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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

This debate focuses on whether, and to what extent, loss of small precapillary 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 (5, 6) have provided conclusive evidence that there is considerable angiogenesis associated with hypoxia-induced pulmonary hypertension. 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 precapillary 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 (24) used fluorescent microbeads to document both breaks and abrupt termination of precapillary 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 neoangiogenesis 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 platelet endothelial cell adhesion molecule (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 (11, 22), 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 precapillary 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 precapillary vessels. One of the ways in which the number of precapillary 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 precapillary arteries is recorded as the number of arteries relative to 100 alveoli or per squared millimeter. Calculating arteries per 100 alveoli makes the assumption that the alveoli are normal in number and calculating squared millimeter 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. With this method, isolated lungs harvested from mice are rinsed of blood and perfused with a physiological salt solution containing 5% bovine serum albumin while being ventilated with a 15% O_2, 6% CO_2, 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 precapillary 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 vWF or PECAM staining of endothelium to landmark arteries accompanying alveolar ducts and alveolar walls down to a precapillary 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 vWF immunostaining techniques to assess precapillary arteries (15). With 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 (3, 12).

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 precapillary, 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 influenced the distensibility characteristics in the pulmonary circulation (9, 10).

We observed that patchy deletion of BMPR1a 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.

First, 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.*

Second, 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 precapillary and capillary circulation.*

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

Fourth, 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

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. With 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 then to 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 three-dimensional data using two-dimensional sections is not as straightforward as it 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