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Essential role of L-arginine uptake and protein tyrosine kinase activity for NO-dependent vasorelaxation induced by stretch, isometric tension and cyclic AMP in rat pulmonary arteries

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1 The NO-dependent component of cyclic AMP-induced vasorelaxation in rat pulmonary arteries is critically dependent on extracellular L-arginine but independent of endothelial cell intracellular [Ca^{2+}]. We examined whether L-arginine uptake was also essential for NO production induced by passive stretch or isometric tension, processes also reported to be Ca^{2+}-independent.

2 The passive length-tension curve was depressed by physiological concentrations of L-arginine (400 μM; $P<0.05$). Inhibition of the y^+ transporter with 10 mM L-lysine, NO synthase with L-NAME (100 μM), or protein tyrosine kinase with erbstatin A (30 μM) caused identical upward shifts ($P<0.001$), alone or in combination. Tyrphostin 23 was similar to erbstatin A, whilst the inactive analogue tyrphostin A1 and genistein were without effect.

3 L-arginine (400 μM) shifted the $PGF_{2\alpha}$ concentration-response curve under isometric conditions to the right ($P<0.05$), whereas L-NAME or L-lysine caused a leftward shift ($P<0.001$). Tyrphostin 23 (30 μM) more than reversed the L-arginine-induced suppression of $PGF_{2\alpha}$-induced tension; subsequent addition of L-NAME had no effect. The L-lysine-sensitive component of CPT cyclic AMP-induced vasorelaxation was abolished by erbstatin A.

4 ACh-induced vasorelaxation was ~80% inhibited by L-NAME, but was not affected by L-lysine or 400 μM L-arginine. Erbstatin A reduced the vasorelaxation by only ~25%.

5 We conclude that activation of NO production by stretch, isometric tension, or cyclic AMP in rat pulmonary arteries is critically dependent on the presence and uptake of physiological concentrations of extracellular L-arginine, and protein tyrosine kinase activity. This directly contrasts with ACh-induced vasorelaxation, which was independent of extracellular L-arginine, and relatively unaffected by tyrosine kinase inhibition.

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Keywords: Pulmonary artery; passive stretch; isometric tension; cyclic AMP; nitric oxide; nitric oxide synthase; L-arginine; arginine paradox; protein tyrosine kinase

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 75 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; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PSS, physiological salt solution

Introduction

Endothelium-derived NO plays a vital role in the modulation of pulmonary vascular resistance, and defects in its synthesis during lung disease contribute to the development of pulmonary hypertension. L-arginine is a key substrate for the endothelial cell NO synthase (eNOS, NOS III) (Moncada *et al*., 1991). The normal intracellular concentration of L-arginine in the endothelial cell has been reported to be as high as 800 μM (Baydoun *et al*., 1990), whereas the EC_{50} for activation of eNOS by L-arginine is substantially less, at ~3 μM (Pollock *et al*., 1991; Su *et al*., 1997). It has therefore been assumed that L-arginine availability is not a limiting factor for full activation of eNOS, and few studies on isolated tissues include L-arginine in the external medium. Indeed, classical endothelium-dependent vasodilators such as acetylcholine (ACh) do not require the presence of extracellular L-arginine (e.g. Peng *et al*., 1996; Hucks & Ward, 2000). It is well known however that in various systemic and pulmonary vascular diseases application of extracellular L-arginine can restore NO-dependent vasorelaxation, even though intracellular L-arginine is not decreased (e.g. Eddahibi *et al*., 1992; Creager *et al*., 1992). This unexplained effect has been termed the 'arginine paradox'.

It is therefore of interest that we recently found that the NO-dependent component of isoprenaline and cyclic AMP-induced vasorelaxation in pulmonary arteries of the rat is critically dependent on uptake of extracellular L-arginine, with an EC_{50} of ~1–7 μM (Hucks & Ward, 2000). Full activation required an extracellular concentration of ~100 μM, not substantially less than that found in the plasma (~160 μM; Peng *et al*., 1996). Classical receptor-dependent vasodilators such as ACh and bradykinin activate eNOS *via* a Ca^{2+}-calmodulin-dependent pathway (Zheng *et al*., 1994). We have shown however that cyclic AMP activates eNOS in HUVECs without a rise in intracellular Ca^{2+} (Ferro *et al*., 1999), and recently Butt *et al*., (2000) have demonstrated that eNOS is both activated and made Ca^{2+}-independent following phosphorylation by cyclic AMP. Mechanical stimuli such as shear stress and isometric

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contraction are also reported to activate eNOS *via* a Ca^{2+}-calmodulin-independent pathway which requires protein tyrosine phosphorylation, though not necessarily of eNOS itself (Ayajiki *et al*., 1996; Fleming *et al*., 1998; 1999). We speculated that if Ca^{2+}-independent activation of eNOS by cyclic AMP was critically dependent on uptake of extracellular L-arginine, then so might be activation by mechanical stimuli.

We therefore investigated the effect of inhibition of L-arginine uptake on eNOS activation by passive stretch or isometric contraction in rat pulmonary arteries. We also examined whether cyclic AMP-induced activation is similarly suppressed by protein tyrosine kinase inhibition. Our results show that activation of eNOS by stretch or isometric contraction is indeed critically dependent on L-arginine uptake in this preparation, and furthermore that activation by cyclic AMP is also abolished by inhibition of protein tyrosine kinase, suggesting a possible common pathway. In contrast, ACh-induced vasorelaxation was independent of L-arginine uptake, and only marginally affected by inhibition of tyrosine kinase inhibition.

Methods

Adult, male Wistar rats (250–350 g) were killed by anaesthetic overdose (intraperitoneal injection of pentabarbitone, 50 mg kg^{-1}), as approved by the Home Office Inspector. The heart and lungs 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 arteries (1150 ± 26 μm i.d.) were dissected free of connective tissue, mounted in an isometric small vessel myograph containing PSS, and equilibrated with 5% CO_2 in air, (pH 7.4, 37°C). The arteries were then stretched to give an equivalent transmural pressure of 30 mmHg, the peak of the active length-tension curve, as previously described in detail (Leach *et al*., 1989; Priest *et al*., 1999). The distance between the myograph jaws under these conditions will be referred to as the initial length, or L_{30}. The presence of a functioning endothelium was determined by application of ACh (10 μM) following agonist induced contraction. After 60 min equilibration 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) (Priest *et al*., 1999). Arteries producing less than 1 mN mm^{-1} were discarded. After washing with PSS the arteries rapidly returned to baseline tone.

Length-tension relationship

The gap between the myograph jaws was initially reduced to 50% of L_{30}. Under these conditions tension was zero for all arteries. After 10 min, the gap was increased to 60% of L_{30}, and tension allowed to stabilize for 10 min before recording (passive tension). The arteries were then briefly challenged with KPSS for 2 min before being returned to PSS; tension stabilized to its original value within 10 min, at which time the next increment in length was made. This procedure was found to result in a more stable and repeatable preparation. This sequence was repeated in increments of 10% of L_{30} up to 120% L_{30}. We examined the passive length-tension relationship in the absence and presence of 400 μM L-arginine, and in the presence of L-arginine following inhibition of the y^+ transporter with 10 mM L-lysine, NO-synthase with 100 μM L-N^G-nitroarginine methyl ester (L-NAME), or protein tyrosine kinase with erbstatin A (30 μM). Preparations were preincubated for 10 min with each inhibitor, which was then present throughout the rest of the experiment. In separate experiments arteries were stretched to 120% of L_{30}, and the effects of two other tyrosine kinase inhibitors, genistein (30 μM) and tyrphostin 23 (30 μM), and the inactive analogue tyrphostin A1 (100 μM), were examined.

Agonist-induced isometric tension

We have suggested that $PGF_{2\alpha}$ increases NO production in rat pulmonary arteries *via* an indirect mechanism involving increased artery wall stress (Priest *et al*., 1999). Recently it has been shown that increased isometric tension induced by $PGF_{2\alpha}$ in rabbit aorta stimulates NO production by a Ca^{2+}-calmodulin-independent pathway, similar to that invoked by shear stress (Fleming *et al*., 1999). We therefore examined whether inhibition of L-arginine uptake abolished the NO-dependent suppression of $PGF_{2\alpha}$-induced isometric tension. Cumulative concentration-response curves were constructed for $PGF_{2\alpha}$ in the absence and presence of 400 μM L-arginine, and in the presence of L-arginine following addition of L-NAME (100 μM), L-lysine (10 mM) or both combined, with 10 min preincubation. In a separate experiment, arteries were constricted with 10 μM $PGF_{2\alpha}$ ($\sim EC_{50}$), and tension allowed to stabilize. The L-arginine concentration in the bath was increased cumulatively to 30, 100 and 400 μM, after which 30 μM tyrphostin 23, followed by 100 μM L-NAME, were added to the bath. Tension was allowed to stabilize after each addition.

Protein tyrosine kinase and cyclic AMP-induced relaxation

If similar pathways were involved in the stimulation of NO-production by both cyclic AMP and mechanical stimuli, it would be predicted that inhibition of protein tyrosine kinases would also diminish or abolish the L-arginine dependent and NO-mediated component of vasorelaxation to cyclic AMP. Arteries were therefore preconstricted with 50 μM $PGF_{2\alpha}$ ($\sim$60% response to KPSS), and cumulative concentration-response curves constructed for CTP cyclic AMP (8-(4-chloro-phenylthio)-adenosine 3′,5′-cyclic monophosphate), a membrane permeant analogue of cyclic AMP, in the absence and presence of 400 μM L-arginine, and in the presence of L-arginine following incubation with erbstatin A (30 μM), L-lysine (10 mM), or both combined.

ACh-induced vasorelaxation

Arteries were preconstricted with 50 μM $PGF_{2\alpha}$ and cumulative concentration-response curves constructed for ACh in the absence and presence of 400 μM L-arginine, and in the presence of 10 mM L-lysine, 30 μM erbstatin A, or 100 μM L-NAME.

Chemicals, solutions and data analysis

All drugs were obtained from Sigma, U.K., with the exception of $PGF_{2\alpha}$ (Upjohn Pharmaceuticals Ltd., Crawley, U.K.), L-NAME, CTP cyclic AMP, erbstatin A, and tyrphostins 23 and A1 (Calbiochem, Notts., U.K.). Other chemicals were of Analar quality (BDH, Southampton, U.K.). Drugs were prepared as stock solutions using PSS

except for erbstatin A, genistein and the tyrphostins, which were dissolved in DMSO. The final concentration of DMSO in the bath was $<0.3\%$, which had no perceptible effect on its own. PSS was made up for each experiment using water freshly drawn from a reverse osmosis-deionization plant with UV irradiation (Elgastat, Elga Ltd, U.K.).

Passive tension is shown in mN. Concentration-response curves to $PGF_{2\alpha}$ are expressed as a percentage of the response to KPSS (80 mM KCl, equimolar 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 Student's *t*-test or ANOVA with a Student-Newman-Keuls *post hoc* test as appropriate (SigmaStat, Jandel Scientific, U.S.A.).

Results

The passive length-tension relationship for pulmonary arteries is shown in Figure 1. In the presence of 400 μM L-arginine, inhibition of L-arginine uptake with L-lysine (10 mM) caused a significant upwards shift in the passive length-tension relationship ($P<0.05$). This effect was identical to that induced by inhibition of NO synthase with L-NAME (100 μM), or inhibition of protein tyrosine kinase with erbstatin A (30 μM) (Figure 1). There was no further shift if all three inhibitors were combined (e.g. tension at 120% L_{30}: 10.0$\pm$1.4 mN, $n=4$; not shown in figure for clarity). In the absence of added L-arginine the length-tension curve lay approximately midway between that in the presence of L-arginine alone and those following addition of the inhibitors. This is consistent with our recent report on cyclic AMP-mediated vasorelaxation, and the suggestion that even in the absence of L-arginine in the PSS there is sufficient extracellular L-arginine adjacent to the endothelium, derived from endogenous sources, to partially activate the mechanism (Hucks & Ward, 2000).

In a separate experiment four arteries were stretched to 120% L_{30} in the absence of L-arginine, producing a force of 8.4$\pm$0.6 mN ($n=4$). Subsequent addition of genistein (30 μM) had no effect (8.4$\pm$0.5 mN), whereas tyrphostin 23 (30 μM) caused a significant increase in force (10.4$\pm$0.4 mN, $P<0.05$), equivalent to that caused by erbstatin A in Figure 1; tyrphostin A1 was ineffective ($n=4$). Addition of L-NAME (100 μM) after tyrphostin 23 had no further effect (10.6$\pm$0.4 mN). Higher concentrations of genistein (100 μM) either had no effect (one artery) or caused a slow decrease in force (two arteries).

Figure 2 shows cumulative concentration-response curves to $PGF_{2\alpha}$ in the absence and presence of L-arginine, and following addition of L-lysine or L-NAME in the presence of

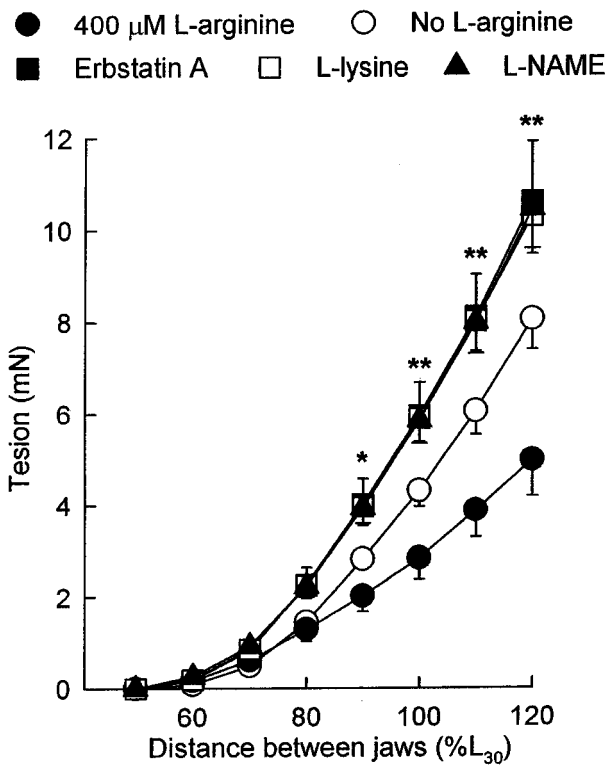

Figure 1 Passive length-tension relationship of pulmonary arteries. Except where noted, all experiments were performed in the presence of 400 μM L-arginine. The curves for L-lysine (10 mM), L-NAME (100 μM) and erbstatin A (30 μM) were all significantly different from those in the presence of L-arginine alone ($P<0.001$; ANOVA), but were not different from each other. *$P<0.05$, **$P<0.01$ *post hoc* test; same significance for all inhibitors against control. The relationship in the absence of L-arginine was significantly different both from 400 μM L-arginine ($P<0.01$) and all inhibitors ($P<0.05$). Each point is the mean of 4–9 experiments, and symbols are mean$\pm$s.e.mean; where no error bar is shown, the error is smaller than the symbol.

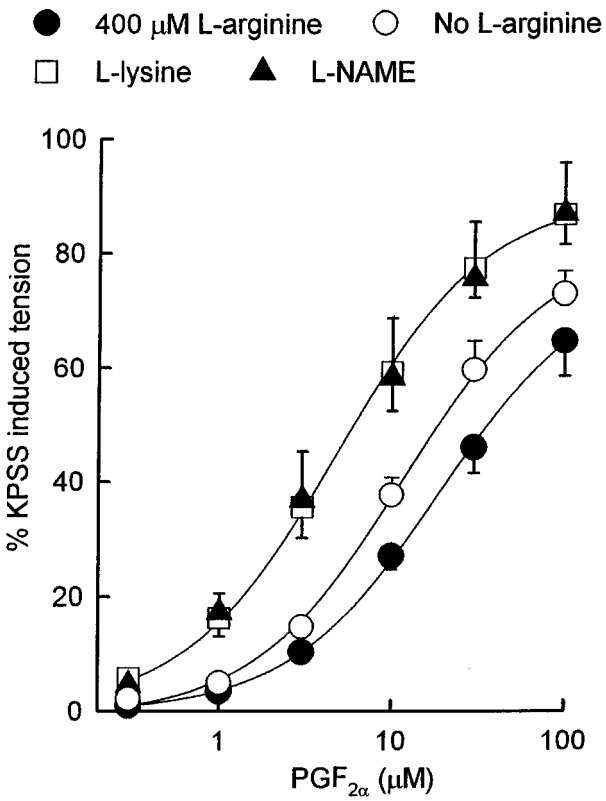

Figure 2 Cumulative concentration-response relationship for $PGF_{2\alpha}$ in pulmonary arteries. Except where noted, all experiments were performed in the presence of 400 μM L-arginine. Each point is the mean of 5–8 experiments, and symbols are mean$\pm$s.e.mean; where no error bar is shown, the error is smaller than the symbol. Statistics were performed on fitted parameters (see text).

L-arginine. L-arginine caused a significant rightward shift in the response curve (EC_{50}: No L-arginine: 12.6 [−1.8, +2.1] μM, $n=8$; 400 μM L-arginine: 19.7 [−5.1, 6.8] μM, $n=7$; $P<0.05$), though there was no change in extrapolated maximum tension (No L-arginine: 83±5% KPSS induced tension; 400 μM L-arginine: 79±8%). L-NAME (100 μM) in the presence of L-arginine caused a significant leftward shift of the response curve (5.2 [−1.8, +2.7] μM; $n=5$; $P<0.001$ versus both presence and absence of L-arginine), but again without a significant increase in extrapolated maximum tension (90±9%, NS). This shift is equivalent to that previously reported for eNOS inhibition in the absence of L-arginine (Priest *et al*., 1999). Blockade of L-arginine uptake with L-lysine had an identical effect to L-NAME (EC_{50}: 5.3 [−1.7, +2.5] μM; maximum tension: 91±4%, $n=8$; $P<0.001$); no further effect was observed when both were combined ($n=4$; not shown). This was similar to the effects observed for the length-tension relationship (Figure 1), and as previously described for cyclic AMP (Hucks & Ward, 2000).

The effects of lower concentrations of L-arginine on constriction induced by 10 μM $PGF_{2\alpha}$, and subsequent tyrosine kinase inhibition by tyrphostin 23, are shown in Figure 3. Cumulative addition of 30, 100 and 400 μM L-arginine caused a significant fall in tension compared to control ($P<0.05$); 400 μM had no further effect over 100 μM. Subsequent addition of tyrphostin 23 (30 μM) caused a very significant increase in tension ($P<0.001$), which was not further increased by 100 μM L-NAME. The tensions developed to this single dose of 10 μM $PGF_{2\alpha}$ and following the cumulative addition of 400 μM L-arginine and L-NAME were not significantly different from the equivalent tensions shown in Figure 2. Neither 100 μM tyrphostin A1 nor 30 μM genistein had any effect on tension induced by 10 μM $PGF_{2\alpha}$ ($n=3$ for each). Preliminary experiments with lower concentrations of L-arginine ($\leqslant$ 10 μM) were unable to show any consistent or significant effect on tension ($n=6$).

Figure 4 shows the effect of erbstatin A on CPT cyclic AMP-induced vasorelaxation of $PGF_{2\alpha}$ constricted arteries. As we have previously described for phenylephrine constricted arteries (Hucks & Ward, 2000), 10 mM L-lysine caused a significant rightward shift of the concentration-response curve (EC_{50}: 400 μM L-arginine: 33 [−6, +7] μM, $n=8$; L-lysine + L-arginine: 101 [−25, +33] μM, $n=9$; $P<0.001$), without affecting maximum relaxation (L-arginine: 115±2% initial tension; L-lysine: 121±12%). Again, erbstatin A in the presence of L-arginine had an identical effect to L-lysine (EC_{50}: 102 [−43, +73] μM, $n=7$, $P<0.001$ compared to L-arginine; maximum relaxation: 123±5%). There was no further inhibition of vasorelaxation when erbstatin A and L-lysine were combined (EC_{50}: 112 [−43, +69] μM; maximum relaxation: 127±13%; $n=9$). In the absence of L-arginine the CPT cyclic AMP response curve again lay mid-way between those for 400 μM L-arginine alone, and in the presence of the inhibitors (EC_{50}: 55 [−19, +29] μM, $P<0.05$ versus both L-arginine alone and in the presence of inhibitors; maximum relaxation: 119±3%, NS; $n=6$).

As previously described (Hucks & Ward, 2000), neither L-arginine nor L-lysine had any effect on ACh-induced vasorelaxation (Figure 5). The majority of ACh-induced vasorelaxation was however blocked by 100 μM L-NAME. In contrast to its effects on the response to the mechanical stimuli and cyclic AMP, erbstatin A (30 μM) had only a small effect on the response to ACh, reducing the maximum relaxation from 67±3% (no L-arginine, $n=6$) to 59±2% (erbstatin A, $n=6$, $P<0.05$), with no significant effect on the EC_{50} (no L-arginine: 261 [−87, +130] nM; 404 [−87, +110] nM, NS).

Discussion

Our results clearly suggest that in pulmonary arteries of the rat uptake of L-arginine from the extracellular space is an

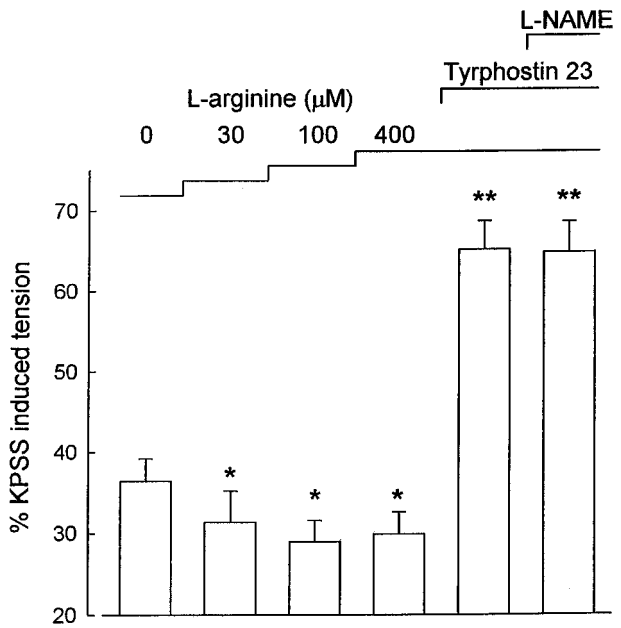

Figure 3 Effect of cumulative addition of L-arginine, followed by 30 μM tyrphostin 23 and 100 μM L-NAME, on the tension in five pulmonary arteries constricted with 10 μM $PGF_{2\alpha}$. Bars represent the mean±s.e.mean. * $P<0.05$, ** $P<0.001$, Student's paired *t*-test.

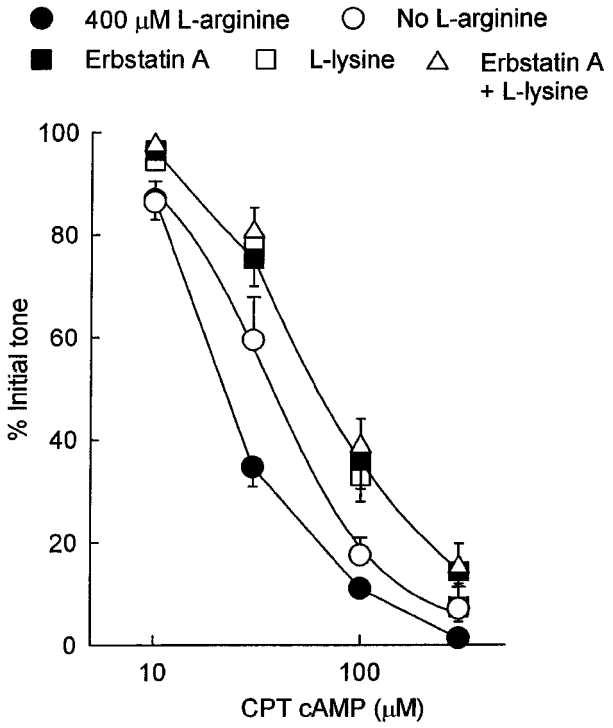

Figure 4 Cumulative concentration-response relationship for CPT cyclic AMP in pulmonary arteries. Except where noted, all experiments were performed in the presence of 400 μM L-arginine. Each point is the mean of 6–9 experiments, and symbols are mean±s.e.mean; where no error bar is shown, the error is smaller than the symbol. Statistics were performed on fitted parameters (see text).

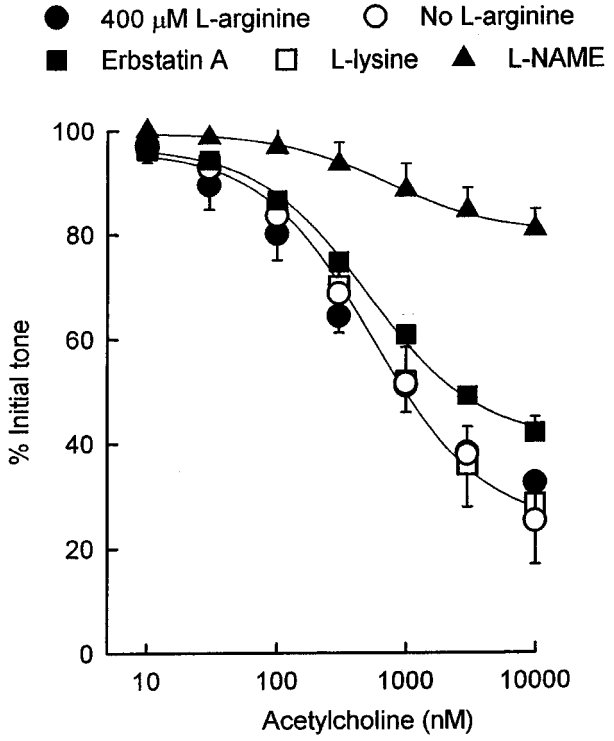

Figure 5 Cumulative concentration-response relationship for ACh in pulmonary arteries. Except where noted, all experiments were performed in the presence of 400 μM L-arginine. Each point is the mean of 4–6 experiments, and symbols are mean ± s.e.mean; where no error bar is shown, the error is smaller than the symbol. Statistics were performed on fitted parameters (see text).

essential requirement for activation of eNOS by stretch and isometric tension. This is directly analogous to our recent report that cyclic AMP-induced, NO-dependent vasorelaxation is critically dependent on extracellular L-arginine (Hucks & Ward, 2000), and our previous study showing that vasorelaxation to tetramethylpyrazine, a cyclic AMP-specific phosphodiesterase inhibitor, was potentiated by physiological concentrations of L-arginine (Peng *et al*., 1996). In contrast, vasorelaxation induced by the classical endothelium-dependent agent ACh was, as previously described (Hucks & Ward, 2000) independent of extracellular L-arginine and unaffected by inhibition of L-arginine uptake.

The limited study on the concentration-dependency for L-arginine implies that 100 μM L-arginine is maximally effective for this mechanism, at least for isometric tension induced by 10 μM $PGF_{2\alpha}$. This is consistent with our previous study on the NO-dependent component of isoprenaline- and cyclic AMP-mediated vasorelaxation in rat pulmonary arteries, where we derived an EC_{50} of 5–10 μM L-arginine, with a maximally effective concentration of 100 μM (Hucks & Ward, 2000). This is close to the normal plasma L-arginine concentration in rats (~150 μM) (Peng *et al*., 1996), which suggests that under normal conditions *in vivo* the mechanisms underlying activation of NO production by cyclic AMP and mechanical stimuli are not limited by availability of extracellular L-arginine. The close approximation between the maximally effective and plasma concentrations of L-arginine however imply that a relatively small suppression of L-arginine uptake could have a significant effect on NO-dependent vasorelaxation stimulated by these mechanisms. This may be of particular relevance in hypoxia and lung disease, as hypoxia is known to inhibit L-arginine uptake into pulmonary artery endothelial cells (Zharikov *et al*., 1997).

In contrast to the *in vivo* situation, most experimental studies on vascular function do not include L-arginine in the extracellular medium as it is assumed that intracellular L-arginine is in excess. The EC_{50} for activation of eNOS by L-arginine is reported to be ~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). Moreover, a large number of previous studies using classical, Ca^{2+}-calmodulin- and endothelium-dependent vasodilators have not reported any requirement for extracellular L-arginine, including studies in the pulmonary vasculature (e.g. Peng *et al*., 1996; Hucks & Ward, 2000). The requirement for extracellular L-arginine for Ca^{2+}-independent mechanisms may also be obfuscated by the phenomenon described in our previous report, namely that even in the nominal absence of L-arginine in the extracellular medium there is still a significant component that is inhibited by L-lysine, though L-lysine had no effect on ACh-induced relaxation (Hucks & Ward, 2000).

We have suggested that in the nominal absence of extracellular L-arginine diffusion or reverse transport of L-arginine from within the surrounding cells may maintain a localized concentration between the endothelium and smooth muscle that is sufficient to allow part activation of the mechanisms described in this and our previous study (Hucks & Ward, 2000). This would be entirely consistent with our current results in the absence of L-arginine, as shown in Figures 1 to 4. We previously estimated that in this preparation the concentration of L-arginine derived from endogenous sources was ~5 μM (Hucks & Ward, 2000), though it is likely that this may vary widely depending on the experimental conditions. Five μM is very close to the EC_{50}, and therefore the steep part of the response curve, and it is noticeable in this respect that the response in the absence of added L-arginine was approximately midway between those in the presence of maximally effective L-arginine (400 μM) and when L-arginine uptake was blocked with L-lysine (see Figures 1, 2, and 4). Note that we have previously shown that 10 mM mannitol, used as an osmotic control for L-lysine, has no effect (Hucks & Ward, 2000). It is therefore possible that certain studies performed in the absence of extracellular L-arginine may underestimate the role of NO, as the system would not be fully activated. It is also possible that the dependence on L-arginine uptake is specific to the pulmonary circulation, as we have found no equivalent effect for cyclic AMP in mesenteric arteries (Hucks & Ward, 2000), although preliminary studies in rat femoral artery have suggested they behave in a similar but less pronounced fashion to pulmonary arteries (own unpublished observations)

We have also demonstrated that inhibition of protein tyrosine kinase with erbstatin A or tyrphostin 23 abolishes both the activation of eNOS by passive stretch or agonist-induced isometric tension (Figures 1 and 3), and the L-lysine-sensitive component of cyclic AMP-induced vasorelaxation (Figure 4), which we have previously shown to be entirely due to NO in pulmonary arteries of the rat (Hucks & Ward, 2000). Genistein however was without effect, as was the inactive analogue of tyrphostin 23, tyrphostin A1. Tyrosine phosphorylation, though not necessarily of the eNOS protein itself, has previously been shown to be instrumental in the Ca^{2+}-calmodulin-independent activation of eNOS by shear stress and isometric tension (Ayajiki *et al*., 1996; Fleming *et al*., 1998; 1999), although it has been reported not to be involved in activation by receptor dependent agonists such as

ACh (Fleming *et al*., 1998). The latter is entirely consistent with the very limited effect of erbstatin A on ACh-induced vasorelaxation in pulmonary arteries, as shown in Figure 5. Whereas inhibition of tyrosine kinase completely abolished the NO-dependent component of the responses to stretch, isometric tension and cyclic AMP, it caused a reduction of only about 25% in the NO-dependent component of ACh-induced vasorelaxation.

In contrast to erbstatin A and herbimycin A (another tyrosine kinase inhibitor), genistein has previously been shown to be ineffective in inhibiting mechanical stimulation of eNOS in systemic arteries (Fleming *et al*., 1998; 1999). This is entirely consistent with our findings in pulmonary arteries for both mechanical and cyclic AMP induced stimulation. There are various possible reasons for the lack of effect of genistein, including involvement of a genistein-insensitive protein tyrosine kinase. However the most likely explanation involves a recently described tyrosine kinase-independent effect of genistein. Unlike erbstatin A, herbimycin A or tyrphostin 23, genistein and its inactive (in terms of tyrosine kinases) analogue daidzein are phytoestrogens, and have both been shown to cause endothelium- and NO-dependent vasorelaxation of rat aorta and pulmonary artery, with an EC_{50} for genistein of $\sim 6\ \mu M$ (Mishra *et al*., 2000). Estradiol has been reported to activate eNOS *via* a rise in intracellular [Ca^{2+}] (Goetz *et al*., 1999), and if, as seems likely, genistein does the same, concomitant activation of this pathway could account for the apparent lack of inhibitory effect against Ca^{2+}-independent activation mechanisms.

Classical endothelium-dependent vasodilators such as ACh increase endothelial cell intracellular [Ca^{2+}], and activate eNOS *via* binding of Ca^{2+}-calmodulin and subsequent dissociation of eNOS from caveolin 1 (Zheng *et al*., 1994; Ghosh *et al*., 1998; Feron *et al*., 1998). Mechanical stimuli however are reported to be Ca^{2+}-calmodulin-independent (Ayajiki *et al*., 1996; Fleming *et al*., 1998; 1999; Marchenko & Sage, 2000), and we have shown that activation of eNOS by cyclic AMP is also Ca^{2+}-independent in HUVECs (Ferro *et al*., 1999), and in rat pulmonary artery endothelial cells (own unpublished observations). Consistent with this, a recent study has demonstrated that phosphorylation of eNOS by cyclic AMP or cyclic GMP causes both activation of the enzyme and development of Ca^{2+}-independence (Butt *et al*., 2000). The close similarities shown between the responses to mechanical stimuli and cyclic AMP, but not ACh, imply that an element of the transduction pathways that lead to activation of eNOS by these stimuli may be common to both. As previously suggested (Fleming *et al*. 1998), this pathway would appear to be separate from that involved in Ca^{2+}-calmodulin-dependent activation.

The question arises as to why uptake of L-arginine should be so vital for Ca^{2+}-independent activation of eNOS, whereas it is not required for Ca^{2+}-dependent activation. A possible explanation derives from a study by McDonald *et al*. (1997), where it was shown that eNOS, caveolin 1, and CAT1 (the cationic amino acid transporter associated with system y^+) all co-locate in pulmonary artery endothelial cells, and that eNOS and CAT1 form a complex within the caveolae. This would allow directed delivery of extracellular L-arginine to the eNOS protein (McDonald *et al*., 1997). In contrast, receptor dependent agonists and Ca^{2+} are reported to cause translocation of eNOS into the cytosol (Prabhakar *et al*., 1998; Goetz *et al*., 1999), presumably dislocating this arrangement and allowing access to the cytosolic L-arginine pool. It is notable in this regard that inhibitors of tyrosine phosphatase, which promote tyrosine phosphorylation, have been found to increase the proportion of eNOS found in the cytoskeletal fraction of endothelial cells (Fleming *et al*., 1998), possibly suggesting translocation to the caveolae associated with the sarcolemma.

It should be noted that cyclic AMP (Ferro *et al*., 1999), shear stress (Posch *et al*., 1999a) and bradykinin and ATP (Bogle *et al*., 1991) are all reported to increase L-arginine uptake in endothelial cells. Notwithstanding the recent demonstration that cyclic AMP can directly activate eNOS (Butt *et al*., 2000), this raises the question as to whether an increase in L-arginine uptake could itself potentiate eNOS activation, or whether it merely facilitates activity during periods of high substrate use.

In summary, we have shown for the first time that uptake of extracellular L-arginine is critical for activation of eNOS by stretch, isometric contraction, and cyclic AMP in pulmonary arteries of the rat. If similar mechanisms are also present in other vascular beds, the possible consequences for disease could be profound. For example, both hypoxia in the pulmonary circulation (Zharikov *et al*., 1997) and lipoproteins in the systemic circulation (Vergnani *et al*., 2000; Posch *et al*., 1999b) have been reported to reduce uptake of L-arginine into endothelial cells. If the mechanisms described in this paper were indeed active, this could lead to a defect in mechanical stimuli- and β-adrenergic-induced NO production. Raising plasma L-arginine might be expected however to enhance uptake, and so mitigate the effects of the defect. Although this prediction remains to be tested, it could therefore at least partly account for the 'arginine paradox' in some vascular beds.

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