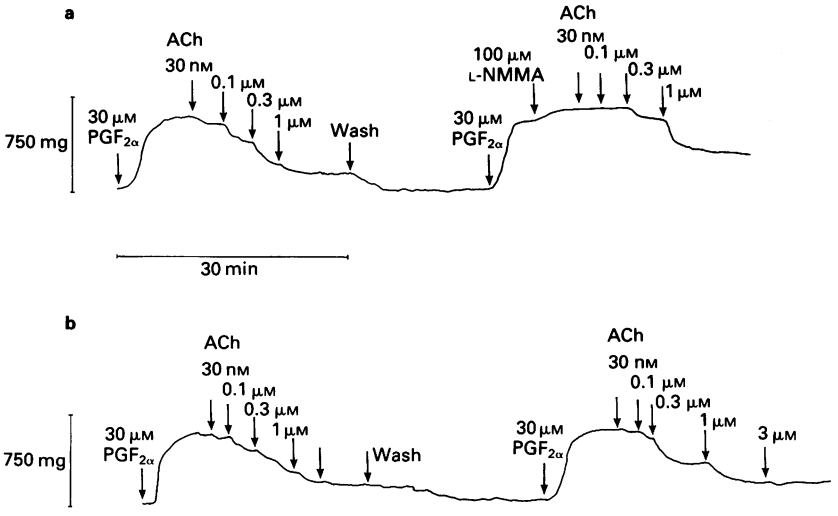

Figure 3 Traces showning the effect of N^G-monomethyl-L-arginine (L-NMMA, 100 μM) pretreatment on the acetylcholine-induced relaxation in human pulmonary arteries (a) preconstricted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), compared to a time-matched control vessel (b).

vessels = 42 ± 7 nM). L-NMMA caused a substantial contraction of preconstricted tissues at all concentrations used for pretreatment (>100% increase above baseline in some tissues), whereas D-NMMA (100 μM) was without effect. L-Arginine (100 μM) pretreatment prevented this contraction to L-NMMA completely, and exposure of vessels contracted by L-NMMA to L-arginine (100 μM) resulted in complete reversal of the contraction. D-Arginine (100 μM) was ineffective. By contrast L-NMMA had no effect on baseline tone at any concentration tested. Neither L-arginine nor L-histidine induced a relaxation of preconstricted tissues even at concentrations as high as 1 mM. Their benzoyl derivatives caused substantial relaxation (40% and 30% respectively at 0.3 mM) in both endothelium-intact and denuded vessel rings. Unlike L-arginine, both N-α-benzoyl-L-arginine and N-α-benzoyl-L-histidine caused relaxations independent of the presence of an L-NMMA contraction. L-Histidine was without effect in endothelium-intact and denuded vessels, and did not reverse contractions to L-NMMA. This indicates that both compounds cause a relaxation by mechanisms unrelated to the action of L-arginine and L-NMMA.

Endothelium removal completely abolished both the relaxant response to ACh and the contraction by L-NMMA in preconstricted tissues.

Human small pulmonary artery ACh (10 nM–10 μM) caused a dose-dependent relaxation of human small pulmonary artery rings; EC_{50} = 0.28 μM (Figure 3). Pretreatment with L-NMMA (100 μM) caused a contraction of all preconstricted tissues (mean increase = 46 ± 11%, n = 5), whereas baseline tensions were unaffected. Figure 4 shows the inhibition by 100 μM L-NMMA of the acetylcholine-induced relaxation (n = 3). D-NMMA (100 μM) failed either to inhibit the acetylcholine-induced relaxation or to cause a further contraction in preconstricted vessel rings, demonstrating the stereoselectivity of the L-NMMA effect on human small pulmonary arteries. As in the rat pulmonary artery, the relaxation to sodium nitroprusside was unaffected by L-NMMA.

Contraction studies

In all contractile studies involving PE, rat pulmonary vessels were pretreated with propranolol (10 μM) to block β-adrenoceptor-mediated vasodilatation.

Pretreatment of the tissues with L-NMMA (100 μM) significantly decreased the EC_{50} of PE (85 ± 23 nM vs 24 ± 5 nM after L-NMMA pretreatment), and resulted in a significant increase in maximum tension generation (355 ± 35 mg vs 425 ± 25 mg after L-NMMA, n = 5, $P < 0.05$) and a significant increase in tension generation at the lowest effective PE concentration (30 ± 10 mg vs 130 ± 40 mg, $P < 0.05$ after treatment). The increased contraction was greatest at low concentrations but was maintained at higher PE concentrations. Endothelium removal mimicked this effect of L-NMMA. The tensions generated by 55 mM KCl did not significantly differ between control and L-NMMA-pretreated tissues (Figure 5).

Pretreatment with L-NMMA (100 μM) significantly increased the response to a single dose of PE at all concentrations tested. When expressed as a % of the initial consistent response to PE, the potentiation was greater at the lowest PE concentration tested with an increase of 212 ± 33% at 30 nM compared to 143 ± 9% at 1 μM PE. Pretreatment with L-arginine (100 μM) had no significant effect on the contractile response.

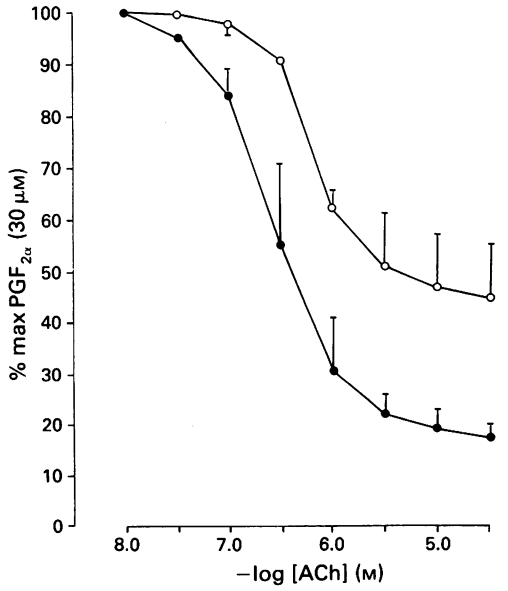

Figure 4 Concentration-response curves for the relaxation induced by acetylcholine (ACh, expressed as a % of the contraction induced by 30 μM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in human pulmonary artery rings; control rings (●), and rings pretreated with 100 μM N^G-monomethyl-L-arginine (○) are shown. Each point represents the mean (n = 3) and vertical lines show s.e.mean.

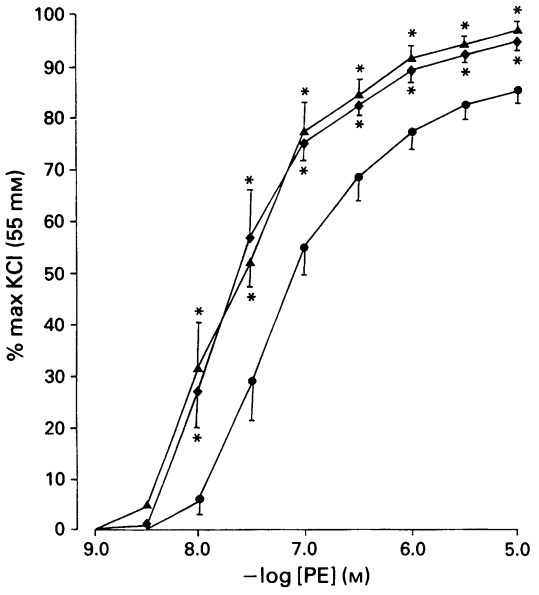

Figure 5 Concentration-response curves for the contraction induced by phenylephrine (PE) in rat pulmonary artery rings; control vessels (●), vessels pretreated with 100 μM N^G-monomethyl-L-arginine (◆), and vessels denuded of endothelium (▲). * $P < 0.05$, as compared to control values. Mean observations are shown ($n = 6$) and vertical lines indicate s.e.mean. Tensions generated by KCl did not differ significantly between the groups.

Discussion

We have demonstrated that inhibition of EDRF synthesis with L-NMMA reduces the endothelium-dependent relaxation in rat and human pulmonary arteries, indicating that EDRF is involved in this response. We have also shown that EDRF modulates the contractility of pulmonary arteries. The inhibition of ACh relaxation by L-NMMA but not D-NMMA, and the reversal of this inhibition by L-arginine but not D-arginine provides evidence that EDRF in rat and human pulmonary arteries is likely to be EDRF derived from L-arginine. The lack of effect of L-NMMA on sodium nitroprusside-induced relaxation in both species demonstrates that the site of action of L-NMMA is the endothelium.

It is of interest that L-NMMA did not completely abolish the ACh-induced relaxation in either rat or human vessels. There are several possible explanations for this. Perhaps relaxant factors other than EDRF are released from pulmonary endothelial cells, or EDRF could be produced from a source unaffected by L-NMMA. The most likely explanation, however, is that L-NMMA is not a highly potent inhibitor of EDRF production from L-arginine.

It has been suggested that L-arginine does not uniquely elicit endothelium-dependent relaxation and that the basic amino acids such as arginine, lysine, glutamine and histidine induce a non-specific relaxation at high concentrations (Thomas *et al.*, 1989b). In our study, only L-arginine reversed the contraction caused by L-NMMA, whereas histidine and its benzoyl derivatives were without effect. Furthermore the effect of arginine was stereoselective since D-arginine was without effect. Similarly, only L-arginine was effective in reversing the inhibitory effect of L-NMMA on ACh-induced relaxation. Unlike with L-arginine, increasing the concentration of ACh failed to overcome the inhibition of endothelium-dependent relaxation by L-NMMA. All of our conclusions rely on the assumption that L-NMMA does indeed specifically inhibit EDRF production in rat and human pulmonary arteries. In the rabbit aorta, the release of NO by ACh infusions (detected by chemiluminescence) is inhibited dose-dependently by 3–300 μM L-NMMA (IC_{50} = 15 μM) (Rees *et al.*, 1989a). Also, the release of NO from bradykinin-stimulated porcine aortic endothelial cells is inhibited by 0.3–10 μM L-NMMA (IC_{50} = 3 μM) (Palmer *et al.*, 1988b). We have shown that L-NMMA, but not D-NMMA, only caused a contraction in tissues which were preconstricted, and never caused a contraction at baseline tensions in any experiment in either rat or human pulmonary arteries. This is consistent with the observation that increments in flow through arteries *in vitro* cause vasodilatation due to the release of EDRF (Rubanyi *et al.*, 1986), in that when the tissue is at baseline tension *in vitro*, no EDRF is released. Once tension is increased with the α-adrenoceptor agonist PE, EDRF is released and thus L-NMMA causes a further contraction by removing the EDRF relaxant brake. It has been suggested that L-NMMA is a non-specific inhibitor of vascular relaxation (Thomas *et al.*, 1989a). However, this is based on the observation that L-NMMA reversed a 30–50% vasodilatation of a number of unrelated compounds. Because none of the tissues were at baseline tension when L-NMMA was applied, the shear stress resulting from the remaining tension would itself generate EDRF release and hence L-NMMA would cause contraction by removing the EDRF brake, as in our preparations.

L-NMMA may also be useful in demonstrating the nature of EDRF release from pulmonary arteries *in vitro*. L-NMMA pretreatment did not alter the lowest effective concentration of PE as expected, because EDRF is only released (and thus removed) when tension is generated by the agonist. At all concentrations of PE tested, the tension generated was significantly increased by L-NMMA pretreatment. This increase in tension was greater at low concentrations but was seen over the whole range of the dose-response curve. This indicates that either EDRF release rises with increased tension, or that a fixed amount of EDRF is released which is more effective as a functional antagonist at lower tensions. Single dose applications of PE support this finding in that the potentiation (as a % of the original contraction) is greater at lower PE concentrations (and hence low tensions). At high PE concentrations the potentiation is less but significantly and consistently maintained. Since stretch-activated ion channels exist in porcine endothelial cells as mechanotransducers (Lansman *et al.*, 1987), it seems more probable that EDRF release rises with increases in tension. Both L-NMMA pretreatment and endothelium removal significantly increased the response attained at any given PE concentration. Responses obtained after endothelium removal were identical to those seen after L-NMMA pretreatment, indicating that EDRF is probably the only relaxing factor released under these conditions from endothelial cells of rat pulmonary artery.

Physiologically we have demonstrated that EDRF can act as a brake which is most effective at lower tensions and, perhaps, provides a protective mechanism against inappropriate vasoconstriction. It would be interesting to investigate the efficacy of this brake in pulmonary vascular disease. Any impairment in EDRF generation would predispose to excessive pulmonary vasoconstriction and thus pulmonary hypertension.

We thank Dr S. Moncada for the kind gift of N^G-monomethyl-L-arginine and N^G-monomethyl-D-arginine. We thank the Chest, Heart and Stroke Association and British Heart Foundation for support.

References

CHAND, N. & ALTURA, B.M. (1981). Acetylcholine and bradykinin relax intrapulmonary arteries by acting on endothelial cells: role in lung vascular disease. *Science*, **213**, 1376–1379.

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288**, 373–376.

GREENBERG, B., RHODEN, K. & BARNES, P.J. (1987). Endothelium-dependent relaxation of human pulmonary arteries. *Am. J. Physiol.*, **252**, H434–H438.

LANSMAN, J.B., HALLAM, J.H. & RINK, T.J. (1987). Single stretch-

activated ion channels in vascular endothelial cells as mechanotransducers. *Nature*, **325**, 811–813.

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., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524–526.

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.

REES, D.D., PALMER, R.M.J., HODSON, H.F. & MONCADA, S. (1989a). A specific inhibitor of nitric oxide formation from L-arginine attenuates endothelium-dependent relaxation. *Br. J. Pharmacol.*, **96**, 418–424.

REES, D.D., PALMER, R.M.J. & MONCADA, S. (1989b). Role of endothelium-derived nitric oxide in the regulation of blood pressure. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 3375–3378.

RUBANYI, G.B., ROMERO, J.C. & VANHOUTTE, P.M. (1986). Flow-induced release of endothelium-derived relaxing factor. *Am. J. Physiol.*, **250**, H1145–H1149.

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

THOMAS, G., COLE, E.A. & RAMWELL, P.W. (1989a). N^G-monomethyl L-arginine is a non-specific inhibitor of vascular relaxation. *Eur. J. Pharmacol.*, **170**, 123–124.

THOMAS, G., HECKER, M. & RAMWELL, P.W. (1989b). Vascular activity of polycations and basic amino acids: L-arginine does not specifically elicit endothelium-dependent relaxation. *Biochem. Biophys. Res. Commun.*, **158**, 177–180.

THOMAS, G. & RAMWELL, P.W. (1988). Vasodilatory properties of mono-L-arginine containing compounds. *Biochem. Biophys. Res. Commun.*, **154**, 332–338.

(Received January 18, 1990
Revised May 1, 1990
Accepted May 16, 1990)