British Journal of Pharmacology (2000) 130, 997–1004

www.nature.com/bjp

Critical dependence of the NO-mediated component of cyclic AMP-induced vasorelaxation on extracellular L-arginine in pulmonary arteries of the rat

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1 A component of isoprenaline-mediated vasorelaxation in pulmonary arteries is mediated by nitric oxide (NO). We examined the effects of physiological concentrations ($\leqslant 400$ μM) of L-arginine on isoprenaline-induced relaxation in rat pulmonary arteries, and following inhibition of L-arginine uptake with L-lysine. In addition, we examined the role of the endothelium, and whether L-arginine affected acetylcholine (ACh)-induced relaxation.

2 Isoprenaline-induced relaxation was potentiated by 400 μM L-arginine in pulmonary arteries; maximum relaxation was increased from $83 \pm 4\%$ of initial tone to $94 \pm 4\%$ ($P < 0.05$). L-lysine (10 mM) not only abolished the potentiation by L-arginine, but suppressed relaxation compared to control ($70 \pm 4\%$, $P < 0.05$), even in the absence of L-arginine added to the bath. Blockade of NO synthase with 100 μM L-NMMA or removal of the endothelium inhibited isoprenaline-induced relaxation to the same extent as L-lysine, and under these conditions the presence or absence of 400 μM L-arginine made no difference. L-lysine had no additional effect when applied in combination with L-NMMA.

3 The effect of extracellular L-arginine was concentration dependent, with an apparent EC_{50} of ~ 1–7 μM.

4 Relaxation to the membrane permeant cyclic AMP analogue CPT cyclic AMP was also potentiated by L-arginine and inhibited by L-lysine. There was however no difference in relaxation induced by acetylcholine (ACh) in the presence of L-arginine or L-lysine, and isoprenaline-induced relaxation of mesenteric arteries was unaffected by L-arginine or L-lysine.

5 These results strongly suggest that extracellular L-arginine is critically important for development of the NO- and endothelium-dependent component of cyclic AMP-induced vasorelaxation in rat pulmonary arteries, but is not required for ACh-induced relaxation. As the apparent EC_{50} for this effect is in the low micromolar range it is likely to be fully activated *in vivo*, as plasma L-arginine is > 150 μM.

British Journal of Pharmacology (2000) **130,** 997–1004

Keywords: Pulmonary artery; isoprenaline; cyclic AMP, β-adrenoceptors; nitric oxide; nitric oxide synthase; L-arginine

Abbreviations: ACh, acetylcholine; cyclic AMP, adenosine 3′,5′-cyclic monophosphate; CPT cyclic AMP, 8-(4-chlorophenylthio)-adenosine 3′,5′-cyclic monophosphate; HUVEC, human umbilical vein endothelial cells; KPSS, physiological salt solution containing 80 mM KCl, equimolar substitution for NaCl; L-NAME, L-N^G-nitroarginine methyl ester; L-NMMA, L-N^G-monomethylarginine; NO, nitric oxide; PDE, phosphodiesterase; PE, phenylephrine; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PSS, physiological salt solution

Introduction

It is becoming clear that in many vascular beds, including the pulmonary, vasodilation to β-adrenergic agonists is mediated at least in part *via* nitric oxide (NO) (Gray & Marshall, 1992; Graves & Poston, 1993; Delpy *et al*., 1996; Priest *et al*., 1997; Ferro *et al*., 1999). It has been proposed that this is due to activation of the endothelium NO synthase (eNOS, NOS III) by cyclic AMP, and does not require an increase in endothelial cell intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) (Gray & Marshall, 1992; Priest *et al*., 1997; 1999; Ferro *et al*., 1999).

L-arginine is an essential substrate for eNOS and the production of NO (Moncada *et al*., 1991). It is generally assumed that under normal physiological conditions L-arginine is in excess, as the EC_{50} for activation of eNOS is $\sim$1-10 μM (Palmer & Moncada, 1989; Su *et al*., 1997), yet the intracellular concentration of L-arginine in the endothelial cell is significantly greater than 100 μM (Baydoun *et al*., 1990; Block *et al*., 1995), and sufficient to maximally activate the enzyme (Su *et al*., 1997). Most studies on isolated preparations do not therefore include L-arginine in the extracellular medium. However application of extracellular L-arginine has been shown to increase or restore NO-dependent relaxation in both pulmonary and systemic circulations in certain conditions and disease states, although there may be no significant decline in endothelial cell L-arginine content (Eddahibi *et al*., 1992; Creager *et al*., 1992; Taylor & Poston, 1994). Moreover, we have shown in isolated pulmonary arteries of the rat that the endothelium- and NO-dependent component of relaxation induced by tetramethylpyrazine, a putative cyclic AMP-specific PDE inhibitor (Lin *et al*., 1993), is substantially potentiated by extracellular L-arginine, with an EC_{50} of ~ 1 μM (Peng *et al*., 1996). In contrast, acetylcholine-induced (ACh) relaxation of pulmonary arteries was unaffected by addition of extracellular L-arginine, suggesting that intracellular L-arginine was not depleted (Peng *et al*., 1996). It is not clear however whether the requirement for L-arginine was related to the cyclic AMP raising activity of tetramethylpyrazine, or another non-specific action.

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We therefore investigated the effect of externally applied L-arginine, in physiologically relevant concentrations, on isoprenaline- and cyclic AMP-induced relaxation of rat pulmonary and mesenteric arteries. Our results suggest that in pulmonary arteries extracellular L-arginine and L-arginine uptake is required for NO-dependent relaxation induced by cyclic AMP, but not for ACh-induced relaxation.

Methods

Tissue preparations

Adult, male Wistar rats (250–350 g) were killed by anaesthetic overdose (intraperitoneal injection of pentabarbitone, 50 mg/kg), as approved by the Home Office Inspector. The heart and lungs and a section of large intestine were excised and placed in a physiological salt solution (PSS) containing (in mM): NaCl 118; $NaHCO_3$ 24; $MgSO_4$ 1; NaH_2PO_4 0.435; glucose 5.56; Na-pyruvate 5; $CaCl_2$ 1.8, and KCl 4. First branch pulmonary (1393 ± 18 μm i.d.) and mesenteric arteries (306 ± 14 μm) were dissected free of connective tissue and mounted in a small vessel myograph as previously described (Priest *et al*., 1997; 1999), and equilibrated with 5% CO_2 in air, (pH 7.35-7.40, 37°C). In some experiments the endothelium was disrupted *in situ* by gently rubbing the luminal surface of the artery with a 40 μm wire or human hair. The presence of a functioning endothelium was determined by application of acetylcholine (ACh; 10 μM) following agonist induced contraction. After 60 min equilibration the arteries were subjected to a standard run up procedure of three 4 min exposures to PSS containing high K^+ (KPSS, 80 mM [K^+], equimolar substitution for NaCl) (Leach *et al*., 1992; Priest *et al*., 1999). Arteries producing less than 1 mN mm^{-1} were discarded. After washing with PSS the arteries returned to baseline tone.

Experimental protocols

Influence of L-arginine on vasorelaxation to isoprenaline Cumulative concentration-response relationships were constructed for the vasorelaxant action of the non-selective β-adrenoceptor agonist isoprenaline in pulmonary arteries, following stable pre-contraction with either $PGF_{2\alpha}$ (50 μM) or the α_1-adrenergic agonist PE (10 μM), and in the presence and absence of 400 μM L-arginine or D-arginine. This concentration of L-arginine had no effect on the pH of the PSS. Both $PGF_{2\alpha}$ and PE were investigated as we have previously shown quantitative differences between isoprenaline-induced relaxation of pulmonary arteries constricted with these agents (Priest *et al*., 1999). The agonist concentrations were chosen so as to elicit the same degree of tension (~85% KPSS), and therefore match any stimulation of NO induced by stretch alone. Similar experiments were performed on mesenteric arteries for isoprenaline, following preconstriction with $PGF_{2\alpha}$ only. We have previously demonstrated that isoprenaline concentrations above 100 nM cause a small but significant α-adrenoceptor-mediated vasoconstriction in pulmonary arteries of the rat (Priest *et al*., 1997). With the exception of experiments using PE as the vasoconstrictor, studies involving isoprenaline were therefore performed in the presence of 10 μM phentolamine (Priest *et al*., 1997). Tension was allowed to stabilize following every addition, and is expressed in terms of the initial induced tension. The role of nitric oxide (NO) and NO synthase was investigated following pre-incubation for 20 mins with L-N^G-monomethylarginine (L-NMMA; 100 μM), or in some cases L-N^G-nitroarginine methyl ester (L-NAME). Experiments were also performed following removal of the endothelium. The requirement for L-arginine uptake was examined by addition of 10 mM L-lysine as a competitive inhibitor of both Na^+ independent (system y^+) and Na^+ dependent transport (Greene *et al*., 1993); 10 mM mannitol was used as an osmotic control. Experiments were time matched in separate arteries.

Concentration-dependence of the action of L-arginine We have previously shown that potentiation of the NO-dependent component of tetramethylpyrazine by external L-arginine has an EC_{50} of about 1 μM (Peng *et al*., 1996). We therefore examined the concentration-dependence of L-arginine on isoprenaline-induced vasorelaxation. Cumulative concentration-response relationships were constructed for the vasorelaxant action of isoprenaline in pulmonary arteries, following stable pre-contraction with $PGF_{2\alpha}$ (50 μM), in the presence of 1–400 μM L-arginine.

Effect of L-arginine and L-lysine on CPT cyclic AMP-induced relaxation β-adrenoceptors agonists act *via* adenylate cyclase to increase cyclic AMP. We therefore investigated whether 400 μM L-arginine affected vasorelaxation induced by CTP cyclic AMP (8-(4-chlorophenylthio)-adenosine 3′,5′-cyclic monophosphate), a membrane permeant analogue of cyclic AMP, and in the presence of 10 mM L-lysine. Experiments were performed as described above.

Effect of L-arginine on ACh-induced relaxation in pulmonary arteries We have previously demonstrated that extracellular L-arginine does not affect vasorelaxation induced by the classical endothelium-dependent vasorelaxant ACh (Peng *et al*., 1996). In order to determine whether competitive inhibition of L-arginine uptake with L-lysine had any effect on ACh-induced relaxation, cumulative concentration response relationships were constructed for ACh following preconstriction with $PGF_{2\alpha}$ (50 μM) in the presence of 400 μM L-arginine or L-arginine plus 10 mM L-lysine.

Effect of L-arginine on isoprenaline-induced relaxation of mesenteric arteries It is well known that pulmonary arteries differ in their response to both cyclic AMP- and NO-dependent vasorelaxants compared to systemic arteries. We therefore examined the effects of L-arginine and L-lysine on isoprenaline-induced relaxation of mesenteric arteries. Cumulative concentration-response relationships were constructed for the vasorelaxant action of isoprenaline following stable pre-contraction with $PGF_{2\alpha}$ (50 μM), and in the presence of 400 μM L-arginine or L-arginine plus 10 mM L-lysine.

Chemicals and solutions

All drugs were obtained from Sigma, U.K. with the exception of $PGF_{2\alpha}$ (Upjohn Pharmaceuticals Ltd., Crawley, U.K.), L-NMMA and CPT cyclic AMP (Novabiocem, Notts., U.K.). Other chemicals were of Analar quality (BDH, Southampton, U.K.). Drugs were prepared as stock solutions using PSS. PSS was made up for each experiment using water freshly drawn from a reverse osmosis-deionisation plant with UV irradiation (Elgastat, Elga Ltd, U.K.).

Data and statistical analysis

Initial developed tensions are given as mN mm^{-1} artery length. Concentration-response curves to the vasoconstrictors are expressed as a percentage of the response to 80 mM KCl (iso-osmolar substitution for NaCl). Relaxation is expressed as a

percentage of the initial tension. The EC_{50} and extrapolated maximum response were estimated for individual concentration-response curves using non-linear least-squares regression (SigmaStat, Jandel Scientific, U.S.A.) where appropriate. EC_{50} values were converted to negative logarithmic values (PD_2) for all statistical analysis, although for ease of comprehension EC_{50} values [$\pm$95% confidence limits] are given in the text. All other values are given as mean$\pm$s.e.mean. Data were compared using an unpaired Student's *t*-test or ANOVA with a Student-Newman-Keuls *post hoc* test as appropriate (SigmaStat, Jandel Scientific, U.S.A.). Differences were considered significant at $P<0.05$.

Results

Initial tensions

There was no difference between the tension developed in pulmonary arteries in response to 50 μM $PGF_{2\alpha}$ and 10 μM PE alone, or in the presence of either 400 μM L-arginine or 10 mM L-lysine (Table 1).

Effect of L-arginine on isoprenaline-induced relaxation in pulmonary arteries

L-arginine (400 μM) potentiated isoprenaline-induced relaxation in arteries constricted with either PE or $PGF_{2\alpha}$ (Figures 1 and 2, and Table 2). For PE, maximum relaxation was significantly increased ($P<0.05$), and the EC_{50} reduced ($P<0.05$). For $PGF_{2\alpha}$ only the increase in maximum relaxation reached significance ($P<0.05$). D-arginine (400 μM) was without effect (Figure 1, $n=6$; data shown for PE only). Surprisingly, when 10 mM L-lysine, a competitive inhibitor of L-arginine uptake, was added in the presence of 400 μM L-arginine, there was a significant inhibition of isoprenaline-induced relaxation for both vasoconstrictors even compared to control (Figures 1 and 2, Table 2). The effect of 10 mM L-lysine in the absence of added L-arginine was not significantly different from its inhibitory effect in the presence of L-arginine for both PE and $PGF_{2\alpha}$ (Table 2). Mannitol (10 mM), used as an osmotic control for L-lysine, had no effect on the potentiation of isoprenaline-induced relaxation by L-arginine (Figure 1, $n=7$); it also had no effect on isoprenaline-induced relaxation in the absence of L-arginine (data not shown, $n=3$).

Effect of nitric oxide synthase inhibition

In order to determine whether the potentiation by L-arginine was indeed mediated by NO, we examined the effect of blockade of the nitric oxide synthase with L-NMMA (100 μM) in the presence of 400 μM L-arginine. For both PE and $PGF_{2\alpha}$ constricted pulmonary arteries L-NMMA completely abolished the effect of L-arginine, and moreover shifted the concentration-response curves such that they were no longer significantly different from those in the presence of L-lysine (Figures 1 and 2, Table 2). For PE constricted arteries only, the effects of L-NMMA alone, and in combination with L-lysine and L-arginine were also examined. L-NMMA (100 μM) had the same effect in the absence of L-arginine as in its presence (56.6$\pm$5.0%, $n=5$, not shown in figure for clarity), suggesting both that the potentiating action of L-arginine on isoprenaline-induced relaxation is entirely related to NO, and that this concentration of L-NMMA was sufficient to abolish the effect of 400 μM L-arginine. L-lysine (10 mM) in the presence of both 400 μM L-arginine and 100 μM L-NMMA caused no further inhibition of isoprenaline-induced relaxation compared to L-NMMA alone or L-NMMA plus L-arginine (71.0$\pm$2.2%, $n=4$; data shown in Figure 3 for clarity).

L-NMMA has been shown to compete for the y^+ transporter (Bogle *et al.*, 1992). To address the possibility that the effect of 100 μM L-NMMA was at least partly due to inhibition of L-arginine uptake rather than direct inhibition of NOS, we also examined the effect of a relatively high concentration of L-NAME (1 mM) in the presence of L-arginine, as L-NAME is not carried by the y^+ transporter (Bogle *et al.*, 1992). L-NAME had the same effect on isoprenaline-induced relaxation as 100 μM L-NMMA, with a maximum relaxation of 60.7$\pm$6.6% ($n=5$, Figure 3), suggesting that 100 μM L-NMMA was maximally inhibiting NOS under these conditions, and not just inhibiting the transporter.

Role of the endothelium

Experiments were performed following removal of the endothelium to determine whether the effects of L-arginine were endothelium dependent. In the absence of L-arginine the concentration-response curves for isoprenaline in both PE and

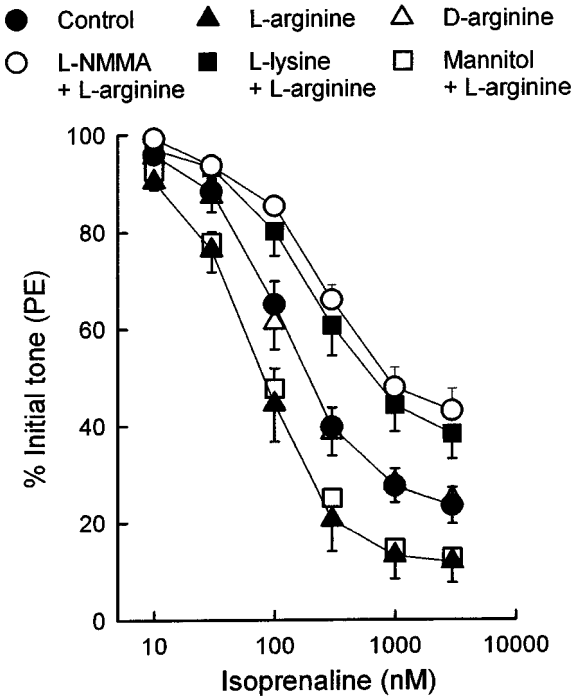

Figure 1 Isoprenaline concentration-response curves in pulmonary arteries constricted with 10 μM PE, and in the presence of L-arginine (400 μM), D-arginine (400 μM), L-arginine plus L-NMMA (100 μM), L-lysine (10 mM) or mannitol (10 mM). Each point is the mean of 6–11 experiments, and symbols are mean$\pm$s.e.mean; where no error bar is shown, the error is smaller than the symbol.

Table 1 Tension (mN mm^{-1}) induced by 10 μM PE and 50 μM $PGF_{2\alpha}$ in pulmonary arteries, and in the presence of 400 μM L-arginine and 10 μM lysine.

	PE (10 μM)	*$PGF_{2\alpha}$* (50 μM)
Control	2.30$\pm$0.15, $n=14$	2.37$\pm$0.14, $n=10$
L-arginine (400 μM)	2.14$\pm$0.11, $n=18$	2.23$\pm$0.18, $n=11$
Lysine (10 mM)	2.37$\pm$0.16, $n=17$	2.50$\pm$0.20, $n=13$

$PGF_{2\alpha}$ constricted denuded arteries were essentially identical to those for endothelium intact arteries in the presence of L-NMMA, L-lysine, or L-lysine, L-NMMA and L-arginine combined (Figures 2 and 3) (PE: 70.6±8.6%, $n=5$; EC_{50}: 282 [−104, +166] nM; $PGF_{2\alpha}$: 45.5±3.3%, $n=9$; EC_{50}: 118 [−48, +81] nM). L-arginine had no effect on isoprenaline-induced relaxation of denuded arteries (Figure 3) (PE constricted denuded arteries: 82±2.6%, $n=5$; EC_{50}: 322 [−92, +129] nM).

Concentration-response of L-arginine

Isoprenaline concentration-response curves were constructed as above for $PGF_{2\alpha}$ constricted pulmonary arteries in the presence of 1, 10 and 100 μM L-arginine. Increasing concentrations of L-arginine caused a concentration-dependent increase in the maximum relaxation calculated from the fitted curve for each individual experiment (1 μM: 64.4±3.3%, $n=7$; 10 μM: 71.0±1.9%, $n=4$; 100 μM: 74.6±1.3%, $n=5$). These data plus the results obtained above for 400 μM L-arginine and control (no added L-arginine) are plotted as the derivative plot shown in Figure 4. These data were fitted assuming Michaelis-Menten kinetics (solid line in Figure 4), giving an apparent EC_{50} of 7±6 μM and a maximum L-arginine dependent component of relaxation of 12±3% of initial tension ($n=31$).

However, the above analysis assumes that the concentration of L-arginine adjacent to the endothelium is zero when L-arginine is omitted from the PSS, as in the control group. This assumption may be incorrect, as under these conditions blockade of L-arginine uptake mechanisms with L-lysine still caused significant inhibition of isoprenaline-induced relaxation (see above), implying that there is an endogenous source of extracellular L-arginine. If this is the case, the magnitude of the L-arginine-dependent component of relaxation would be underestimated, and a more accurate figure for the effect of zero available L-arginine may be provided by the data from experiments performed in the presence of 10 mM L-lysine alone. Substituting this data for the control group resulted in a smaller EC_{50} of 0.9±0.4 μM, and an increased L-arginine-dependent component of relaxation of 23±4% of initial tension ($n=26$).

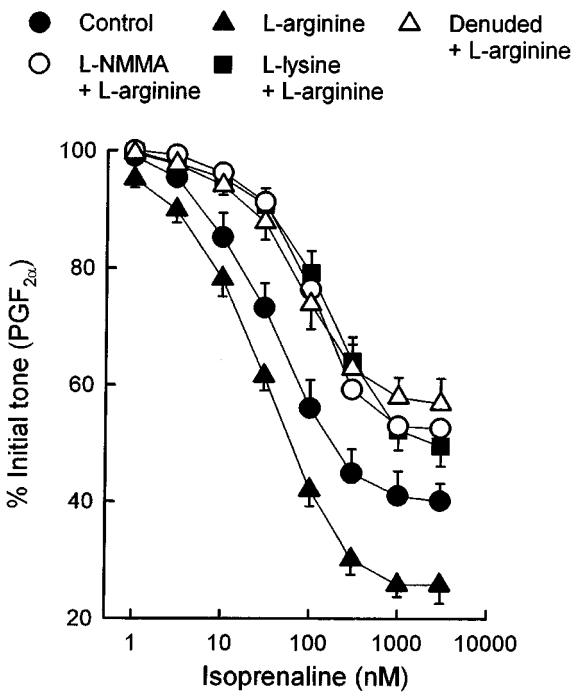

Figure 2 Isoprenaline concentration-response curves in pulmonary arteries constricted with 50 μM $PGF_{2\alpha}$ and in the presence of L-arginine (400 μM), L-arginine plus L-NMMA (100 μM) or L-lysine (10 mM). The response of endothelium-denuded arteries in the presence of L-arginine is also shown. Each point is the mean of 6–10 experiments, and symbols are mean±s.e.mean.

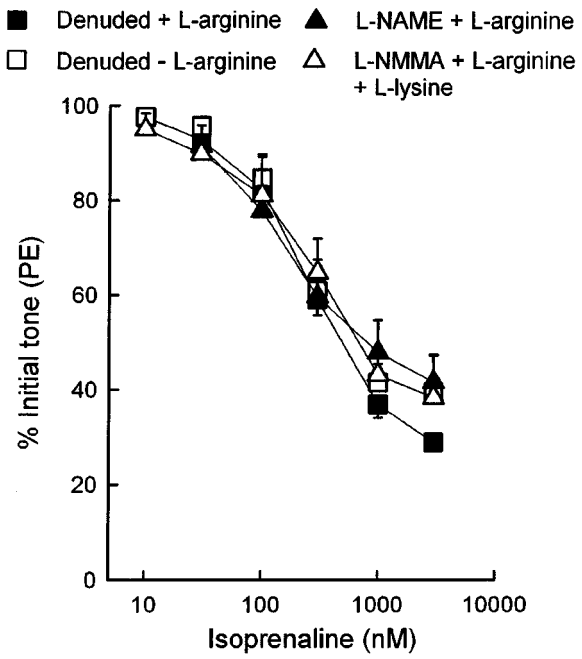

Figure 3 Isoprenaline concentration-response curves in intact pulmonary arteries constricted with 10 μM PE in the presence of L-NAME (1 mM) plus L-arginine (400 μM), and the combined presence of L-arginine (400 μM), L-NMMA (100 μM) and L-lysine (10 mM); and in endothelium-denuded arteries in the presence and absence of L-arginine. Each point is the mean of 4–5 experiments, and symbols are mean±s.e.mean; where no error bar is shown, the error is smaller than the symbol.

Table 2 Maximum relaxation and EC_{50} for isoprenaline in pulmonary arteries. *$P<0.05$, **$P<0.01$ compared to control; †$P<0.01$, ††$P<0.001$ compared to L-arginine (400 μM).

	PE constricted arteries			*$PGF_{2\alpha}$ constricted arteries*		
	Max relaxation (% initial tension)	*EC_{50}* (nM)	n	*Max relaxation* (% initial tension)	*EC_{50}* (nM)	n
Control	82.7±3.7%	138 [−37, +50]	9	63.7±2.6%	38 [−14, +22]	9
L-arginine (400 μM)	94.4±4.0%*	69 [−21, +31]*	8	75.5±2.3%	27 [−5. +6]	6
L-lysine (10 μM)+L-arginine	69.7±4.4%*††	281 [−11, +17]*††	11	54.8±2.9%*††	165 [−72, +130]**††	7
L-lysine alone	72.9±2.6%*†	235 [−63, +86]*††	6	51.7±3.7%*††	207 [−89, +93]**††	4
L-NMMA (100 μM)+L-arginine	64.6±5.4%*††	270 [−75, +104]*††	8	52.6±1.4%**††	117 [−21, +25]**††	8

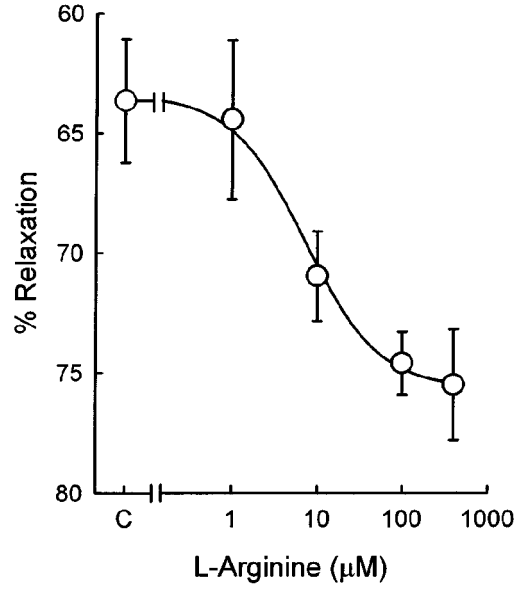

Figure 4 Derivative plot showing concentration dependence of the potentiating effect of L-arginine on the maximum relaxation to isoprenaline. *C* represents the control data (in the absence of added L-arginine). The solid line is the fitted curve, assuming Michaelis-Menten kinetics (see text). Each point is the mean of 4–10 experiments, and symbols are mean ± s.e.mean.

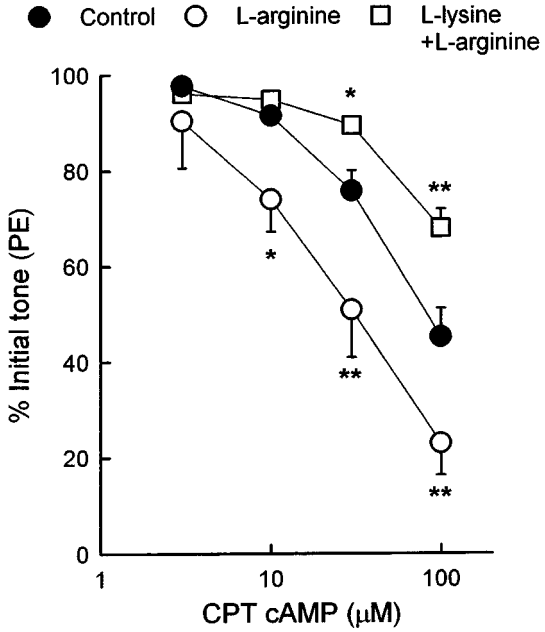

Figure 5 CPT cyclic AMP concentration-response curves in pulmonary arteries constricted with 10 μM PE, and in the presence of L-arginine (400 μM) or L-arginine plus L-lysine (10 mM). Each point is the mean of 7–10 experiments, and symbols are mean ± s.e.mean. $*P<0.05$, $**P<0.01$ compared to control; $\dagger P<0.05$, $\dagger\dagger P<0.001$ L-arginine compared to L-lysine (ANOVA, Student-Newman-Keuls *post hoc*).

Effect of L-arginine and L-lysine on cyclic AMP-mediated relaxation

L-arginine (400 μM) potentiated CPT cyclic AMP-induced relaxation in pulmonary arteries constricted with PE ($P<0.05$), whereas 10 mM L-lysine plus L-arginine inhibited relaxation ($P<0.05$; Figure 5). The data could not be adequately fitted, and comparisons between curves were made using two way ANOVA and a Student-Newman-Keuls *post hoc* test (see Figure 5).

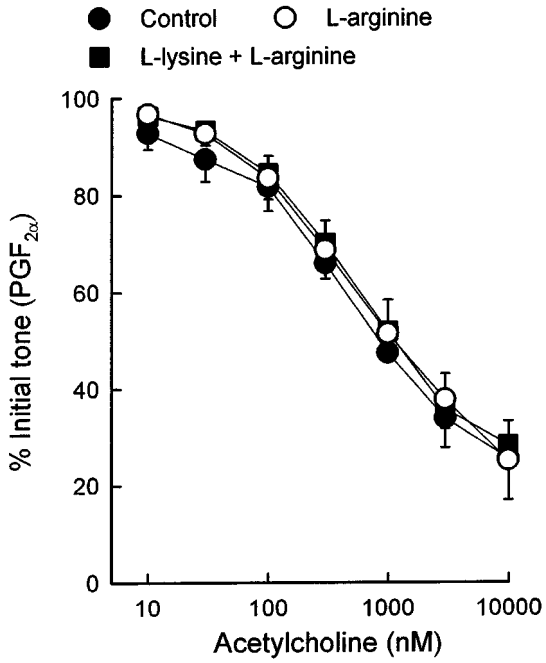

Figure 6 ACh concentration-response curves in pulmonary arteries constricted with 50 μM $PGF_{2\alpha}$, and in the presence of L-arginine (400 μM) or L-arginine plus L-lysine (10 mM). Each point is the mean of five experiments, and symbols are mean ± s.e.mean; where no error bar is shown, the error is smaller than the symbol.

Effect of L-arginine and L-lysine on ACh-induced relaxation

Figure 6 demonstrates that neither 400 μM L-arginine nor 10 mM L-lysine plus 400 μM L-arginine had any effect on the concentration-response to ACh of $PGF_{2\alpha}$-constricted pulmonary arteries ($n=5$ each group).

Effect of L-arginine and L-lysine on isoprenaline induced relaxation of mesenteric arteries

The results are shown in Figure 7. Neither addition of 400 μM L-arginine nor L-arginine plus 10 mM L-lysine had any effect on ACh-induced relaxation in mesenteric arteries ($n=5-6$ for each group). L-lysine alone was also without effect (data not shown, $n=7$).

Discussion

Our results clearly demonstrate that physiological concentrations of extracellular L-arginine potentiate isoprenaline-induced relaxation in pulmonary arteries of the rat. This effect was concentration-dependent, stereospecific, and independent of the vasoconstrictor ($PGF_{2\alpha}$ or PE). It is also consistent with our previous report on the effects of L-arginine on the vasorelaxant properties of the putative cyclic AMP-specific PDE inhibitor tetramethylpyrazine (Peng *et al.*, 1996). L-lysine, used as a competitive inhibitor of L-arginine uptake, not only abolished the potentiation of relaxation by L-arginine but also inhibited isoprenaline-induced relaxation compared to control, that is in the absence of L-arginine added to the bath (Figures 1 and 2). If it can be assumed that L-lysine has no significant non-specific effects, an assumption supported by the fact that L-lysine had no effect on isoprenaline-induced relaxation in denuded pulmonary arteries or mesenteric arteries, or on

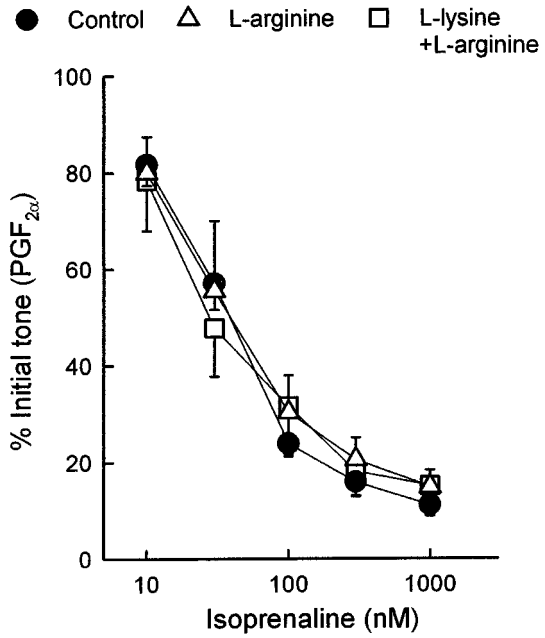

Figure 7 Isoprenaline concentration-response curves in mesenteric arteries constricted with 50 μM $PGF_{2\alpha}$, and in the presence of L-arginine (400 μM) or L-arginine plus L-lysine (10 mM). Each point is the mean of 5–7 experiments, and symbols are mean ± s.e.mean.

ACh-induced relaxation, then these results imply that the preparation had an endogenous source of extracellular L-arginine (see below).

Both blockade of NOS with L-NMMA and removal of the endothelium inhibited isoprenaline-induced relaxation to the same extent as L-lysine, and L-lysine had no further effect when applied in combination with L-NMMA. These results suggest that the response to all of these conditions is due to the same cause, namely removal of the NO-dependent component of relaxation. Consistent with this, L-arginine (400 μM) had no potentiating effect on isoprenaline-induced relaxation under any of these conditions (see Figure 3). These results are consistent with our previous reports that isoprenaline-induced relaxation of pulmonary arteries includes a significant component that is endothelium-dependent and NO-mediated (Priest *et al*., 1997; 1999), and that β-adrenoceptor agonists stimulate NO-production in cultured HUVECs (Ferro *et al*., 1999). They also strongly suggest that the NO-mediated component of isoprenaline-induced relaxation is critically dependent on extracellular L-arginine and L-lysine-sensitive L-arginine uptake, and that the NOS responsible is located in the endothelium, and is not inducible NOS (iNOS) located in the smooth muscle.

We and others have suggested that isoprenaline stimulates eNOS *via* a rise in cyclic AMP (Gray & Marshall, 1992; Rebich *et al*., 1995; Priest *et al*., 1997; Ferro *et al*., 1999). If so, it would be expected that L-arginine and L-lysine would affect relaxation induced directly by CPT cyclic AMP in a similar fashion to that induced by isoprenaline, and this was indeed the case (Figure 5).

The question arises as to whether the potentiating effect of extracellular L-arginine is specific for cyclic AMP-mediated activation of eNOS, or whether it reflects a general requirement by eNOS for extracellular L-arginine in this preparation. We have previously reported that extracellular L-arginine has no effect on ACh-induced relaxation of rat pulmonary arteries (Peng *et al*., 1996), and in the present report have both confirmed this finding and shown that inhibition of L-arginine uptake with L-lysine also has no effect (Figure 6). These results suggest that both intracellular L-arginine in the endothelial cells is not depleted in this preparation, and that the apparent requirement for extracellular L-arginine and L-arginine entry is limited to activation of eNOS by cyclic AMP-dependent pathways, although other Ca^{2+}-independent activation mechanisms remain to be investigated (see below).

Mesenteric arteries

Isoprenaline-induced relaxation in rat systemic arteries has also been shown to be inhibited by removal of the endothelium or inhibition of NO synthesis (Gray & Marshall, 1992; Graves & Poston, 1993; Delpy *et al*., 1996). However in a previous study we found that addition of L-arginine had little effect on tetramethylpyrazine-induced relaxation in rat mesenteric arteries (Peng *et al*., 1996). We now believe that the effects of tetramethylpyrazine are primarily mediated *via* cyclic AMP. We therefore examined whether L-arginine or L-lysine had any effect on isoprenaline-induced relaxation of mesenteric arteries, which has been shown to be partially mediated by NO (Graves & Poston, 1993). In contrast to pulmonary arteries, isoprenaline-induced relaxation in rat mesenteric arteries was completely unaffected by either L-arginine at physiological concentrations or L-lysine (Figure 7). This suggests that whatever mechanism is involved, it may be limited to particular vascular beds. In this respect it is interesting that in preliminary experiments we have found no effect of L-arginine or L-lysine on forskolin-induced relaxation of rat femoral arteries (own unpublished observations).

Concentration dependence of response to L-arginine

The potentiating effect of extracellular L-arginine was concentration dependent. Analysis of derivative plots of maximum isoprenaline-induced relaxation against L-arginine concentration provided an EC_{50} of around 7 μM when the control data was used to estimate the effect of zero L-arginine, and less than 1 μM if this was estimated from the 10 mM L-lysine data (see Results, and Figure 4). Either value is close to the EC_{50} for L-arginine that we have previously reported for potentiation of tetramethylpyrazine-induced relaxation ($\sim$1 μM) (Peng *et al*., 1996), and that for activation of eNOS itself (3–5 μM) in pulmonary artery endothelial cells (Su *et al*., 1997). It would therefore seem likely that the rate limiting step is the concentration of L-arginine delivered to the eNOS enzyme rather than the transport of L-arginine across the membrane, as the most important L-arginine transport mechanism, system y^+, has been reported to have an EC_{50} greater than 50 μM L-arginine in pulmonary artery endothelial cells (Greene *et al*., 1993).

Our results show that there is a significant endothelium- and NO-dependent component of isoprenaline induced relaxation in the absence of L-arginine added to the PSS. The fact that this could be abolished by inhibition of L-arginine uptake with L-lysine implies that some extracellular L-arginine is available to the endothelium even under these conditions. From Figures 1 and 2 it can be seen that the control curves fall approximately mid-way between those for 400 μM L-arginine and 10 mM L-lysine, suggesting that the concentration of this endogenous L-arginine is close to the EC_{50}, or around 1–7 μM (see Results and Figure 4). As the intracellular concentration of L-arginine is at least 100 fold greater than this (Gold *et al*., 1989; Baydoun *et al*., 1990; Block *et al*., 1995; Bogle *et al*., 1996), we can speculate that only a small amount of diffusion and/or reverse

transport of L-arginine from the cells would be needed to maintain such a low concentration of L-arginine, particularly if localised to the extracellular spaces between the smooth muscle and endothelium. In support of this, it is known that L-arginine efflux from endothelial cells does occur (e.g. Bogle *et al*., 1996). Although this hypothesis would be consistent with the data, it is difficult to test directly. It is however unlikely to be of physiological relevance, as normal plasma L-arginine concentration in rats is more than 150 μM (Peng *et al*., 1996), sufficient to cause maximal potentiation of isoprenaline-induced relaxation (see Figure 4).

An alternative hypothesis to explain the inhibitory effect of L-lysine in the absence of L-arginine is that L-lysine interferes directly with the eNOS, or with its ability to access intracellular L-arginine. However, there have been no previous reports suggesting that this might be the case, and we show that ACh-induced relaxation was not affected by L-lysine.

It should be noted that the proportion of total isoprenaline-induced relaxation that is dependent on L-arginine and blocked by L-lysine increases at lower concentrations of isoprenaline (see Figures 1 and 2), so that at 300 nM and below the L-arginine- and NO-dependent component predominates. An equivalent effect is observed for CPT cyclic AMP, where at 30 μM the same component accounts for $\sim$75% of relaxation (Figure 5). This effect is reflected by the shifts in the EC_{50} for isoprenaline caused by the presence of 400 μM L-arginine, L-lysine and L-NMMA (Table 2). These results suggest that whatever the pathway by which cyclic AMP activates eNOS, it is more sensitive to cyclic AMP than those leading to NO-independent relaxation.

Our results imply that when eNOS is activated by a rise in cyclic AMP it only has access to L-arginine that enters across the cell membrane, whereas following activation by ACh it has access to the high concentration of L-arginine in the cytosol of the endothelial cell. A high proportion of eNOS is known to be located in plasma membrane caveolae, which contain structural proteins called caveolins. There is good evidence that at least in resting conditions eNOS is complexed with caveolin 1 (Garcia-Cardena *et al*., 1996). Caveolin 1 binding to eNOS has been shown both to inhibit and be disrupted by binding of Ca^{2+}-calmodulin to eNOS, thereby forming a reciprocal regulatory system for activation (Feron *et al*., 1998; Ghosh *et al*., 1998). However localization of eNOS within caveolae has also been reported to optimise NO production (Liu *et al*., 1996). In an elegant study McDonald *et al*. (1997) have shown that in pulmonary artery endothelial cells the proteins for eNOS, caveolin 1, and CAT1 (the cationic amino acid transporter associated with system y^+) all co-locate, and they provided evidence that eNOS and CAT1 form a complex within the caveolae. This would allow directed delivery of extracellular L-arginine to the eNOS (McDonald *et al*., 1997), which would be entirely consistent with our results concerning the critical dependence of cyclic AMP-induced NO-mediated relaxation on extracellular L-arginine.

It cannot however explain the lack of dependence on extracellular L-arginine exhibited by classical endothelium-dependent vasodilators such as ACh and bradykinin. It has been suggested that release of Ca^{2+} from intracellular stores is the initiating factor for activation of eNOS by these agents, which is then maintained by a sustained phase of Ca^{2+} entry across the membrane (Zheng *et al*., 1994). The increase in Ca^{2+} then results in Ca^{2+}-calmodulin binding and dissociation of eNOS from caveolin 1, allowing activation of the enzyme (Feron *et al*., 1998; Ghosh *et al*., 1998). There is now evidence that agents that increase endothelial cell $[Ca^{2+}]_i$ also cause translocation of eNOS into the cytosol and away from the membrane (Prabhakar *et al*., 1998; Goetz *et al*., 1999), presumably also dissociating from CAT1 and allowing access to the cytosolic L-arginine.

We have shown that isoprenaline-induced activation of eNOS in HUVECs is not associated with a rise in $[Ca^{2+}]_i$ (Ferro *et al*., 1999), and in similar preliminary studies have also found no effect of isoprenaline on $[Ca^{2+}]_i$ of rat cultured pulmonary artery endothelial cells (own unpublished observations). There is increasing evidence for Ca^{2+}-independent mechanisms of eNOS activation, specifically during shear stress and stretch (Ayajiki *et al*., 1996; Fleming *et al*., 1998; Fleming & Busse, 1999). Shear stress and Ca^{2+}-independent activation of eNOS have been associated with tyrosine phosphorylation and mimicked by tyrosine phosphatase inhibitors (Ayajiki *et al*., 1996; Fleming & Busse, 1999). eNOS is known to be phosphorylated in response to various stimuli (Fleming & Busse, 1999), and it has recently been shown that the serine/threonine protein kinase Akt (PKB) both phosphorylates and activates eNOS (Fulton *et al*., 1999). It is not known however whether cyclic AMP causes PKA-dependent phosphorylation of eNOS or of any associated regulatory proteins, although cyclic strain, which also stimulates NO production (Fleming & Busse, 1999), has been reported to activate adenylate cyclase with a subsequent increase in PKA activity (Cohen *et al*., 1997).

In conclusion, we have shown that in rat pulmonary arteries the NO- and endothelium-dependent component of relaxation to isoprenaline and cyclic AMP is critically dependent on the extracellular L-arginine concentration. This would be consistent with the reported close association between eNOS and CAT1 within the caveolae. There is sufficient evidence to allow us to speculate that cyclic AMP-mediated activation of eNOS may involve similar processes to those occurring during other types of Ca^{2+}-independent activation, for example shear stress, and that these might therefore also be dependent on extracellular rather than cytosolic L-arginine. This may however be tissue specific, as isoprenaline-induced relaxation of rat mesenteric arteries showed no dependence on extracellular L-arginine. These results also highlight potential dangers inherent in the common practice of excluding physiological concentrations of L-arginine in the bathing medium for studies on the role of NO.

We thank the Special Trustees of St Thomas' Hospital (DH) for support.

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(Received December 13, 1999
Revised February 17, 2000
Accepted March 27, 2000)