

Point:Counterpoint: Chronic Hypoxia-Induced Pulmonary Hypertension Does / Does Not Lead to Loss of Pulmonary Vasculature

Point: Chronic Hypoxia-Induced Pulmonary Hypertension Does Lead to Loss of Pulmonary Vasculature

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This debate focuses on whether and to what extent, loss of small pre-capillary arteries is associated with the elevation in pulmonary artery pressure and resistance that accompanies chronic hypoxia-induced pulmonary hypertension. The issue has become the focus of much debate for a number of reasons. Papers from McLoughlin and his group have provided conclusive evidence that there is considerable angiogenesis associated with hypoxia induced pulmonary hypertension (6) (5). In addition, rho kinase inhibitors normalize pulmonary pressure in chronic hypoxia presumably by a pure vasodilatory action (7).

The fact that chronic hypoxia can stimulate neoangiogenesis does not negate the fact that chronic hypoxia can also result in loss of **pre-capillary** arteries. It is now generally accepted that loss of distal arteries in the clinical and experimental setting of pulmonary

hypertension is the result of apoptosis of both endothelial cells and pericytes. This has been well documented in the monocrotaline model of pulmonary hypertension (24). Using this model, Stewart and colleagues used fluorescent microbeads to document both breaks and abrupt termination of pre-capillary vessels, a feature associated with severe pulmonary hypertension. The authors went on to show how infusion of endothelial progenitor cells that synthesize endothelial nitric oxide synthase can reverse the pulmonary hypertension in association with rebuilding the distal vasculature that had been interrupted. In the clinical setting, a fulminant form of neo-angiogenesis is associated with end-stage primary and secondary forms of pulmonary hypertension (4). This does not produce an effective increase in pulmonary flow through conduit channels, but rather represents structurally and functionally dysregulated vessels that resemble tumor vessels (4). These abnormal angiogenic channels are thought to arise because of the proliferation of 'apoptosis resistant' local endothelial cells or from the seeding of the lumen with circulating progenitor endothelial cells (20).

A variety of pulmonary hypertension inducing stimuli used in the experimental setting or related to clinical disease are associated with histological evidence of loss of distal pulmonary arteries, assessed either by PECAM staining of endothelial cells or by barium gelatin infusion of the lungs. Experimental stimuli in addition to chronic hypoxia that induce loss of vessels in association with pulmonary hypertension have included injection of the toxin monocrotaline (23), monocrotaline and pneumonectomy (17), exposure to chronic hyperoxia (22) (11), and creation of aortopulmonary shunts (19). In the clinical setting conditions associated with pulmonary hypertension and loss of arteries include congenital heart defects (18), lung developmental abnormalities(2) and idiopathic pulmonary hypertension (16). Improved resolution of current imaging techniques might, in the future, detect loss of pre-capillary arteries in association with pulmonary hypertension in the clinical setting. Loss of arteries reflecting loss of vascular reserve might be reflected in heightened pulmonary artery pressure and resistance with exercise or changes in pulmonary vascular impedance, which most accurately represents the total right ventricular afterload, including both steady and pulsatile right ventricular work requirements(21).

Unfortunately, only the minority of clinical or experimental studies of chronic hypoxia induced pulmonary hypertension report whether there is loss of pre-capillary vessels. One of the ways in which the number of pre-capillary vessels is precisely determined is by barium gelatin infusion. This barium permits radiographic assessment of the circulation and the gelatin does not allow the contrast to pass into the capillary bed. Thus it is easy to count barium filled peripheral arteries at alveolar duct and wall level on microscopic examination of lung tissue sections. Usually the number of pre-capillary arteries is recorded as the number of arteries relative to 100 alveoli or per mm^2 . Calculating arteries per 100 alveoli makes the assumption that the alveoli are normal in number and calculating per mm^2 makes the assumption that the number and size of alveoli are normal.

In addition to distensibility analysis(21), microCT, (8) can be used to support loss of filling of distal arteries following exposure to chronic hypoxia using the barium gelatin method or perfluorooctyl bromide (PFOB) as an intravascular X-Ray contrast agent. Using this method, isolated lungs harvested from mice are rinsed of blood and perfused with a physiologic salt solution containing 5% bovine serum albumin while being ventilated with a 15% O₂, 6% CO₂, balance nitrogen mixture. Papavarine is added to the perfusate and recirculated prior to imaging to remove residual muscle tone. The perfusate is then replaced with PFOB which is trapped at the pre-capillary level and only fills the arterial vasculature. High resolution planar images are taken at an airway pressure of 6 mmHg for a range of intravascular pressures (between 6 and 17 mmHg).

Alternatively, one can use PECAM staining of endothelium to landmark arteries accompanying alveolar ducts and alveolar walls down to a pre-capillary size of 20 μm and to express those arteries relative to alveoli. This technique runs the risk of including venules in the assessment, but venules can generally be differentiated from arterioles since they are surrounded by loose connective tissue, they run in connective tissue septae in the lung and they often have prominent branches. One of our recent studies has shown excellent correlation between the barium gelatin and PECAM immunostaining techniques

to assess pre-capillary arteries (15). Using these techniques, a reduction in the number of arteries relative to alveoli has been documented in rodents with chronic hypoxia induced pulmonary hypertension in our laboratory (13, 14) and in that of others (12) (3).

Studies using transgenic mice have taught us that there can be tremendous discrepancies between the hemodynamic assessments of pulmonary artery pressure and resistance and the remodeling response of the distal circulation in terms of muscularization of distal vessels, hypertrophy of more proximal arteries and loss of arteries relative to alveoli. For example, in mice with overexpression of S100A4/Mts1 a baseline increase in pulmonary artery pressure is further augmented by chronic hypoxia relative to controls, but we were unable to identify an increase in the muscularization of distal vessels, in the loss of distal vessels landmarked as pre-capillary or in the wall thickness of normally muscular arteries that would explain this discrepancy. We did, however, document marked changes in the elastin matrix(14, 15). that might have influence the distensibility characteristics in the pulmonary circulation(9, 10).

We have observed that patchy deletion of BMPRIa in smooth muscle cells, and others have reported that haploinsufficiency of BMPRII, results in equivalent pulmonary artery pressures found in wild type mice exposed to chronic hypoxia, but less structural remodeling of the distal circulation (1). New studies are considering the contribution of the extracellular matrix, where an increase in aberrantly distributed elastin and collagen could be associated with reduced compliance (9, 10) and thus increased impedance even when resistance is unchanged.

So, the following might summarize what we believe is the basis for the difference of opinion regarding hypoxia-mediated loss of distal arteries.

1. Pulmonary hypertension is reversed with rho kinase inhibitors. *However, this does not negate the loss of vessels, only that under basal conditions, the loss of distal arteries does not impair resting steady hemodynamics of the pulmonary circulation.*

2. Stereology shows increased angiogenesis and increased capillary length in hypoxia. *This is well documented but does not tell us about aberrant or 'lost' connections between the pre-capillary and capillary circulation.*

3. Loss of arteries is not always seen in hypoxia. *Certain methodologies like barium-gelatin injection are designed to facilitate assessment of the distal pre-capillary pulmonary vasculature, but this method can be technically challenging particularly in murine lungs. However, PECAM immunostaining validate the loss of vessels when used in the same series of experiments and this should also be possible with microCT with PFOB.*

4. Certain murine species may not show loss of arteries. *This may be true despite the fact that other features of remodeling of the pulmonary circulation are observed. Also, we need to look beyond the vascular changes we have typically observed in chronic hypoxia induced pulmonary hypertension into those that affect the total right ventricular afterload, including both steady and pulsatile right ventricular work.*

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Counterpoint: Chronic hypoxia-induced pulmonary hypertension does not lead to loss of pulmonary vasculature

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Exposure of native sea-level dwellers to chronic hypoxia due to migration to high altitude leads to the development of increased pulmonary vascular resistance causing pulmonary hypertension. In some susceptible individuals this progressively worsens causing right ventricular failure and ultimately death. The increased pulmonary vascular resistance has previously been attributed to two structural changes in the pulmonary vascular bed: inward remodelling of the pulmonary arterioles leading to narrowing of the lumen and loss of pulmonary blood vessels, i.e., vascular rarefaction. It is the latter change that is the focus of this debate. While many groups have reported loss of vessels during exposure of rodents to chronic hypoxia (10, 11, 15, 16, 18, 25, 26, 29), several other groups could find no such loss (3, 5, 6, 14, 17, 19) and more recently some have provided evidence of new vessel formation in the pulmonary vasculature in response to sustained hypoxia (1, 12, 13, 22).

The evidence that hypoxia-induced pulmonary hypertension causes loss of vessels from the pulmonary circulation may be categorized under three broad headings: histological data, angiographic data and functional data demonstrating the “fixed” nature of the increased pulmonary vascular resistance; we will consider each of these in turn. When using histological techniques the ability to detect loss of blood vessels in any tissue depends critically on the method used to identify them. A method commonly used in the pulmonary circulation has been to isolate the lung post mortem and to then perfuse a barium-gelatin mixture into the pulmonary artery at high pressure. As this mixture cools it solidifies within the arterial side of the circulation so that arterial vessels may be easily identified microscopically. Although intuitively attractive this approach is fraught with difficulties as the distance to which the barium-gelatin mix penetrates is a complex

function of the vascular resistance, the rate of cooling of the mixture during infusion and its viscosity. Increased resistance to flow of the mix, whether due to vasoconstriction or structural alterations of the vessels, will reduce the number of vessels filled and identified (6). Indeed it has been reported that, if the perfusion pressure used in chronically hypoxic lungs is elevated to compensate for their elevated vascular resistance, no evidence of vascular loss can be found (6). A further problem with the barium-gelatin method is that it only permits identification of arterial vessels while excluding the capillary and venous beds. These latter two segments are major sites of new vessel formation in the systemic circulation suggesting that their exclusion when assessing the pulmonary circulation may be misleading (23). Thus, vascular density data obtained using barium-gelatin injection must be interpreted with caution. Alternative ways of identifying pulmonary vessels include the use of elastin stains, immunostaining with endothelial cell markers or the use of resin embedding permitting thin sectioning and reliable morphometric identification. Interestingly, results obtained using these methods frequently do not show vessel loss (3, 12-14, 22).

Once tissue sections have been obtained, the extent of the vascular bed must then be quantified. Obtaining reliable 3-dimensional data using 2-dimensional sections is not as straightforward as might at first appear (2, 9, 28). A commonly used approach has been to take a single transverse section of the left lung at the level of the hilum, to count the number of vessels and alveoli observed and to compute the ratio of these two or alternatively to compute the number of vessels per unit area of the section; the resultant value has been loosely called "vessel density". The first problem with this approach is that the section is not representative of the lung as a whole. A second problem is that the

number of intersections between a section and blood vessels is not a unique function of vessel length but also depends on the relative orientation of the plane of section and the vessels (2, 9, 28). Thus, a single section (or multiple sections of a single fixed orientation) does not allow reliable estimation of vessel length. Perhaps most importantly, this method is not sensitive to the increases in lung volume caused by hypoxia (4, 10, 12-14, 24, 25). For example vessel density as described above could remain unchanged if the lung enlarged and grew new vessels proportionately.

Use of stereological techniques allows unbiased quantitative analysis of the three-dimensional structure of the lung vasculature. Important aspects of the method are the use of systematic random sampling from throughout the lung, to ensure that the data obtained are representative of the whole organ, and quantification of changes in lung volume. This allows absolute quantities to be measured even in circumstances where the total lung volume changes (2, 9, 28). Use of stereology demonstrates new vessel formation in the pulmonary circulation in response to chronic hypoxia, not vessel loss (1, 12, 13). This finding is supported by previous work in which the pulmonary vascular volume, estimated by filling it with a solution containing tritiated albumin, was found to be increased in chronically hypoxic lungs (5).

Angiographic techniques form the second category of methods used to examine the structural changes in the pulmonary circulation following chronic hypoxia and have frequently been reported as showing a loss of pulmonary vessels. However, the problem is again that filling of the blood vessels is influenced by pulmonary vascular resistance and is therefore not a reliable method for identifying vessels. For example, it has been

shown that the extent of the vascular bed revealed by such methods critically depends on the perfusion pressure (6).

The final category of evidence that is used to support the view that structural changes underlie hypoxic pulmonary hypertension is functional in nature. Once chronic hypoxic pulmonary hypertension has become established abrupt return to normoxic conditions does not cause an immediate substantial fall in pulmonary arterial pressure (7, 8). Moreover, most vasodilator agents only produce small acute falls in pulmonary arterial pressure and vascular resistance remains substantially elevated above control values. This has been interpreted as showing that ongoing hypoxic vasoconstriction is not a significant contributor to the established pulmonary hypertension and that “fixed” structural changes, including inward remodelling and the loss of pulmonary vessels must be the dominant mechanism. However, more recently it has been appreciated that the RhoA-Rho kinase pathway, through its effect on myosin light chain phosphatase activity and thus sustained vascular smooth muscle contraction, has a very important role in the chronically hypoxic lung. This pathway is upregulated in the pulmonary vascular smooth muscle in hypoxic pulmonary hypertension and acute administration of inhibitors of Rho kinase to chronically hypoxic hypertensive lungs immediately reduces pulmonary vascular resistance to values that are close to normal (13, 20, 21). Such an immediate return of pulmonary vascular resistance to near control values is not compatible with a major role for structural changes, including either hypertrophic luminal encroachment or vessel loss, as the basis for chronic hypoxic pulmonary hypertension. A recent editorial has addressed the likely role of sustained vasoconstriction in the apparent structural inward remodelling of hypoxia-induced hypertensive rat pulmonary arteries (27).

Whether or not the pulmonary arterial wall thickening has an important negative impact on vascular distensibility in the chronically hypoxic lung remains to be determined.

In summary, we conclude that structural data obtained using rigorous stereological techniques and functional evidence obtained with recently developed inhibitors of the RhoA-Rho kinase pathway demonstrate that chronic alveolar hypoxia, acting in the absence of any lung disease, does not cause loss of pulmonary vessels.

Acknowledgements: P McLoughlin is funded by Programme grants from the Health Research Board (Ireland) and PRTL Higher Education Authority (Ireland). **I McMurtry receives funding from the National Institutes of Health (NHLBI HL-14985).**

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Rebuttal

Rabinovitch, Chesler, and Moulthen

Three issues raised in the article by McLoughlin and McMurtry claiming ‘no loss of arteries’ in hypoxia have been addressed in our original previous article (i) loss of arteries has always referred to pre-capillary arteries rather than veins or capillaries (ii) restoration of pressure to normal values can occur in the face of a remodeled vascular bed with fewer vessels, witness minimal elevation in mean pulmonary artery pressures even after pneumonectomy, and (iii) there may be rodent strains in which loss of arteries may not be a feature of hypoxia induced vascular remodeling. Among the papers cited to support the authors’ contention that loss of precapillary arteries following chronic hypoxia cannot be, are only two utilizing comprehensive morphometric analysis but these evaluated Wistar (2, 3) not Sprague Dawley rats. So it is possible that Wistar rats do not show this

feature in hypoxia. The reference by Kay et al (5) reported failure to show loss of arteries either in response to chronic hypoxia or following monocrotaline injection but only by measuring vessels of a certain diameter range and therefore would have included veins. Now there is ample evidence from many groups that supports loss of pre-capillary arteries in monocrotaline induced pulmonary hypertension as shown in studies by Zhao et al, using fluorescent microspheres (10). References by Meyrick and Reid (6) and Hislop and Reid (4) do document a loss of barium filled arteries in chronic hypoxia. The reference by Mooi and Wagenvoort only questions arterial loss, but without evidence. There is no evidence of failure to find loss of vessels in the reference by Brusselmans(1) and in fact the Carmeliet group has reported loss of distal pulmonary arteries in hypoxia in another publication as cited. As the authors point out, the number of groups reporting loss of vessels in rodents following exposure to hypoxia, outweigh those groups that fail to observe this abnormality. Additional studies are cited claiming that in younger animals the loss of arteries may not consider a change in alveolar number, but previous studies have incorporated morphometric analyses of total alveolar number and still find a chronic hypoxia induced loss of pre-capillary arteries in developing lungs (8). , Finally we provide evidence to show specifically that 'quantitative models of the rat pulmonary arterial tree morphometry applied to hypoxia-induced arterial remodeling' show a significant decrease in pulmonary arterial distensibility in the (7) (9) .

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Rebuttal

McLoughlin and McMurtry

We read with great interest the arguments put forward by Drs. Rabinovitch, Chesler and Molthen to support their case (1). They raise a number of important issues that warrant further investigation. However, not surprisingly, we cannot agree with all that they propose. We discuss here those issues not dealt with in our original submission.

Regarding the limitations of the barium gelatin infusion technique, our opponents argue that studies in which small pulmonary arteries were identified using anti-PECAM immunostaining also report vascular loss. However, others who have used anti-von Willebrand Factor or elastin staining to identify vessels did not detect reduced vascular density in hypoxic hypertensive lungs (2-4).

Dr. Rabinovitch and her co-authors next discuss studies of the pulmonary vasculature undertaken using contrast (PFOB) based microCT imaging. While this elegant approach holds much promise for the future, we do not believe it can at present address the issue of angiogenesis or rarefaction in the lung. First, the resolution of the technique means that the smallest vessel that can be visualised is at best 70 microns in diameter (5).

Second, the principal pathway analysis that this technique uses does not examine parallel distal pathways. Finally, as we have all agreed, full relaxation of vascular tone is essential for angiographic techniques to accurately detect structural changes. It seems unlikely that brief exposure to papaverine, which was used to prepare the lungs for the PFOB-microCT studies (5), could have achieved this. Rho kinase inhibitors appear to be uniquely potent in reducing vascular tone in the chronically hypoxic pulmonary circulation (6, 7), and to our knowledge transient exposure to papaverine has not been shown to be similarly effective. Thus, the PFOB-microCT technique cannot provide unambiguous information regarding distal vessel number.

We agree with Dr Rabinovitch and her colleagues that Rho kinase inhibitors have only been shown to abolish chronic hypoxic pulmonary hypertension at low flow rates and their effects should be examined over a larger range of flows. They suggest that angiogenesis and pre-capillary arteriolar loss may occur simultaneously. Such a mechanism could reconcile seemingly disparate views on this issue and warrants further investigation. Finally, we agree that there can be major discrepancies between hemodynamic changes and structural changes in the vascular bed following chronic hypoxia. Factors such as reduced vascular compliance may contribute importantly to right ventricular overloading, although it should be recognized that, in addition to changes in structure, smooth muscle contraction can also alter compliance (8).

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