British Journal of Pharmacology (1997) 122, 99–102

Response of normoxic pulmonary arteries of the rat in the resting and contracted state to NO synthase blockade

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1 The pulmonary vasculature is normally in a low resting state of tone. It has been hypothesized that this basal tone is actively maintained by the continuous release of a vasodilator in the resting state. However, evidence for basal release of nitric oxide (NO) is inconclusive.

2 We studied the release of NO in arteries from the pulmonary circulation of male Wistar-Kyoto rats by examining the effects of the L-arginine analogue N^G-nitro-L-arginine methyl ester (L-NAME) on resting pulmonary arteries and on vessels pre-contracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$).

3 Rats ($n=21$) were killed by an overdose with pentobarbitone. Pulmonary arteries were dissected (mean internal diameter 459 ± 11 μm) and mounted in a small vessel wire myograph. Resting tensions were set to simulate transmural pressures of 17.5 mmHg.

4 L-NAME (100 μM) was found to produce a contraction of 0.64 ± 0.09 mN mm^{-1} in resting pulmonary arteries when added alone to the myograph bath. This contraction was not produced following removal of the endothelium. Vessel contraction to $PGF_{2\alpha}$ (100 μM) was found to be significantly greater when carried out in the presence of L-NAME (100 μM) -1.37 ± 0.15 mN mm^{-1} compared with 1.96 ± 0.17 mN mm^{-1}. Dilatation following acetylcholine (ACh) (1 μM) was abolished in the presence of L-NAME (100 μM).

5 Rat pulmonary artery contraction in response to the addition of L-NAME and the absence of contraction upon removal of the endothelium provides supportive evidence of the active release of nitric oxide for the maintenance of resting tone.

Keywords: L-NAME; pulmonary artery; nitric oxide

Introduction

The pulmonary vasculature is normally in a low state of tone and action of dilator substances cannot usually be shown unless vessels are preconstricted. Weir (1978) proposed that the low basal tone of the pulmonary system was actively maintained, a theory pre-dating the discovery of endothelial-derived relaxing factor (EDRF) and nitric oxide (NO). A considerable amount of evidence has accrued to show that tone in the systemic circulation is modulated by continuous release of NO (Moncada *et al*., 1991). In animals, both acute (Rees *et al*., 1989; Aisaka *et al*., 1989; Gardiner *et al*., 1990) and chronic (Barer *et al*., 1993; Hampl *et al*., 1993) inhibition of NO synthase has been shown to elevate systemic arterial pressure. Furthermore, acute inhibition of NO synthase may increase systemic arterial tone in the resting state in man (Vallance *et al*., 1989). Evidence for the role of NO as a pulmonary arterial vasodilator is extensive, but the existence of a specific agent for the maintenance of low basal tone has not been proven.

Whilst NO would be the ideal candidate for continuous release to maintain low pulmonary arterial tone, the use of NO analogues has produced no conclusive evidence of such a role. A single acute dose of N^G-nitro-L-arginine methyl ester (L-NAME) added to the perfusate of isolated lungs from normoxic rats was found to have no effect on pulmonary artery pressure, although the same dose raised pressure in chronically hypoxic rats (Barer *et al*., 1993). Robertson *et al*. (1990) found that another NO synthase inhibitor, N^G-monomethyl-L-arginine (L-NMMA), also failed to alter pulmonary artery pressure in the isolated organs of rats. Chronic deficiency of NO, produced by the addition of L-NAME to drinking water over 3 weeks, was found to elevate systemic arterial pressure without altering resting pulmonary arterial pressure (Hampl *et al*., 1993; Emery, 1993).

Whilst these experiments do not support the existence of a role for NO in resting pulmonary tone, there is some contradictory evidence. L-NAME was found to elevate pulmonary artery pressure and pulmonary vascular resistance in rabbit lungs *in vivo* (Wiklund *et al*., 1990). Both L-NAME and methylene blue were shown to elevate basal pulmonary vascular resistance in pig, sheep and human lungs in isolated organ preparations (Cremona *et al*., 1994). Calculated pulmonary vascular resistance increased in adult man following L-NMMA (Stamler *et al*., 1994). In children, pulmonary blood flow velocity fell in a dose-dependent way following L-NMMA and returned to baseline with L-arginine, as assessed by intravascular Doppler measurement and quantitative angiography (Celermajer *et al*., 1994). If NO were to be released under basal conditions, an increase in resistance and flow velocity would be consistent with vasoconstriction induced by inhibition.

The current experiment was established to examine the effect of the NO analogue L-NAME on resting isolated pulmonary vessels by means of the wire myograph. In addition, we investigated the effect of L-NAME on vasoconstriction produced by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$).

Methods

Animals

Male Wistar-Kyoto rats were obtained from the Royal Hallamshire Hospital animal house and maintained on a standard chow and tap water diet. At approximately 12–13 weeks of age, animals were anaesthetized with intraperitoneal pentobarbitone sodium (15 mg 100 g^{-1} body weight) and the heart and lungs removed. The left lung was mounted in a dissection dish with the parietal surface lying inferiorly and an incision was made along the superficial aspect of the bronchus cutting towards smaller bronchioles. Bronchial tissue was then re-

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moved, revealing pulmonary arterioles below. The vessel was lifted free after division of any further surrounding tissue and promptly mounted on a wire myograph.

Myograph system

Experiments were performed on an automated wire myograph (Cambustion Ltd, Cambridge). Vessels were mounted by a standard method previously described, a 2.3 mm length of pulmonary arteriole being left suspended between two tungsten wires of 40 μm diameter (Mulvaney & Halpern, 1977). Contractility of the vessel was measured by means of a sensing jaw attached to a feedback force transducer with a resolution of 0.05 mN and drift of less than 0.05 mN h^{-1}, calibrated weekly. In order to quantify contractility of different vessels, each vessel was pre-tensioned to an equivalent pressure of 17.5 mmHg by use of the Laplace equation, with the normalized lumen diameter being obtained by the procedure described by Mulvaney & Halpern (1977). Vessel rings were studied at 100% internal diameter. A resting tension of 17.5 mmHg was chosen to approximate the pressure in pulmonary arteries (Herget *et al*., 1978) and arterioles (Bhattacharya *et al*., 1982) of normoxic rats *in vivo*.

Experimental protocol

Following normalization and pretensioning, vessels were allowed to equilibrate for 45 min. In order to assess tissue viability, a contraction with a maximal concentration of potassium chloride (KCl 100 mM) was performed, followed by wash-out of the bath with four changes of physiological saline solution (PSS). There was then a rest period of approximately 20 min. A second contraction to KCl (100 mM) was performed followed by a further wash-out and rest period. Vessels failing to contract by more than 0.5 mN mm^{-1} at this stage were assumed to be damaged and were not studied further. The loading routine was then repeated to ensure stable baseline conditions. Values quoted for vessel diameter are those measured after the second vessel loading. These procedures were carried out before each of the following experiments.

First experiment

Vessels were pre-contracted with $PGF_{2\alpha}$ (100 μM) and, once the contraction had stabilized, dilated with ACh (1 μM). The bath was washed with four changes of PSS. After a further rest period, L-NAME (100 μM) was added and the tissue responses to $PGF_{2\alpha}$ (100 μM) and ACh (1 μM) were re-examined.

Second experiment

Vessels were pre-contracted with $PGF_{2\alpha}$ (100 μM) and, after stabilization of the contraction, dilated with ACh (1 μM). Vessels failing to dilate to ACh by more than 15% of the active tension increase to $PGF_{2\alpha}$ were excluded from further analysis, on the basis of probable endothelial damage. A dose-response curve was then performed with additive doses of L-NAME (1–700 μM). Contractions were allowed to stabilize before each addition of L-NAME.

Third experiment

Concentration-response curves were established for $PGF_{2\alpha}$ (1–100 μM) before and after the addition of L-NAME (100 μM).

Fourth experiment

One of each pair of vessels loaded in the myograph bath was depleted of endothelium by the internal surface being rubbed with a human hair. Vessels were pre-contracted with $PGF_{2\alpha}$ (100 μM) and dilated with ACh (1 μM). Endothelium-intact vessel responses were then compared at four additive doses of L-NAME (100 μM, 1 mM, 2 mM, 10 mM) with the responses of arterioles with endothelium removed.

Solutions and drugs

Vessels were bathed in PSS perfused with 95% O_2 and 5% CO_2 at a constant temperature of 37°C. pH was stable at 7.4 throughout. PSS contained (mM): NaCl 120, KCl 4.7, $MgSO_4$ 1.17, $NaHCO_3$ 25, K permanganate 1.18, EDTA 26.9 μM and glucose 5.5 and was made up in de-ionized water. Finally, $CaCl_2$ 0.37 mM was added after the above mixture had been bubbled with 95% O_2/5% CO_2 for 20 min.

$PGF_{2\alpha}$, ACh, KCl, atrial natriuretic peptide (ANP) and L-NAME were obtained from Sigma Chemicals and diluted with 0.9% normal saline where appropriate.

Data analysis

Data were analysed by one-way analysis of variance. A P value less than 0.05 was considered significant. Results are expressed as means $\pm$ s.e.mean. Relaxation of vessels was expressed both as an absolute tension change from maximal contraction and as a percentage change from pre-contraction. To calculate EC_{50} values, concentration-effect curves were drawn for each vessel, a sigmoid function attached and the concentration producing 50% of the maximal response was estimated. Overall mean geometric EC_{50} with 95% confidence intervals (C.I.) was then calculated for each group of vessels.

Results

Rat weight and vessel sizes

A total of 19 rats providing 61 vessels were studied. Mean rat weight was 282 ± 61.7 g with no significant difference between experiments. Mean vessel size over all experiments was 459 ± 11 μm.

Experiment 1

A greater contraction was produced by $PGF_{2\alpha}$ (100 μM) in the presence of L-NAME (100 μM) compared to that produced by $PGF_{2\alpha}$ (100 μM) alone from equivalent baseline tensions. Mean active contraction following $PGF_{2\alpha}$ alone was 1.37 ± 0.15 mN mm^{-1}, whereas contraction following the addition of $PGF_{2\alpha}$ in the presence of L-NAME was 1.96 ± 0.17 mN mm^{-1}, $P = 0.01$ ($n = 6$ rats, 16 vessels).

Following $PGF_{2\alpha}$, ACh (1 μM) produced a mean active relaxation of 0.36 ± 0.06 mN mm^{-1} (mean 32.6% dilatation from peak contraction to $PGF_{2\alpha}$). This dilatation was effectively abolished when ACh was added in the presence of L-NAME, active relaxation being 0.02 ± 0.05 mN mm^{-1} (mean 1.13%), $P = 0.0001$.

Experiment 2

Mean maximum active contraction to L-NAME from baseline was 0.64 ± 0.09 mN mm^{-1}, $P = 0.0004$. Concentration-effect curves were drawn for each vessel and the concentration producing 50% of the maximal response (EC_{50}) was calculated. Overall, the mean EC_{50} for L-NAME was calculated to be 25.6 (95% C.I. 38.1 to 13.1) μM ($n = 5$ rats, 15 vessels). Mean concentration-responses are shown in Figure 1.

Experiment 3

Mean maximum active contraction to $PGF_{2\alpha}$ was 1.65 ± 0.24 mN mm^{-1} compared to 2.15 ± 0.29 mN mm^{-1} ($P = 0.042$) following $PGF_{2\alpha}$ in the presence of L-NAME (100 μM). Concentration-effect curves were drawn for each vessel and EC_{50} for $PGF_{2\alpha}$ with and without L-NAME was calculated. There was a significant difference between mean

EC_{50} for $PGF_{2\alpha}$ alone 9.2 (95% C.I. 10.41 to 7.99) μM and $PGF_{2\alpha}$ in the presence of L-NAME 3.9 (95% C.I. 4.83 to 3.35) μM, $P<0.0001$ ($n=5$ rats, 15 vessels). Mean concentration-effect curves are shown in Figure 2.

Experiment 4

Mean active contraction to $PGF_{2\alpha}$ (100 μM) was 1.07 ± 0.39 mN mm^{-1} in vessels with endothelium compared to 0.84 ± 0.08 mN mm^{-1} in vessels without endothelium. This difference was not significant ($P=0.5$). There was an active relaxation of 0.3 ± 0.04 mN mm^{-1} to ACh (1 μM) in vessels with endothelium compared to a mean active relaxation of 0.006 ± 0.009 mN mm^{-1} in vessels without endothelium. This difference coincided with the difference in response of vessels to L-NAME dependent upon the presence of endothelium. Mean maximum contraction to L-NAME (2 mM) in vessels with endothelium was 0.34 ± 0.06 mN mm^{-1} compared to -0.003 ± 0.02 mN mm^{-1} in vessels without endothelium, $P=0.0001$. ($n=3$ rats, 12 vessels). The different responses are shown in Figure 3.

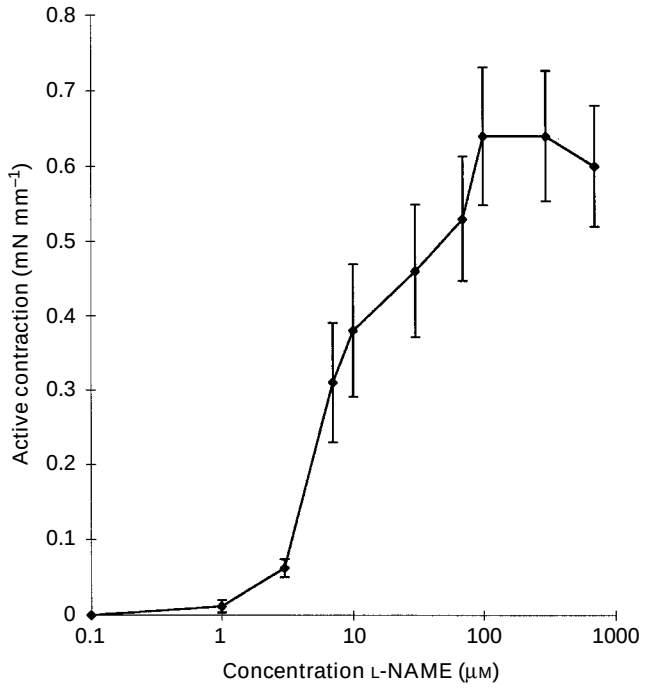

Figure 1 Mean concentration-responses to L-NAME (1 μM–700 μM) in rat pulmonary artery. Vertical lines show s.e.mean.

Discussion

In this study we have shown that L-NAME is capable of eliciting a contractile response in pulmonary vessels mounted in the wire myograph. This contractile effect is abolished by the removal of arteriolar endothelium. This would suggest that basal release of NO from the endothelium may contribute to the low resistance seen in the normal pulmonary circulation. Our findings contrast with experiments in the isolated perfused lung model in the rat (Robertson *et al*., 1990; Barer *et al*., 1993) where no alteration in pulmonary artery pressure in response to NO synthase inhibitors was found. However, an increase in pulmonary vascular resistance has been demonstrated in sheep, pig and human experiments (Cremona *et al*., 1994). Similarly *in vivo* experiments have found an increase in pulmonary vascular resistance in rabbits (Wiklund *et al*., 1990). Flow rates used in the isolated organ experiments vary and at high rates, NO release is known to occur because of shear stress (Busse *et al*., 1993). The variation in responses to NO synthase inhibitors in isolated organ experiments may well reflect differences in the experimental conditions used. In the wire myograph, vessels are held under isometric conditions without luminal flow and should not therefore be exposed to differences in shear force. In addition, in this experiment the responses of a specific size of pulmonary vessel, thought to be representative of 'resistance arteries', were studied as opposed to the response of the lung as a whole. It is known that responses vary according to the size of a vessel and that there may be a difference between the overall resistivity of the lung and the response of certain vessels within (Leach *et al*., 1992). Thus release of NO to maintain basal tone may be a property of selected vessels within the pulmonary circulation.

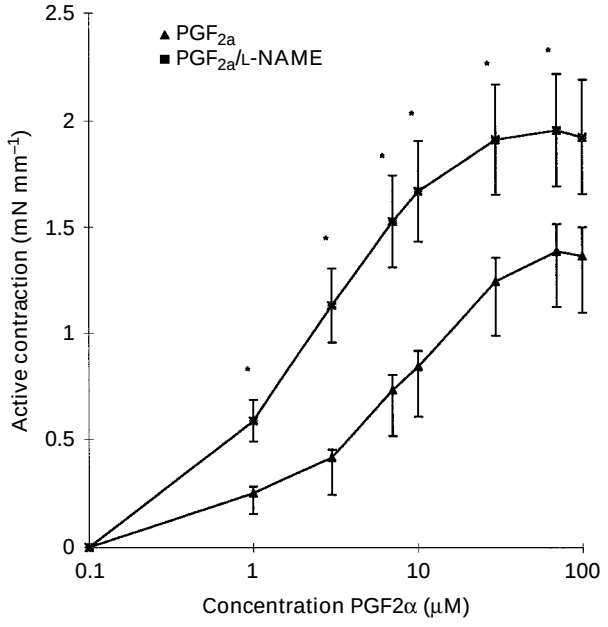

Figure 2 Mean concentration-responses to $PGF_{2\alpha}$ (1 μM–100 μM) before and after the addition of L-NAME (100 μM) in rat pulmonary artery. Vertical lines show s.e.mean; $*P<0.05$.

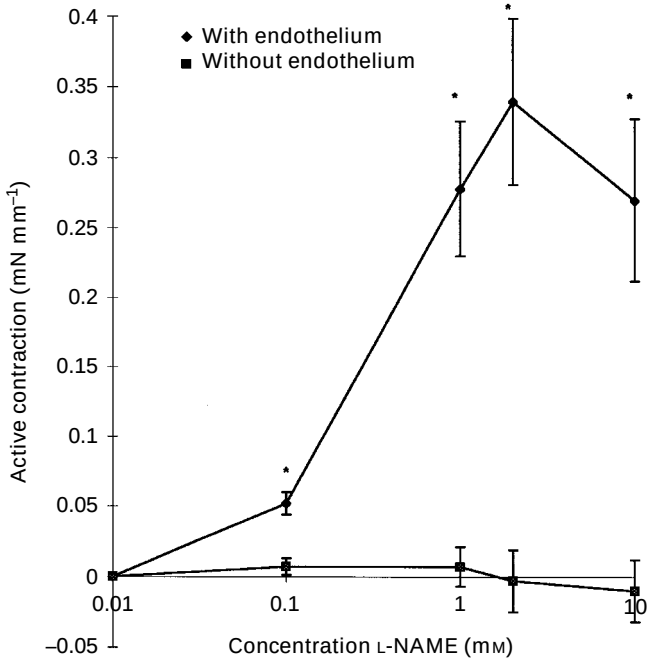

Figure 3 Mean concentration-response to L-NAME (100 μM–10 mM) in vessels with and without endothelium. Vertical lines show s.e.mean; $*P<0.05$.

Several studies have demonstrated that there is an effect of NO synthase inhibition on the precontracted pulmonary circulation. We have shown that L-NAME causes a leftward, non-parallel, shift of the concentration-response curve to $PGF_{2\alpha}$ in the rat isolated pulmonary artery. EC_{50} altered from 9.2 μM (95% C.I. 10.41 to 7.99) to 3.9 μM (95% C.I. 4.83 to 3.35) in the presence of L-NAME. $PGF_{2\alpha}$ is believed to act via endothelial surface receptors and, theoretically, it would be possible either for L-NAME to be an antagonist at this site or to inhibit $PGF_{2\alpha}$ receptor-induced NO production. However, no such interaction has been found and the shift in the concentration-response curve with depression of maximal responses at the higher concentrations seen in Figure 2 would argue against a competitive interaction. A more likely explanation is that $PGF_{2\alpha}$ increased the release of NO and that L-NAME inhibited this, resulting in increased vasoconstriction. It would be possible for NO release to occur either spontaneously from the endothelium as a consequence of the contraction, or for $PGF_{2\alpha}$ to release endothelium-derived NO in addition to producing contraction. Other agonists are believed to stimulate NO release during constriction. Cocks & Angus (1983) found that removal of endothelium from isolated coronary arteries of dogs and pigs resulted in a relative increase in contraction following 5-hydroxytryptamine and noradrenaline. Similarly, endothelin-1 has been shown to augment endothelial NO production during contraction (Thiemermann *et al*., 1989).

In summary, we have shown that inhibition of NO synthesis in isolated pulmonary resistance arteries causes vasoconstriction which is independent of luminal flow and results in an increased potency of contractile agonists. Evidence is provided for a role of spontaneously released NO in maintaining resting tone in the rat pulmonary artery.

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(Received February 27, 1997
Revised May 28, 1997
Accepted June 9, 1997)