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Superoxide and endothelium-dependent constriction to flow in porcine small pulmonary arteries

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1 The aim of this study was to determine the response of porcine small pulmonary arteries to intralumenal flow and to identify the cellular mechanisms and potential mediators involved in the response.

2 Porcine small pulmonary arteries were isolated from a branch of the main intrapulmonary artery of the lower lung lobe and studied in a perfusion myograph system that allowed independent control of transmural pressure and intralumenal flow. At a transmural pressure of 20 mmHg, the baseline internal diameter (BID) of the arteries was 251.2 ± 16.1 μm ($n=16$).

3 Under quiescent conditions or during constriction with U46619 to $\sim 60\%$ of BID, intralumenal flow caused reversible constriction in arteries with endothelium (in the presence of U46619, flow decreased diameter from $60.0 \pm 2.5\%$ to $49.5 \pm 3.0\%$ BID at 10 μl min^{-1}, $n=16$, $P<0.05$) but no change in diameter of arteries without endothelium.

4 In the presence of superoxide dismutase (SOD, 150 u ml^{-1}), the response to flow was converted from constriction to vasodilatation (in presence of U46619 and SOD, flow increased diameter from $54.2 \pm 3.4\%$ to $76.7 \pm 4.5\%$ BID at 10 μl min^{-1}, $n=10$, $P<0.05$). Inhibition of NO synthase with L-NAME (3×10^{-5} M) abolished the flow-induced vasodilatation occurring in the presence of SOD and the flow-induced constriction occurring in the absence of SOD. In arteries with endothelium, L-NAME (3×10^{-5} M) caused significant vasoconstriction, whereas SOD did not alter vasomotor tone.

5 Acetylcholine (10^{-8} to 10^{-6} M) caused endothelium-dependent relaxation of small pulmonary arteries that was not significantly affected by SOD (150 u ml^{-1}) but was inhibited by L-NAME (3×10^{-5} M).

6 These results suggest that in small, porcine, isolated pulmonary arteries, intralumenal flow increases the production of NO but this is obscured by the generation of superoxide which causes vasoconstriction.

Keywords: Shear stress; EDCF; endothelium-derived constricting factor; microcirculation

Introduction

Shear stress exerted by flowing blood regulates endothelial cell structure and function (Nerem *et al*., 1993; Davies, 1995). One of the earliest responses of endothelial cells to an increase in shear stress is increased production of vasoactive mediators (Davies, 1995). In most blood vessels, shear stress causes endothelium-dependent relaxation which is attributed to increased production of endothelium-derived nitric oxide (EDNO) or prostacyclin (Holtz *et al*., 1984; Frangos *et al*., 1985; Koller & Kaley, 1990; Kanai *et al*., 1995). The pulmonary circulation is a low pressure, low resistance vascular bed that displays unique reactivity to chemical, hormonal or haemodynamic stimuli (e.g. Gaine *et al*., 1997). Indeed, a previous preliminary study demonstrated that intralumenal flow caused vasoconstriction, not vasodilatation, of small pulmonary arteries (Bevan, 1993). The present experiments were, therefore, performed to assess the response of small porcine pulmonary arteries to flow and to determine the cellular mechanisms and potential mediators involved in the response.

Methods

Artery preparation

Male pigs (25–30 kg) were anaesthetized with ketamine (700 mg i.m.) followed by pentobarbitone sodium (12.5 mg kg^{-1}, i.v.). The pigs were then killed by exsanguination through the femoral arteries. The lungs were rapidly removed and placed in cold modified Krebs-Ringer bicarbonate solution (in mM): NaCl 118.3, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, glucose 11.1 and edetate calcium disodium 0.016 (control solution). Small pulmonary arteries were isolated from a branch of the main intrapulmonary artery of the lower left lobe. All side branches were tied off under microscopy. In some experiments, endothelial cells were disrupted by placing a wire (70 μm in diameter) into the vessel lumen. Endothelial removal was confirmed during the course of the experiment by the absence of relaxation to acetylcholine. Arteries were placed in a microvascular chamber (Living Systems, Burlington, VT) (Liu & Flavahan, 1997) and cannulated at the proximal end with a glass micropipette and secured with 12-0 nylon monofilament suture. Control solution was then perfused slowly from the tip of the cannula until the artery was filled with solution. The artery was then attached to the second cannula with 12-0 nylon and connected to a reservoir system that allowed independent control of transmural pressure and intralumenal flow (Wiener & Carmines, 1994). The glass micropipettes were connected to pressure transducers in order to provide continuous measurement of upstream and downstream pressures. The small arteries were maintained in no-flow state and were held at a constant transmural pressure of 20 mmHg, the mean pulmonary arterial pressure for normotensive swine (Herity *et al*., 1994; Emil *et al*., 1996). The chamber was superfused (recirculation) with 100 ml of control solution and maintained at 37°C, pH 7.4, and gassed with 16% O_2-5% Co_2-balance N_2.

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The chamber was placed on the stage of an inverted microscope (X20, Nikon TMS-F, Japan) connected to a video camera (Panasonic, CCTV camera, Japan). The vessel image was projected onto a video monitor and internal diameter was continuously determined near the mid-point of the vessels by a video dimension analyser (Living Systems Instrumentation, Burlington VT). Vessel diameter and pressure measurements were continuously monitored by means of a four channel recorder.

Experimental protocol

The internal diameter attained when the arteries were initially brought to a transmural pressure (P_{TM}) of 20 mmHg was designated as the baseline internal diameter (BID). Subsequent exposure of the arteries to vasodilators (papaverine 0.1 mM, sodium nitroprusside, 10 μM) failed to relax the arteries beyond the BID, suggesting that this represented the fully-relaxed diameter of the blood vessels. The pulmonary arteries were allowed to equilibrate for 45–60 min at this pressure before the experiment was performed. KCl (60 mM) was added to the chamber to evoke vasoconstriction and once the response stabilized, chamber superfusate was replaced with fresh control solution and vessel diameter allowed to return to baseline. In most experiments, the small arteries were constricted to $\sim$60% of BID with the thromboxane mimic U46619 (10^{-9} to 10^{-8} M) before the assessment of vasomotor responses. This represented the total level of constriction and included any myogenic tone that the arteries developed during the equilibration period. To study flow-induced responses, the upstream glass micropipette was connected to a syringe pump (Wiener & Carmines, 1994). The resistances of both the upstream and downstream glass micropipettes were equivalent (determined by means of pressure-flow curves in the absence of microvessels) (Carmines & Wiener, 1994). Initiation of flow caused inflow (upstream) pressure to increase (3–5 mmHg at flow of 10 μl min^{-1}) and this was immediately offset by decreasing the outflow (downstream) pressure in order to maintain a constant transmural pressure. Preliminary experiments demonstrated that responses to intralumenal flow (10 μl min^{-1}) stabilized within 5 min and that repeated exposure of the arteries to flow (10 μl min^{-1}, 5 min) evoked reproducible responses. Therefore, in all experiments, the control response of the artery to intralumenal flow (10 μl min^{-1}, 5 min) was determined before the assessment of the influence of inhibitors. When the influence of L-NAME, an inhibitor of NO synthase, was analysed, it was added to the chamber superfusate 30 min before the arteries were exposed to U46619. When the influence of superoxide dismutase (SOD, 150 u ml^{-1}) was analysed, the enzyme was added to the chamber superfusate during the contractile response to U-46619 and 20 min before exposure of the vessel to an increase in flow. When responses to acetylcholine were studied, the concentration of agonist was increased in half-log increments once the response to the preceding concentration had stabilized.

The BIDs of the arteries used in each experimental group were similar: 251.2$\pm$16.1 μm (n=16) for control arteries (with endothelium), 249.2$\pm$20.6 μm (n=10) for SOD-treated arteries, 222.0$\pm$28.7 μm (n=5) for L-NAME-treated arteries, 222.0$\pm$28.7 μm (n=5) for L-NAME + SOD-treated arteries, and 233.5$\pm$48.3 μm (n=4) for endothelium-denuded arteries.

Drugs

Acetylcholine chloride, N^G-nitro-L-arginine-methyl ester (L-NAME), superoxide dismutase (bovine erythrocytes), thromboxane A_2 analogue U-46619 (9,11-dideoxy-9α-(methanoepoxy)$PGF_{2\alpha}$; Cayman Chemical Co, Ann Arbor, MI) were obtained from Sigma Chemical (St. Louis, MO) unless stated otherwise. Stock solutions of drugs were prepared fresh each day and stored at 4°C during the experiment. Drugs were dissolved in distilled water and added to the superfusate in volumes less than 600 μl. All drug concentrations are expressed as final molar concentration (mol l^{-1}) in the chamber superfusate.

Data analysis

Vessel diameter was expressed as a percentage of the baseline internal diameter (BID), the diameter obtained initially when the arteries were brought to a transmural pressure of 20 mmHg. Data are expressed as means$\pm$s.e.mean for n number of experiments, where n equals the number of animals from which blood vessels were studied. Statistical evaluation of the data was performed by Student's t test for either paired or unpaired observations. When more than two means were compared, analysis of variance was used. If a significant F value was found, Scheffe's test for multiple comparisons was employed to identify differences between groups. Values were considered to be statistically different when P was less than 0.05.

Wall shear rate (γ) and wall shear stress (τ) were calculated according to:

$$\gamma = 4Q/\pi R^3 \qquad \tau = \eta\gamma$$

where Q is flow (cm^3 s^{-1}), R is vessel radius (cm) and η is viscosity (poise or g cm^{-1} s^{-1}, 0.01 for control solution). The units for shear rate are s^{-1} and for shear stress are dyn cm^{-2}.

Results

Baseline characteristics

During an initial equilibration period (45 to 60 min), arteries developed spontaneous myogenic tone causing constriction to 82.1$\pm$6.5% BID (n=16) in arteries with endothelium and to 80.0$\pm$7.8% BID (n=4) in endothelium-denuded arteries. In arteries with endothelium, inhibition of NO synthase with L-NAME (3×10^{-5} M) caused significant vasoconstriction, decreasing vessel diameter from 85.5$\pm$6.7% to 72.1$\pm$9.0% BID (change of $-$13.4$\pm$3.6) (n=6, $P<0.05$). Superoxide dismutase (SOD, 150 u ml^{-1}) did not alter artery diameter under baseline conditions (diameters of 77.1$\pm$9.1% and 76.5$\pm$7.5% BID before and after SOD, respectively, n=5, $P>0.05$), or during constriction with U46619 (diameters of 60.0$\pm$5.4% and 60.3$\pm$5.5% BID before and after SOD, respectively, n=5, $P>0.05$).

Response to flow

Quiescent arteries In the absence of an exogenous constrictor stimuli, intralumenal flow (5 to 30 μl min^{-1}) caused significant, graded constriction of small pulmonary arteries, decreasing vessel diameter from 87.1$\pm$11.5% BID under no-flow conditions to 64.2$\pm$10.3% BID at 30 μl min^{-1} intralumenal flow (n=3, $P<0.05$) (Figure 1). The calculated shear stress at each level of flow was 1.2 dyn cm^{-2} at 5 μl min^{-1}, 2.3 dyn cm^{-2} at 10 μl min^{-1}, 4.6 dyn cm^{-2} at 20 μl min^{-1} and 7.0 dyn cm^{-2} at 30 μl min^{-1}.

Further characterization and analysis of the response to intralumenal flow was performed by use of an intermediate flow rate, namely 10 μl min^{-1}. At this rate, intralumenal flow

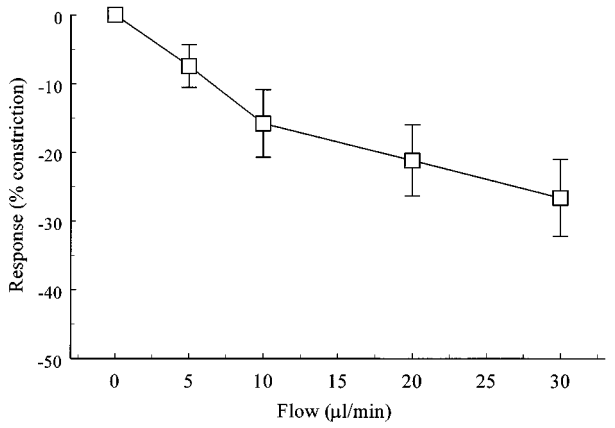

Figure 1 Effect of intralumenal flow (5 to 30 μl min^{-1}) on diameter of porcine small pulmonary arteries. Arteries with endothelium were studied under quiescent conditions (i.e. absence of U46619) and exposed to each level of flow for approximately 5 min until the constriction stabilized, at which point flow was increased. Data are expressed as % of BID and are presented as means with vertical lines indicating s.e.mean ($n=3$).

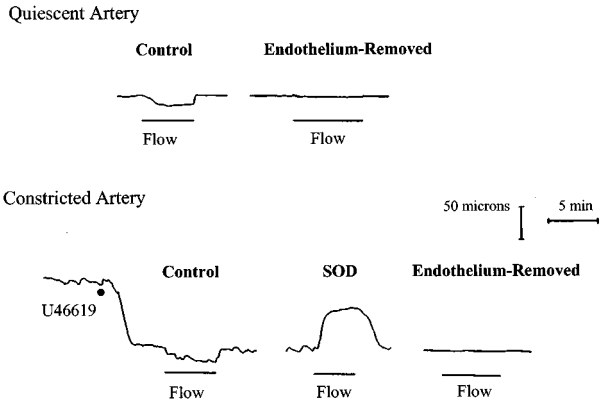

Figure 2 Representative tracing demonstrating the effect of intralumenal flow (10 μl min^{-1}) on diameter of porcine small pulmonary arteries.

caused reversible constriction of pulmonary arteries with endothelium (Figure 2) but no change in tone of arteries denuded of endothelium (diameters of $90.1\pm7.1\%$ and $89.8\pm6.9\%$ BID in the absence and presence of flow, respectively, $n=3$; $P>0.05$). Endothelial removal did not cause non-specific depression in the ability of the arteries to constrict: KCl (60 mM) caused similar constriction in arteries with and without endothelium, decreasing vessel diameter from $95.8\pm2.0\%$ to $46.2\pm4.7\%$ BID ($n=16$) in arteries with endothelium, and from $92.3\pm2.7\%$ to $38.5\pm6.6\%$ BID ($n=5$) in arteries without endothelium.

Arteries constricted with U46619 In order to analyse further the mechanisms underlying flow-induced constriction and to assess whether flow could also cause relaxation, the response to intralumenal flow (10 μl min^{-1}) was assessed in pulmonary arteries constricted to $\sim60\%$ of BID with U46619. As was observed under quiescent conditions, intralumenal flow (10 μl min^{-1}) caused reversible constriction in arteries with endothelium (decrease in diameter from $60.0\pm2.5\%$ to $49.5\pm3.0\%$ BID, change of -10.5 ± 2.0, $n=16$, $P<0.05$) but no change in diameter of arteries without endothelium (Figures 2 and 3). Rapid, endothelium-dependent contractions are often mediated by increased production of superoxide (Katusic & Vanhoutte, 1989; Katusic, 1996). Indeed, in the presence of SOD (150 u ml^{-1}), the endothelium-dependent constriction to flow was abolished and flow (10 μl min^{-1}) caused profound vasodilatation (increase in diameter from $54.2\pm3.4\%$ to $76.7\pm4.5\%$ BID, change of $+22.5\pm5.1$, $n=10$, $P<0.05$) (Figures 2 and 3). Inhibition of NO synthase with L-NAME (3×10^{-5} M) abolished the flow-induced vasodilatation occurring in the presence of SOD (Figure 3). In the absence of SOD, L-NAME (3×10^{-5} M) also abolished the constrictor response to intralumenal flow (Figure 3). Therefore, after L-NAME or L-NAME + SOD, intralumenal flow no longer had any effect on arterial tone (Figure 3).

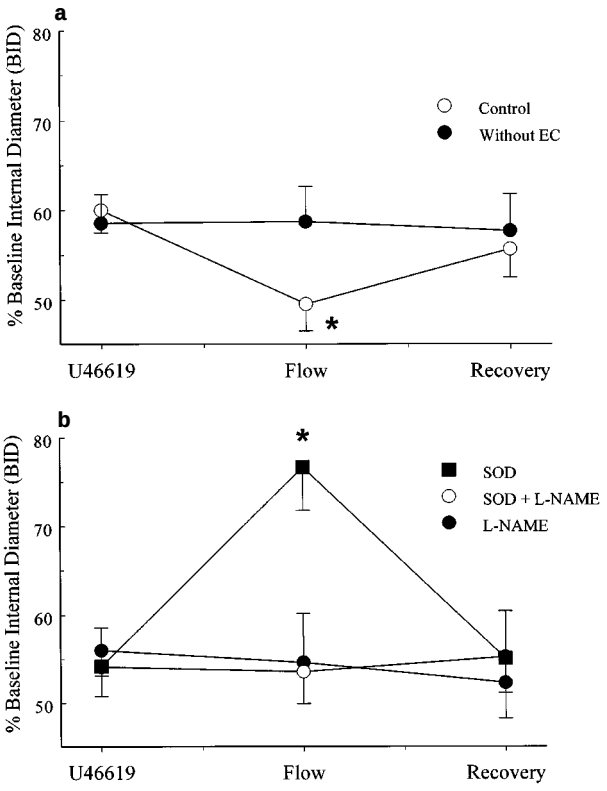

Figure 3 (a) Effect of intralumenal flow (10 μl min^{-1}) on diameter of porcine small pulmonary arteries with (control) and without endothelium. (b) Effect of intralumenal flow (10 μl min^{-1}) on diameter of porcine small pulmonary arteries (with endothelium) treated with superoxide dismutase (SOD, 150 u ml^{-1}), L-NAME (3×10^{-5} M, inhibitor of NO synthase) or SOD (150 u ml^{-1}) + L-NAME (3×10^{-5} M). Arteries were constricted with U46619 to approximately 60% of baseline internal diameter (BID) before flow was started. Exposure to flow was for approximately 5 min after which time the arteries were allowed to recover (recovery). Data are expressed as % of BID and are presented as means $\pm$ s.e.mean. (a) $n=16$ (control) or 4 (without EC); (b) $n=10$ (SOD) or 5 (L-NAME, SOD + L-NAME). *Indicates that the response to intralumenal flow is statistically significant ($P<0.05$).

Response to acetylcholine

In pulmonary arteries (with endothelium) constricted to $\sim60\%$ of BID with U46619, acetylcholine (10^{-8} to 10^{-6} M) caused concentration-dependent relaxation that was not significantly affected by SOD (150 u ml^{-1}) but was virtually abolished by L-NAME (3×10^{-5} M) (Figure 4). In endothelium-denuded arteries that were constricted with U46619, acetylcholine (10^{-6} M) had no effect on arterial diameter (Figure 4).

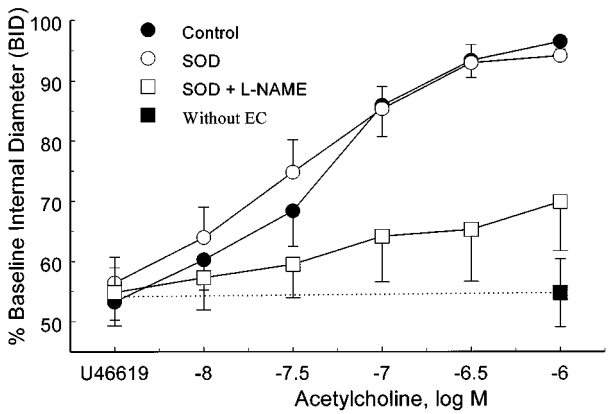

Figure 4 Effect of SOD (150 u ml^{-1}) or SOD (150 u ml^{-1}) + L-NAME (3×10^{-5} M) on relaxations to the endothelium-dependent dilator, acetylcholine, in porcine small pulmonary arteries (with endothelium). For comparison, the response of endothelium-denuded arteries to acetylcholine (10^{-6} M) is also shown. Arteries were constricted with U46619 to approximately 60% of baseline intralumenal diameter (BID) before the responses to acetylcholine were obtained. Data are expressed as % of BID and presented as means with vertical lines showing s.e.mean; $n = 8$ (control), 4 (without EC), 9 (SOD) or 5 (SOD + L-NAME).

Discussion

In the present study, intralumenal flow caused constriction of porcine small pulmonary arteries that was abolished by removal of endothelium, indicating that flow-induced vasoconstriction in this blood vessel is endothelium-dependent. This could result from flow-induced decrease in activity of endothelial dilator mediators (e.g. NO) and/or increase in activity of endothelial constrictor mediators (e.g. superoxide). Indeed, superoxide dismutase (SOD) abolished the flow-induced vasoconstriction and converted the response to flow-induced vasodilatation, suggesting that the constriction was mediated by superoxide radicals. The flow-induced vasodilatation observed following SOD was abolished by L-NAME, an inhibitor of NO synthase, suggesting that the dilatation was mediated by NO. These results suggest that intralumenal flow increased the production of NO, but this was obscured by the generation of superoxide which evoked vasoconstriction.

Superoxide can cause vasoconstriction either by a direct action on smooth muscle cells or by an indirect action to inactivate endothelium-derived NO (Rubanyi & Vanhoutte, 1986; Gryglewski *et al*., 1986; Auch-Schwelk *et al*., 1989; Katusic & Vanhoutte, 1989). In the present study, inhibition of NO synthase by L-NAME caused vasoconstriction suggesting that a significant amount of NO was released under basal, unstimulated conditions. Following L-NAME, the vasoconstrictor response to flow was abolished, consistent with superoxide-mediated inactivation of NO as the cause of flow-induced vasoconstriction. Indeed, vasoconstrictor responses to L-NAME and intralumenal flow (10 $\mu l\ min^{-1}$) were of similar magnitude (absolute changes in BID of $-13.4 \pm 3.6\%$, $n = 6$, and $-14.2 \pm 4.5\%$, $n = 3$ respectively, $P > 0.05$). If superoxide had acted independently of NO, then the vasoconstrictor response would be resistant to inhibition by L-NAME (e.g. Flavahan *et al*., 1991). As was observed in previous studies (Kuo *et al*., 1991; Kanai *et al*., 1995), flow increased the activity of endothelium-derived NO, but in small pulmonary arteries, this response was only observed following inhibition of superoxide with SOD. Superoxide-mediated inactivation of this stimulated production of NO would obviously prevent flow-induced vasodilatation, but it would not be expected to evoke a net constriction. In order to cause a net constriction, the NO activity that was present under basal, no-flow conditions would also have to be inhibited.

Inhibition of superoxide with SOD had no effect on arterial diameter in the absence of intralumenal flow, suggesting that the activity of superoxide was low under static conditions but was increased by flow. Endothelial cells, unlike other cells in the blood vessel wall, are exquisitely sensitive to changes in flow (e.g. Davies, 1995), and are therefore likely to be the cellular source of radical production. However, because superoxide acted by inhibiting an endothelium-derived dilator, endothelium-dependent constriction could also result from a non-endothelial source of radical production. Endothelial cells are capable of generating reactive oxygen species via a number of pathways, including xanthine oxidase, NAD(P)H oxidase and cyclo-oxygenase (Katusic & Vanhoutte, 1989; Bulkley, 1993; Mohazzeb *et al*., 1994). Indeed, when the availability of substrate (arginine) or co-factors (e.g. tetrahydrobiopterin) is limited, NO synthase can also generate reactive oxygen species (Pou *et al*., 1992; Cosentino & Katusic, 1995). Therefore, the inhibitory effect of L-NAME on endothelium-dependent, flow-induced vasoconstriction could have resulted from inhibition of superoxide generation by NO synthase. However, this explanation is considered unlikely. In the absence of endothelial stimulation, L-NAME caused vasoconstriction, whereas inhibition of superoxide with SOD did not alter vasomotor tone. Furthermore, when the activity of NO synthase was increased by acetylcholine, inhibition of superoxide by SOD did not alter the NO-dependent vasodilator response. This suggests that under quiescent or stimulated conditions, NO synthase was not associated with the initiation of superoxide-mediated vasoconstriction. Therefore, the data suggests that shear stress, but not acetylcholine, activates a superoxide-generating system in endothelial cells, consistent with these stimuli activating distinct signalling pathways in the endothelium (Hutcheson & Griffith, 1994; Wellman & Bevan, 1995).

Most previous studies have demonstrated that intralumenal flow causes vasodilatation by increasing the production of endothelium-derived prostacyclin and NO (Holtz *et al*., 1984; Frangos *et al*., 1985; Koller & Kaley, 1990; Kanai *et al*., 1995). However, vasoconstrictor responses to flow have also been observed in several arteries of the rabbit including ear, pial and pulmonary arteries (Bevan & Joyce, 1988; Garcia-Rolden & Bevan, 1990; Bevan, 1993). Because flow-induced contraction in ear arteries was still observed following endothelial removal, it has been assumed that flow-induced contraction is an endothelium-independent event (Bevan, 1993). However, at least for porcine isolated small pulmonary arteries, this constriction is endothelium-dependent and is mediated by increased production of superoxide radicals. In the present study, flow-induced vasoconstriction of porcine small pulmonary arteries was observed at calculated wall shear stresses of 1 to 7 dyn cm^{-2}. Therefore, flow-induced constriction displayed similar sensitivity to other endothelial cell responses to shear stress, including shear-stress induced production of NO (Kuchan & Frangos, 1994; Kanai *et al*., 1995), activation of K^+-channels (Alevriadou *et al*., 1993), cytosolic acidification (Ziegelstein *et al*., 1992), actin depolymerization (Morita *et al*., 1994), calcium mobilization (Davies, 1995), activation of transcription factors (Lan *et al*., 1994; Resnick & Gimbrone, 1995) and induction of shear stress-responsive genes, including NO synthase (Ranjan *et al*., 1995). Although not evident in previous functional studies, increased production

of reactive oxygen species may represent a generalized endothelial response to shear stress. Laurindo *et al*. (1994) demonstrated that femoral artery endothelial cells generated superoxide in response to flow under *in vitro* and *in vivo* conditions. The enzymatic source of the radical was not identified but it was not associated with NO synthase or cyclo-oxygenase activity. Furthermore, shear stress rapidly increased the DNA binding activity of the oxidant-sensitive transcription factors nuclear factor-κB (NF-κB) and AP-1 in nuclear extracts of endothelial cells (Lan *et al*., 1994). These factors are thought to mediate, in part, the transcriptional regulation of shear stress-responsive genes, e.g. NO synthase and PDGF-B (Davies, 1995; Khachigian *et al*., 1995). Therefore, superoxide and other reactive oxygen species may be important intracellular and extracellular mediators of endothelial responses to intralumenal flow and shear stress.

The present study demonstrates that intralumenal flow causes endothelium-dependent vasoconstriction in isolated pulmonary arteries, mediated by superoxide-induced inactivation of endothelium-derived NO. The role of flow-induced vasoconstriction in the physiological or pathophysiological regulation of the pulmonary circulation *in vivo* is not known. Although the oxidant response could be an artifact of this *in vitro* preparation, we consider this unlikely. Superoxide activity was associated only with flow-induced activation of the endothelial cells and was not evident during acetylcholine-induced responses. Moreover, in parallel experiments on similar-sized porcine coronary arteries, we demonstrated flow-induced, endothelium-dependent vasodilatation mediated by NO (data not shown). In contrast to flow-induced vasodilatation, which by decreasing the shear stress at the surface of the endothelial cell can be self-limiting, flow-induced constriction will increase endothelial shear stress and could potentially act as a positive-feedback, pathological mechanism. In systemic vascular beds, increases in pressure augment vascular resistance (autoregulation) and this is restrained by flow-induced vasodilatation (Griffith & Edwards, 1990; Hecker *et al*., 1993). In contrast, increases in pressure in the pulmonary circulation actually decrease vascular resistance due to pressure-induced vessel distension or vessel recruitment. Therefore, as occurs in systemic vascular beds, the response of the pulmonary endothelium to increased flow may function to reduce or modulate the vascular response to increasing pressure.

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