REVIEW

The structural basis of pulmonary hypertension in chronic lung disease: remodelling, rarefaction or angiogenesis?

Natalie Hopkins and Paul McLoughlin

Department of Human Anatomy and Physiology, Conway Institute of Biomolecular and Biomedical Research and Dublin Molecular Medicine Centre, University College, Earlsfort Terrace, Dublin 2, Ireland

Abstract

Chronic lung disease in humans is frequently complicated by the development of secondary pulmonary hypertension, which is associated with increased morbidity and mortality. Hypoxia, inflammation and increased shear stress are the primary stimuli although the exact pathways through which these initiating events lead to pulmonary hypertension remain to be completely elucidated. The increase in pulmonary vascular resistance is attributed, in part, to remodelling of the walls of resistance vessels. This consists of intimal, medial and adventitial hypertrophy, which can lead to encroachment into and reduction of the vascular lumen. In addition, it has been reported that there is a reduction in the number of blood vessels in the hypertensive lung, which could also contribute to increased vascular resistance. The pulmonary endothelium plays a key role in mediating and modulating these changes. These structural alterations in the pulmonary vasculature contrast sharply with the responses of the systemic vasculature to the same stimuli. In systemic organs, both hypoxia and inflammation cause angiogenesis. Furthermore, remodelling of the walls of resistance vessels is not observed in these conditions. Thus it has been generally stated that, in the adult pulmonary circulation, angiogenesis does not occur. Prompted by previous observations that chronic airway inflammation can lead to pulmonary vascular remodelling without hypertension, we have recently shown, using quantitative stereological techniques, that angiogenesis can occur in the adult pulmonary circulation. Pulmonary angiogenesis has also been reported in some other conditions including post-pneumonectomy lung growth, metastatic disease of the lung and in biliary cirrhosis. Such angiogenesis may serve to prevent or attenuate increased vascular resistance in lung disease. In view of these more recent data, the role of structural alterations in the pulmonary vasculature in the development of pulmonary hypertension should be carefully reconsidered.

Key words angiogenesis; chronic inflammation; endothelium; hypoxia; nitric oxide; pulmonary hypetension; remodelling.

Introduction

Sustained pulmonary hypertension is a common complication of chronic lung diseases including chronic obstructive pulmonary disease, cystic fibrosis and bronchiectasis. Particularly in the presence of hypoxaemia, such secondary pulmonary hypertension is strongly associated with increased morbidity and reduced survival (Semmens & Reid, 1974; Ryland & Reid, 1975; MacNee, 1994a,b). Furthermore, the presence of cor pulmonale in these conditions is an independent predictor of increased mortality (Incalzi et al. 1999; Skwarski et al. 1991), suggesting that pulmonary hypertension contributes directly to increased mortality.

The poor prognosis observed in untreated primary pulmonary hypertension supports the view that secondary pulmonary hypertension increases mortality. Primary pulmonary hypertension is a syndrome

Correspondence

Professor P. McLoughlin, Department of Human Anatomy and Physiology, Conway Institute of Biomolecular and Biomedical Research, University College, Earlsfort Terrace, Dublin 2, Ireland. Tel. 353 1 716 7310; fax: 353 1 716 7417; e-mail: paul.mcloughlin@ucd.i.e.

characterized by chronically increased pulmonary vascular resistance in the absence of a known cause, which, if untreated, usually leads to death within four years (Rubin, 1997; Haworth, 1998; Archer & Rich, 2000). Furthermore, successful treatment, which reduces pulmonary arterial pressure and vascular resistance, leads to long-term survival (Archer & Rich, 2000; Barst et al. 1996; Rubin, 1997). These findings demonstrate that pulmonary hypertension *per se* can shorten life.

Taken together, this evidence suggests that secondary pulmonary hypertension contributes directly to premature mortality in human lung and cardiac diseases and causes substantial added morbidity. Because of this, intensive research efforts are focused on investigating the underlying mechanisms of this condition in order to identify potential novel therapeutic interventions.

Altered vascular structure in pulmonary hypertension

Secondary pulmonary hypertension results from sustained vasoconstriction and structural alterations to the pulmonary vascular bed. The major stimuli that are responsible for these changes are chronic alveolar hypoxia, chronic inflammation and excessive shear stress (Voelkel & Tuder, 1995). Most commonly, these stimuli act together to produce increased pulmonary vascular resistance (Voelkel & Tuder, 1995). Regardless of the stimuli that cause pulmonary hypertension, the structural changes that are thought to underlie the increased vascular resistance can be broadly classified into two processes: first, remodelling of the pulmonary resistance vessels and, second, a reduction in the total number of blood vessels in the lung, sometimes termed rarefaction or pruning.

Remodelling is a process that causes thickening of the arterial walls and is thought to increase resistance by causing the vessel walls to encroach into the lumen and reduce its diameter. A prominent feature of vascular remodelling is medial thickening, a change that is of particular interest because of the ability of medial smooth muscle to alter lumen size by contraction and relaxation. In previously muscularized, more proximal pulmonary vessels, medial enlargement is caused by hypertrophy and hyperplasia of the pre-existing vascular smooth muscle cells (Meyrick & Reid, 1978, 1979a; Meyrick et al. 1980; Meyrick & Brigham, 1986). In addition, the smooth muscle cells elaborate extracellular matrix proteins, which also contribute to medial enlargement (Poiani et al. 1990). In previously non-muscular precapillary arterioles, the *de novo* development of a muscular media is observed. The smooth muscle cells, which form this new media, are derived by differentiation of intermediate cells that are present in the normal walls of these vessels (Meyrick & Reid, 1978; Jones, 1992). Smooth muscle cells are also formed by migration and differentiation of interstitial fibroblasts (Jones, 1992). A new internal elastic lamina is formed in these remodelled vessels so that their structure comes to resemble that of the resistance vessels of the systemic circulation (Fig. 1). Adventitial hypertrophy is also a prominent feature of pulmonary hypertension. Resident fibroblasts proliferate and produce matrix proteins in response to hypoxia and chronic inflammation (Meyrick & Reid, 1979b).

Reduction in the total number of blood vessels in a vascular bed will increase vascular resistance by reducing the number of paralleled pathways through that circulation. Rarefaction of vessels in the pulmonary circulation has been reported in human subjects with pulmonary hypertension (Ryland & Reid, 1975; Rabinovitch et al. 1979) and in animal models (Hislop & Reid, 1976, 1977; Meyrick & Reid, 1979a; Meyrick et al. 1980; Meyrick & Brigham, 1986; Jones & Reid, 1995; Partovian et al. 2000). This loss of blood vessels has been detected as a reduction in the ratio of the number of blood vessels to the number of alveoli in the intra-acinar (gas exchange) regions of the lung. A similar process of blood vessel loss is well recognized in systemic hypertension and contributes significantly to increased peripheral vascular resistance (Bohlen, 1989; Prewitt et al. 1982; Greene et al. 1989; Hansen-Smith et al. 1990; Schiffrin, 1992; Hernandez et al. 1999).

Hypoxia and vascular remodelling

The evidence that chronic hypoxia leads to pulmonary hypertension is extensive and has been reviewed previously (Fishman, 1985; Heath et al. 1973; Rabinovitch et al. 1979; Grover et al. 1983; Meyrick & Reid, 1983; MacNee, 1994a; Jones & Reid, 1995). In brief, exposure of humans and a wide variety of animal species to hypoxic environments causes pulmonary hypertension associated with right ventricular hypertrophy and pulmonary vascular remodelling. There is a great variation in the magnitude of this response in different species and in individuals within species (Fishman, 1985; Grover
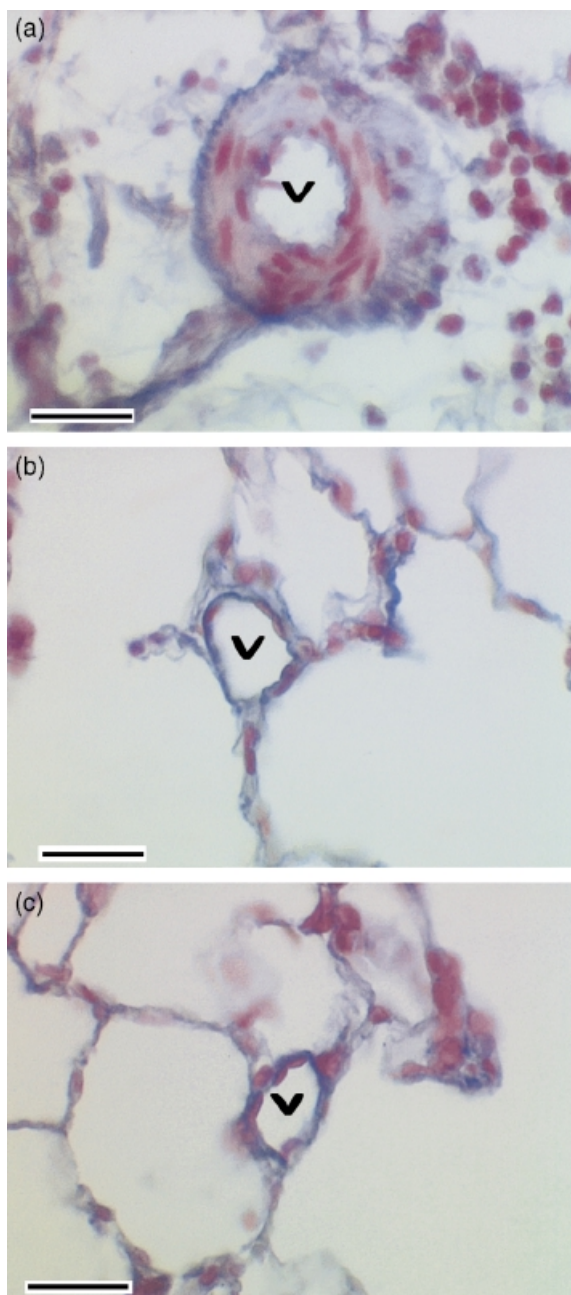
Fig. 1 Photomicrographs showing intra-acinar pulmonary blood vessels (v) from (a) a rat lung in which chronic airway infection had been induced by inoculation of *Pseudomonas aeruginosa* in agar beads, (b) lung inoculated with sterile agar beads alone, and (c) lung that had not been inoculated. Sections were stained with Miller's stain, which shows elastin as blue, and counter stained with haematoxylin to demonstrate nuclei. Note well-developed tunica media in chronically infected lungs with an internal and external elastic lamina, whereas no tunica media is seen and there is a single elastic lamina in non-inoculated lung and in lung inoculated with sterile agar beads. Scale bar = 40 μm in all panels. (Figure reproduced with permission from Hopkins et al. *Journal of Applied Physiology*, 91: 919–928, 2001.)

et al. 1983). In some susceptible individuals, exposure to high altitude leads to progressively worsening pulmonary hypertension, right ventricular failure and ultimately death, e.g. chronic mountain sickness in human children and Brisket disease in cattle (Hecht et al. 1962; Sui et al. 1988). Return to an environment with a normal partial pressure of oxygen reverses these changes in normal and susceptible individuals (Heath et al. 1973; Hislop & Reid, 1977; Grover et al. 1983; Fishman, 1985).

During the early period of hypoxic exposure, vascular resistance is elevated largely due to hypoxic vasoconstriction. However, following sustained hypoxia return to a normal pO_2 causes an immediate small fall in pulmonary arterial pressure to a value that is substantially above normal (Fried et al. 1983; Sime et al. 1971; Lockhart et al. 1976), suggesting that structural changes in the pulmonary vascular bed become a major determinant of vascular resistance. Most attention has focused on the well-described remodelling of the precapillary resistance vessels as the cause of pulmonary hypertension. These structural changes include muscularization of previously non-muscular arterioles, increased medial thickness of previously partially and completely muscular arterioles and deposition of additional matrix components, including collagen and elastin, in the vascular walls, as described above (Fishman, 1985; Rabinovitch et al. 1979; Grover et al. 1983; Stenmark & Mecham, 1997; Rabinovitch, 1999, 2001).

It is interesting to contrast this response of the pulmonary circulation with that of the systemic circulation. In rats, exposure to chronic hypoxia leads to reduced vascular response to vasoconstrictors both *in vivo* and in isolated systemic vessels (Doyle & Walker, 1991). Chronic hypoxia has also been shown to reduce systemic arterial blood pressure and increase maximal vascular conductance in normal rats (Smith & Marshall, 1999). Furthermore, when spontaneously hypertensive rats are maintained in chronic hypoxia, systemic hypertension is abrogated (Henley & Tucker, 1987). Studies of humans show that high altitude dwellers in the Andes have a lower incidence of systemic hypertension than age, socio-economic and racially matched sea-level residents (Ruiz & Penaloza, 1977). Marticorena et al. (1969) reported that long-term residence of native low-landers at high altitude leads to a reduction in systemic blood pressure Thus, the evidence from both animal and human studies demonstrates that chronic hypoxia leads to reduction in systemic blood pressure in direct

contrast to the sustained hypertension observed in the pulmonary circulation.

Chronic inflammation and vascular remodelling

The second major stimulus to the development of pulmonary hypertension is the presence of chronic inflammation in the lungs. One of the most common forms of chronic lung inflammation is chronic airway inflammation, such as that produced by cigarette smoking or chronic airways infection. Experimentally induced chronic airway inflammation in rats leads to remodelling of pulmonary resistance vessels (Cash et al. 1978; Herget et al. 1981; Graham et al. 1990; McCormack & Paterson, 1993; Cadogan et al. 1999) and the development of pulmonary hypertension (Herget et al. 1981; McCormack & Paterson, 1993). The vascular remodelling induced by chronic airway inflammation is structurally similar to that observed in chronic hypoxic pulmonary hypertension (Fig. 1). Most notably it causes marked medial hypertrophy suggesting that encroachment of the vessel wall on the vascular lumen contributes to increased resistance, in a manner similar to that described in chronic hypoxia. Airway inflammation caused by long-term inhalation of cigarette smoke also causes pulmonary vascular remodelling in rats (Sekhon et al. 1994). In human subjects who are cigarette smokers, the pulmonary vascular remodelling that is observed is disproportionate to the degree of arterial hypoxaemia, suggesting that it results in large part from chronic airway inflammation. Thus structural abnormalities of pulmonary vessels are observed in asymptomatic smokers (Hale et al. 1984; Peinado et al. 1999) and medial thickness in patients with COPD is unrelated to arterial pO_2 (Barbera et al. 1994). Furthermore, vascular remodelling is observed in COPD even when arterial pO_2 is greater than 8 kPa (Wright et al. 1983; Magee et al. 1988; Peinado et al. 1999), whereas in normal individuals chronic hypoxia in isolation does not cause pulmonary hypertension unless arterial pO_2 is less than 8 kPa (Grover et al. 1983). Collectively, these data suggest that chronic airway inflammation leads to pulmonary vascular remodelling and contributes to the development of pulmonary hypertension by mechanisms that are independent of hypoxia.

Inflammation of the pulmonary vasculature and diffuse interstitial inflammatory disease can also lead to remodelling and pulmonary hypertension. In animals, interventions that lead to vascular inflammation, including monocrotaline injection, Crotalaria ingestion and repeated injection of endotoxin, cause remodelling of resistance vessels and pulmonary hypertension (Kay et al. 1967; Heath, 1969; Hislop & Reid, 1974; Meyrick et al. 1980; Meyrick & Reid, 1982; Meyrick & Brigham, 1986; Meyrick & Perkett, 1989; Reindel et al. 1990). Diffuse interstitial inflammatory disease of the lung can also cause pulmonary vascular remodelling and hypertension in animal models (Bishop et al. 1990; Lippmann, 1977; Michel et al. 1988; Champion et al. 1999) and in human disease conditions, including adult respiratory distress syndrome (Katz et al. 1984; Zapol & Snider, 1977; Snow et al. 1982; Tomashefski et al. 1983; Leeman, 1999; Wyncoll & Evans, 1999), cryptogenic fibrosing alveolitis (Giaid et al. 1993) and systemic autoimmune diseases (Gurubhagavatula & Palevsky, 1997).

Shear stress and vascular remodelling

It is well recognized that abnormal haemodynamic shear stress is a potent stimulus to vascular remodelling and the development of pulmonary hypertension. Congenital heart diseases that cause left to right shunt, including patent ductus arteriosus, atrial and ventricular septal defects, chronically augment vascular shear stress in the lung. If left uncorrected, these conditions frequently lead to pulmonary vascular remodelling, increased pulmonary vascular resistance, pulmonary hypertension, shunt reversal, right heart failure and death (Heath & Edwards, 1958; Rabinovitch et al. 1981; Meyrick & Reid, 1983). In animal models, it has been shown that increasing pulmonary blood flow, by the creation of left to right shunts, leads to vascular remodelling, increased resistance and pulmonary hypertension (Bousamra et al. 2000; Friedli et al. 1975; Rendas et al. 1979; Everett et al. 1998).

Increased shear stress may be a significant contributory stimulus to the development of pulmonary hypertension in circumstances in which hypoxia and inflammation are the primary initiating stimulus. In a uniform rigid cylinder, shear stress (τ) at the interface between fluid and vessel wall is given by

$$\tau = 4Q\eta/\pi r^3$$

where Q is flow rate, η is the viscosity of the fluid and r is the lumen radius. Since the pulmonary circulation

must normally accommodate the whole cardiac output, total pulmonary blood flow must remain constant following luminal narrowing whether due to vasoconstriction or remodelling of the vessel wall. This means that shear stress at the vessel wall will increase markedly as it is inversely related to the third power of the radius. The importance of this contribution is clearly illustrated by the work of Rabinovitch et al. (1983). They reduced the vascular shear stress in hypoxic lungs by constricting the left main branch of the pulmonary artery with a 'band' and demonstrated marked reduction of medial hypertrophy and the degree of extension of vascular smooth muscle into normally non-muscular vessels (Rabinovitch et al. 1983). Conversely, increased shear stress produced by pneumonectomy worsens monocrotaline-induced pulmonary hypertension in rats (Okada et al. 1997). In contrast to the pulmonary vascular response, long-term increases in blood flow in systemic vessels causes increased luminal diameter and reduced resistance (Langille & O'Donnell, 1986; Gibbons & Dzau, 1994).

Altered endothelial function and vascular remodelling

The pulmonary endothelial cell produces a number of important mediators that modulate pulmonary vascular smooth muscle tone and have important influences on vascular smooth muscle cell proliferation and wall remodelling. Amongst the more important vasodilators produced by the endothelium are nitric oxide (NO) and prostacyclin (PGI$_2$), which are also potent inhibitors of wall remodelling. The endothelium-derived vasoconstrictor endothelin is a key mediator of resistance vessel wall remodelling (MacLean, 1999). Other mediators that also stimulate smooth muscle proliferation include prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) and platelet-derived growth factor-*B*. Furthermore, the endothelium has a key role in the uptake and catabolism of mediators that can promote remodelling, such as serotonin (MacLean, 1999). Alteration in endothelial production or catabolism of vasoactive mediators can play a central role in vascular remodelling.

Considerable attention has been focused on the role of altered NO production in the development of hypoxia-induced pulmonary hypertension. In chronically hypoxic rat lungs, impaired endothelium-dependent relaxation has been demonstrated (Adnot et al. 1991; Maruyama & Maruyama, 1994) and a similar impairment has been demonstrated in patients with chronic hypoxic lung disease (Dinh-Xuan et al. 1991), suggesting that impaired endothelium-dependent relaxation may contribute to the increased pulmonary vascular resistance of chronic hypoxia. Expression of endothelial nitric oxide synthase is increased in experimental hypoxic pulmonary hypertension (Le Cras et al. 1996, 1998; Tyler et al. 1999) but, despite this, NO production is reduced (Le Cras & McMurtry, 2001). Hypoxic vascular remodelling and pulmonary arterial pressures are increased in endothelial nitric oxide synthase (eNOS) knockout mice (Steudel et al. 1998; Fagan et al. 1999b) while chronic administration of inhaled NO attenuates hypoxic vascular remodelling (Kouyoumdjian et al. 1994; Roberts et al. 1995). Stimulation of endogenous NO production by dietary supplementation with L-arginine also protects against hypoxia-induced vascular remodelling (Mitani et al. 1997; Fagan et al. 1999a). Taken together, these data suggest that impaired endothelial production of NO may contribute to the development of chronic hypoxic pulmonary hypertension.

The potential role of altered endothelial NO synthase expression and activity in chronic inflammatory lung disease has received much less attention. We have found that chronic airway infection in rats leads to reduced eNOS expression in the pulmonary vasculature (Fig. 2) and that these chronically infected lungs are hypersensitive to the vasodilator effects of NO (Cadogan et al. 1999). Furthermore, isolated pulmonary arteries from these lungs demonstrate impaired endothelium-dependent relaxation (Fig. 3). In human subjects with chronic inflammatory airway disease secondary to cigarette smoking, who are not hypoxic, pulmonary vascular endothelium-dependent relaxation is also impaired and eNOS expression is reduced (Peinado et al. 1998; Barbera et al. 2001). Taken together, these data demonstrate that chronic airway inflammation leads to reduced eNOS expression and activity and suggest that reduced NO production may contribute to the vascular remodelling observed in these conditions.

In other forms of chronic lung inflammation, the behaviour of endothelial NO synthase is controversial. Tyler et al. (1999) reported that in monocrotaline-induced pulmonary hypertension in rats, eNOS expression is reduced. In support of these findings, lipopolysacharide and pro-inflammatory cytokines reduced endothelial NO expression and endothelium-dependent relaxation in pulmonary arteries in organ
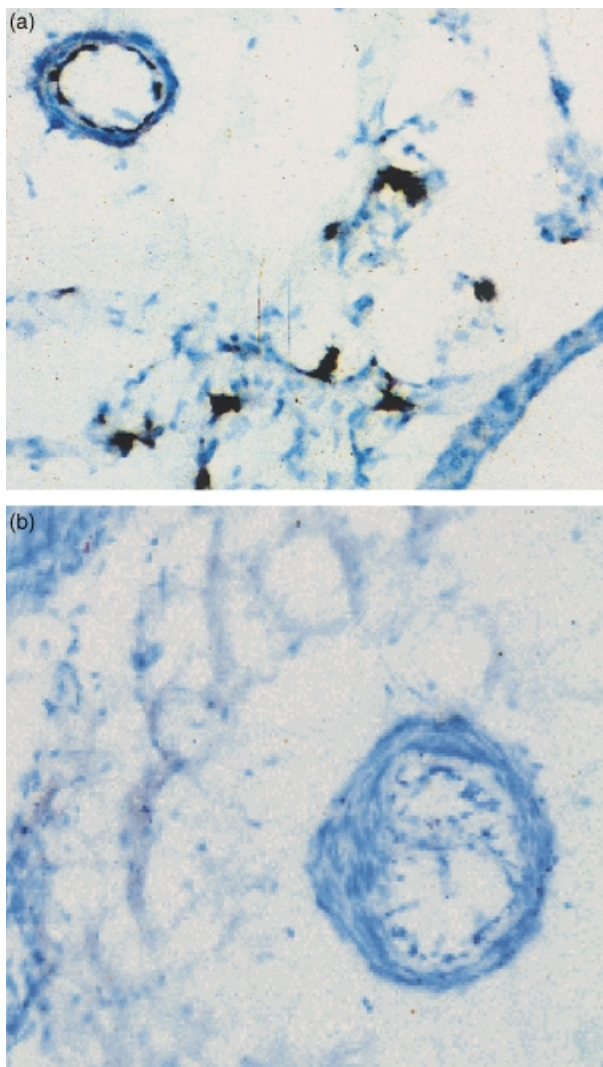
Fig. 2 Immunoperoxidase staining of endothelial NOS in control lungs, and in lung with chronic airway infection following inoculation with *Pseudomonas aeruginosa* in agar beads. (a) Photomicrograph showing staining of eNOS in endothelium of blood vessel of control lung and patchy staining in alveolar walls (blue–black colour). (b) Photomicrograph showing absence of staining of eNOS in endothelium of blood vessel of *Pseudomonas*-inoculated lung. All original magnifications ×380. (Figure reproduced with permission from Cadogan et al. *American Journal of Physiology*, 277: L616–627, 1999.)

culture (Ziesche et al. 1996). However, others have reported that monocrotaline causes increased eNOS expression and increased endothelium-dependent relaxation in rat lungs (Resta et al. 1997). It has also been reported that hyperoxia-induced lung inflammation reduced endothelial NO synthase expression (Steudel et al. 1999). Thus, the role of endothelial NO synthase

in these conditions is not as clear as its role in chronic airway inflammation and requires further investigation.

The position of the vascular endothelium, immediately adjacent to the blood vessel lumen, means that it must be the site at which alterations in shear stress are detected and transduced. Acute increases in shear stress can immediately activate shear stress-dependent channels, elevate intracellular inositol 1,4,5-triphosphate and diacylglycerol, activate the mitogen-activated and stress-activated protein kinase pathways and cause cytoskeletal reorganization in endothelial cells (Ballermann et al. 1998; Fisher et al. 2001). Change in shear stress also alters production of vasoactive mediators, including NO, prostacyclin and endothelin in cultured endothelial cells (Frangos et al. 1985; Grabowski et al. 1985; Buga et al. 1991; Malek & Izumo, 1992; Malek et al. 1993; Korenaga et al. 1994). Shear stress response elements have been identified in the sequence of genes encoding important vascular growth factors (Ballermann et al. 1998). Furthermore, there is evidence that increased shear stress alters endothelial cell membrane potential and increases NO production in the isolated intact lung and in the whole organism (Nakache & Gaub, 1988; Hakim, 1994; Storme et al. 1999; Tworetzky et al. 2000; Ogasa et al. 2001).

While these data provide good evidence that shear stress can regulate endothelial cell function, they largely demonstrate changes, such as increased NO and PGI_2 synthesis, that might be expected to minimize pulmonary vascular resistance, inhibit wall remodelling and prevent the development of pulmonary hypertension. However, prolonged excessive shear stress can lead to endothelial cell damage, reduce the production of vasodilator agents that inhibit wall remodelling, promote the production of mediators that lead to vascular wall remodelling, and increase the expression of adhesion molecules and cytokines that promote an inflammatory response in the vascular wall (Esterly et al. 1968; Reidy & Bowyer, 1977; Zhu et al. 1997; Nomura et al. 2001). Chronic increase in shear stress caused by left to right shunting leads to impaired endothelium-dependent relaxation in pulmonary arteries (Fullerton et al. 1996). In support of these findings, endothelium-dependent arterial relaxation is attenuated and circulating endothelin and thromboxane concentrations are increased in patients with Eisenmengers syndrome (Dinh Xuan et al. 1990; Yoshibayashi et al. 1991; Cacoub et al. 1993; Celermajer et al. 1993; Tweddell et al. 1994). Thus, chronic excessive shear stress may promote the
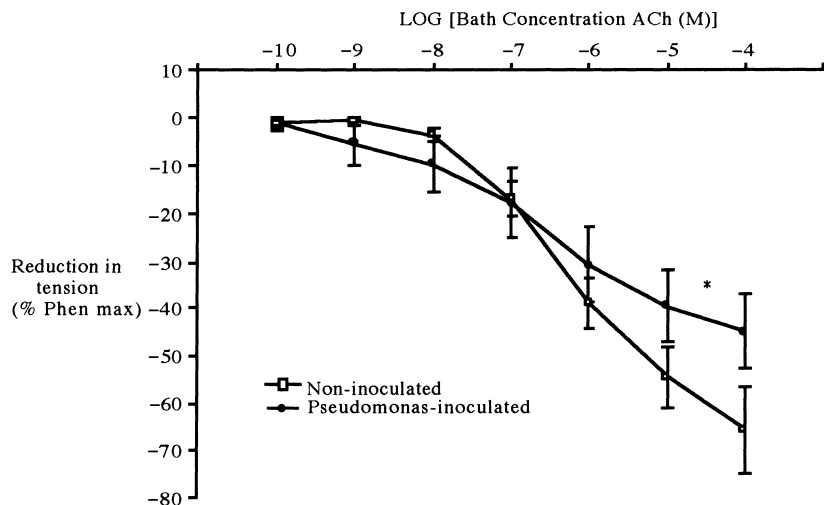
LOG [Bath Concentration ACh (M)]

Fig. 3 Mean (± SEM) reduction in wall tension in response to increasing concentrations of acetylcholine (ACh) in preconstricted pulmonary arterial rings isolated from control (*n* = 9) and chronically infected (*n* = 6) rats. Asterisk indicates significant difference between the two groups (*P* < 0.05, ANOVA).

Reduction in tension (% Phen max)

Non-inoculated
Pseudomonas-inoculated

Vascular rarefaction in pulmonary hypertension

Intriguingly, in the pulmonary circulation it is reported that chronic hypoxia leads to a reduction in the pulmonary vascular bed in adult animals and man, rather than stimulating angiogenesis. In animal models of chronic hypoxic lung disease, the ratio of pulmonary arterioles to pulmonary alveoli is reduced, suggesting loss of these blood vessels (Hislop & Reid, 1976; Rabinovitch et al. 1979; Jones & Reid, 1995; Partovian et al. 2000). No remnants of these lost vessels could be found (Hislop & Reid, 1976; Rabinovitch et al. 1979) and on re-introduction of animals to normoxic environments, some but not all of these 'lost' vessels were restored, suggesting a permanent reduction in the pulmonary vascular bed (Hislop & Reid, 1977). Additionally, in human subjects suffering from congenital heart disease and pulmonary hypertension, a similar alteration in alveolar to arterial ratio has been observed in the lungs (Rabinovitch et al. 1979). Thus, it has been postulated that rarefaction is an important component of the structural basis for hypoxic pulmonary hypertension.

This hypoxia-induced loss of blood vessels in the pulmonary circulations is in stark contrast to the marked angiogenesis observed in the systemic circulation in hypoxia (LaManna et al. 1992; Smith, 1997; Deveci et al. 1998; Marti & Risau, 1999; Smith & Marshall, 1999;

Griffioen & Molema, 2000) and has led to the suggestion that the pulmonary circulation in the adult is incapable of angiogenesis. Sobin et al. (1983), using transmission electronic microscopy specifically sought, but could not find, evidence of new capillary formation in chronically hypoxic rat lungs. Furthermore, adenovirally mediated over-expression of the specific endothelial cell mitogen vascular endothelial growth factor (VEGF) in the lung did not alter the ratio of vessels to alveoli (Partovian et al. 2000), in contrast to its potent angiogenic effects in systemic vascular beds.

Rarefaction is also thought to contribute significantly to the development of hypertension in chronic inflammatory conditions of the lung. Pulmonary hypertension produced by crotalaria ingestion led to a reduction in the ratio of arterioles to alveoli in the rat (Meyrick & Reid, 1979a; Meyrick et al. 1980), while repeated endotoxin infusion in sheep led to a similar reduction (Meyrick & Brigham, 1986). In human subjects suffering from cystic fibrosis-induced chronic airway infection, it has also been reported that the arterial to alveoli ratio is reduced (Ryland & Reid, 1975). These findings have been interpreted to indicate a loss of blood vessels, a response that is again markedly different from the response of the systemic vasculature in chronic inflammation, where angiogenesis is prominent and plays a key role in the disease process (Griffioen & Molema, 2000; Sullivan et al. 2000). Schraufnagel and colleagues used scanning electron microscopy to seek evidence of angiogenesis in moncrotaline-induced inflammation and pulmonary hypertension in rats. While they reported extensive new vessel formation in the peribronchial region and

on the pleural surface arising from systemic vessels, they could find no evidence of new vessels in the alveolar walls, again suggesting that angiogenesis cannot occur in the adult pulmonary circulation (Schraufnagel et al. 1986; Schraufnagel, 1990).

However, not all investigators agree that rarefaction occurs in pulmonary hypertension. Several studies of pulmonary hypertension in the rat report that the number of blood vessels in the lung was unaltered following chronic hypoxia (Meyrick & Reid, 1979b; Emery et al. 1981; Kay et al. 1982; Finlay et al. 1986) and following chronic inflammatory lung disease (Kay et al. 1982).

Pulmonary vascular remodelling without pulmonary hypertension

A close association between remodelling of pulmonary resistance vessels and the development of hypertension is not invariably observed. We and others have reported that, in chronically infected lungs in rats, pulmonary hypertension did not occur and right ventricular hypertrophy was not observed, despite the development of thickened walls in pulmonary vessels (Graham, 1990; Cadogan et al. 1999). In a similar observation in human subjects with chronic obstructive pulmonary disease, Wright et al. (1983) have reported marked thickening of the walls of pulmonary arterial vessels in the absence of pulmonary hypertension.

One possible explanation of these findings is that wall thickening occurred in an outward direction so that the size of the vascular lumen was not compromised. This phenomenon, termed compensatory enlargement, is well recognized in the systemic circulation (Glagov et al. 1993) but not in the pulmonary vasculature. We tested this hypothesis in chronically infected rat lungs produced using a well-described method of intratracheal inoculation of *Pseudomonas aeruginosa* organisms that have been incorporated into agar beads. To obtain estimates of the absolute dimensions of maximally dilated, intra-acinar resistance vessels, we used quantitative stereological techniques (Hopkins et al. 2001). These demonstrated that chronic infection did not cause a reduction in mean lumen radius although wall thickness was increased. Thus compensatory hypertrophy had occurred, which may account, at least in part, for the absence of increased pulmonary vascular resistance in such chronically infected lungs (Cadogan et al. 1999).

A second possible mechanism that might have prevented the development of pulmonary hypertension is that angiogenesis had occurred in the pulmonary circulation in response to chronic airway infection. To test this hypothesis, we determined total vessel length in the gas exchange (intra-acinar) region of the lung, excluding capillaries, and the number of vascular branch points in those vessels in chronically infected rat lungs. These vessels included the arterioles accompanying respiratory bronchioles, those running in the alveolar walls and all the intra-acinar venules; the diameter of such vessels is approximately 35–45 μm. We observed a substantial increase in total vessel length (Fig. 4) and in the total number of branch-points (Fig. 5) in the pulmonary circulation, suggesting that angiogenesis had occurred (Hopkins et al. 2001). In particular, the evidence of increased branching suggests that angiogenesis led to formation of new arteriolar and venular vessels in addition to the pre-existing pathways. In the systemic circulation, capillary angiogenesis is accompanied by formation of new arterial vessels, so-called arteriogenesis (Carmeliet, 2000). Our evidence
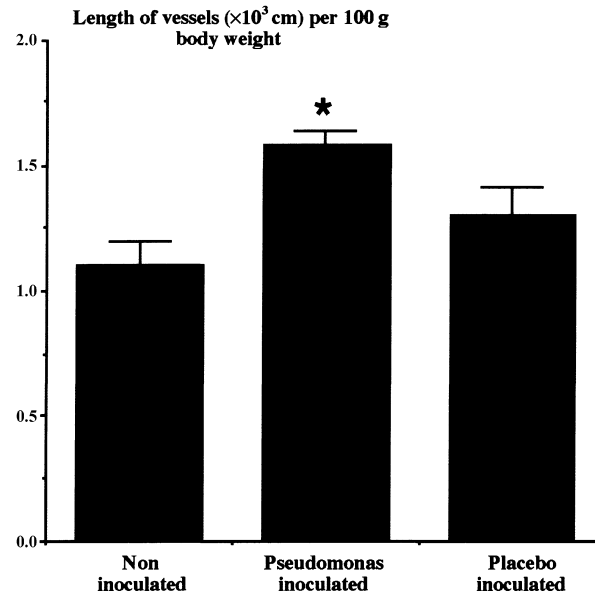

Fig. 4 The mean (± SEM) total length of intra-acinar pulmonary blood vessels in the left lungs of normal control lungs (*n* = 8), lungs with chronic airway infection following inoculation with *Pseudomonas aeruginosa* in agar beads (*n* = 10), and lungs inoculated with sterile agar beads alone (*n* = 6). Asterisk indicates significant difference from non-inoculated and placebo-inoculated groups (*P* < 0.05, ANOVA). (Figure reproduced with permission from Hopkins et al. *Journal of Applied Physiology*, 91: 919–928, 2001.)

No. of branch-points ($\times 10^5$) per 100 g body weight

Fig. 5 The mean (± SEM) number of branch-points of intra-acinar pulmonary blood vessels in the left lungs of control rats (*n* = 8), lungs with chronic airway infection following inoculation with *Pseudomonas aeruginosa* in agar beads (*n* = 10), and lungs inoculated with sterile agar beads alone (*n* = 6). Asterisk indicates significant difference from non-inoculated and placebo-inoculated groups ($P < 0.05$, ANOVA). (Figure reproduced with permission from Hopkins et al. *Journal of Applied Physiology*, 91: 919–928, 2001.)
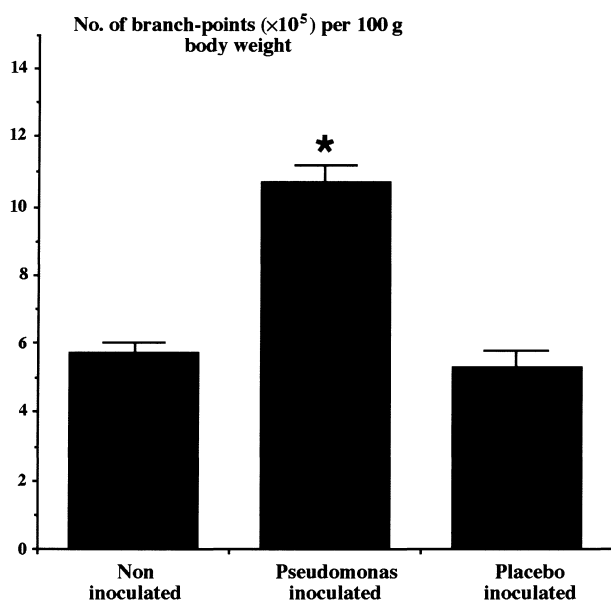
suggests that a similar behaviour can occur in the pulmonary circulation and may contribute to the maintenance of a normal low pulmonary vascular resistance.

More recent evidence supports our findings of new vessel formation in the pulmonary circulation. Hsia et al. (1994) have shown that in adult dogs following right pneumonectomy there is proliferation of new alveolar septa and alveolar capillaries in the residual left lung. Other reports demonstrate that endothelial cells of the pulmonary circulation can replicate in a variety of circumstances. Following pneumonectomy, the total number of endothelial cells in the remaining lung increases significantly in rats (Thet & Law, 1984). Sekhon et al. (1994) have shown that airway inflammation caused by cigarette smoke stimulated endothelial cell proliferation in the lung as demonstrated by labelling of endothelial cell nuclei with bromodeoxyuridine. Endothelial cell proliferation in the pulmonary circulation has also been observed during the development of monocrotaline-induced pulmonary hypertension in rats (Meyrick & Reid, 1982). Schraufnagel et al. (1997) have reported evidence of pulmonary angiogensis in

rat lung following the induction of biliary cirrhosis. Studies in humans with lung disease have also provided evidence of angiogenesis in the pulmonary circulation. Schraufnagel et al. (1996), using scanning electron microscopic techniques, found evidence of new vessel formation in the lungs of a patient with pulmonary veno-occlusive disease. In addition, it has been demonstrated radiologically that the majority of metastatic tumours in the lung receive their blood supply either exclusively (46% of metastases) or predominantly (36% of metastases) from the pulmonary circulation (Milne & Zerhouni, 1987). Taken together, these reports suggest that pulmonary endothelial cells can proliferate and form new vessels in the adult lung.

The role played by vascular remodelling in the development of pulmonary hypertension in chronic lung infection is complex. Our data suggest that, under certain conditions, wall remodelling may be not result in reduction of the mean lumen diameter (Hopkins et al. 2001). Furthermore, the addition of parallel vascular pathways through the lungs may serve to oppose any increase in total pulmonary vascular resistance that might result from reductions in vascular lumen diameter in some vessels. If these two processes occur together, vascular resistance may not increase despite thickening of the vessel wall. However, in other circumstances, such as more persistent or more severe lung disease, remodelling of the vascular wall, including the wall of newly formed vessels, might lead to significant reductions in mean lumen diameter. Under such conditions, the increase in resistance caused by narrowed vessels might outweigh the extent to which new vessel formation could reduce resistance and the net effect might then become an increase in total pulmonary vascular resistance.

Quantification of vascular structure in the lung

It is clear that there are directly conflicting reports regarding rarefaction and angiogenesis in the pulmonary circulation in a variety of circumstances. The possibility that these apparent discrepancies might arise because of methodological problems is worthy of consideration. The normal lung is a highly vascular structure with a vast vascular network, which is required to provide adequate surface area for gas exchange. Given the extent of the normal vasculature when compared with other organs, it is extremely difficult to detect newly formed blood vessels in the lung. This

problem has been reviewed and commented upon previously (Mooi & Wagenvoort, 1983; Schraufnagel et al. 1996). In view of this, it is interesting to note that those studies that have clearly demonstrated angiogenesis in the pulmonary circulation have used the techniques of quantitative stereology (Thet & Law, 1984; Hsia et al. 1994; Hopkins et al. 2001). Stereology allows statistical inferences about the three-dimensional structural parameters of objects based on two-dimensional information such as that provided by histological images. In addition, by using strict random sampling protocols, stereological approaches ensure that the data obtained are unbiased and representative of the whole lung. A further feature of stereology is that it carefully defines and quantifies a reference volume within which a particular parameter is to be measured. This step allows absolute quantities to be measured, e.g. the total length of intra-acinar resistance vessels in the lung.

The task of identifying angiogenesis provides an example of the importance of these issues. Angiogenesis in the lung would increase the total length of vessels in the lung. If the lung increased in volume during the course of our experimental intervention (e.g. hypoxia), but the length of vessel per unit volume of lung were to remain constant, then the number of vessel transections per unit area of cross-section would remain constant, i.e. the number of vessels observed per microscopic field of view would be unaltered. A conventional approach such as this would conclude that no angiogenesis had taken place. However, a stereological approach, by recognizing the altered reference volume (total lung volume), would identify the increase in total vessel length (Bolender et al. 1993; Gundersen et al. 1988a,b). Use of inappropriate or undefined reference spaces can lead to significant errors. For example, an increased ratio of wall thickness to lumen diameter in a vessel might arise either because lumen diameter had reduced, or because wall thickness had increased without change in the lumen size. This implies that changes in this ratio do not allow any direct inference to be made about changes in vascular resistance.

Conclusions

Standard teaching suggests that secondary pulmonary hypertension results, in large part, from two major structural alterations in the pulmonary circulation. The first is thickening of the walls of resistance vessels that causes a reduction of the vascular lumen. The second structural alteration is a reduction in the total number of pulmonary vessels or rarefaction. Recent evidence demonstrates that vascular wall thickening can occur without structural alteration in lumen diameter. Furthermore, angiogenesis can occur in the pulmonary circulation and may serve to prevent or attenuate increased vascular resistance in lung disease. Quantitative stereological approaches are required to allow determination of the three-dimensional parameters that characterize this vascular bed. In view of these more recent data, the potential role of structural alterations in the pulmonary vasculature in the development of pulmonary hypertension should be carefully reconsidered.

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