Tetrahydrobiopterin in Pulmonary Hypertension

Pulmonary Hypertension in Guanosine Triphosphate-Cyclohydrolase–Deficient Mice

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The regulation of pulmonary vascular tone is a complex process and represents a balance between constrictor and dilator influences. In pulmonary hypertension, whether caused by hypoxia or flow- and pressure-induced remodeling, the balance is tilted predominantly toward vasoconstriction. Despite decades of research, the initiating events in most patients with primary pulmonary hypertension remain unknown. The causes are probably diverse, and clinical recognition of the disease often occurs after the process is fairly advanced. On presentation, initiating events are obscured by the adaptations that occurred in the pulmonary circulation in response to the hypertension. Thus, the clinically evident disease may be quite distant from the initial cause(s) of pulmonary hypertension. Laboratory investigations of specific biochemical defects that can lead to pulmonary hypertension and vascular remodeling should help us understand these proximate causes. In this issue of Circulation, 2 independent studies report that a defect in the synthesis of tetrahydrobiopterin (BH₄), a cofactor required for the synthesis of nitric oxide (NO) through the catalytic activity of NO synthase (NOS), results in pulmonary hypertension. The studies confirm that a defect in endothelial NOS (eNOS) function can be an initiating event leading to both pulmonary hypertension and pulmonary vascular remodeling. Their results are consistent with observations of impaired NOS activity and NOS-dependent vasodilation in both patients and animal models with pulmonary hypertension. Such studies provide proof of concept that a specific biochemical defect can cause eNOS uncoupling to remodel the pulmonary vasculature.

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The mechanisms involved in the opposing responses of pulmonary and systemic vascular beds to hypoxia and oxygen remain unknown; however, rapid progress has been made in understanding the vascular biology of the pulmonary circulation since the discovery of how NO plays a protective role in the circulation nearly 2 decades ago. All 3 NOS isoforms are expressed in the lung. The tonic regulation of pulmonary vessels is dependent on both eNOS and neuronal NOS (nNOS) in the airway epithelium. Nitric oxide is likely involved in lung growth and development, according to reports that showed that eNOS-knockout mice have defective lungs with a poorly developed air–blood barrier that simulates alveolar-capillary dysplasia. Expression and activity of eNOS are developmentally regulated, with large increases in both during late gestation. A number of studies demonstrate that NOS plays a central role in regulating pulmonary vascular tone during normoxia as well as in response to hypoxia, pressure, or flow.

With eNOS playing such a varied and critical role in pulmonary function during and after development, one might predict that defects in eNOS function lead to pulmonary disease. To understand how eNOS dysfunction leads to vascular disease, it is necessary to take a closer look at the catalytic activity of NOS. NOS is an oxidoreductase that is dynamically regulated by essential cofactors, chaperones, and phosphorylation/dephosphorylation events. Calcium/calmodulin activation causes a conformation change in the tertiary structure of the enzyme leading to electron flow through the reductase domain. Phosphorylation at Ser1177 (human) increases electron flux from NADPH through flavin adenine dinucleotide, and flavin mononucleotide in the reductase domain for delivery to heme in the arginine oxygenase domain. Electron transfer to O₂ bound on heme generates an activated O₂ that, in coupled activity, is used for the oxidation of arginine to generate NO and citrulline. Failure of the activated O₂ to react with arginine results in uncoupled activity and generation of the superoxide anion (O₂⁻), a radical species that scavenges NO to attenuate NO-dependent physiological responses. It is at this critical step that BH₄ and heat shock protein 90 (hsp90) appear to promote coupled activity, in that depletion of BH₄ or inhibition of hsp90-dependent signaling leads to the uncoupling of eNOS activity and increased O₂⁻ generation. Both NO and O₂⁻ may have distinct physiological roles in the pulmonary vascular bed, and the relative amounts of NO and O₂⁻ generated by NOS may be developmentally regulated. The biological effects of NO and O₂⁻ balance are influenced by other factors, such as relative levels of superoxide dismutase (SOD), which dismutates O₂⁻ to H₂O₂, another oxidant that has been shown to promote vasodilation.

The studies reported by Khoo et al1 and Nandi et al2 show that BH₄ plays a pivotal role in eNOS function, which has an impact on the pulmonary circulation. Their conclusions are...
supported by data from morphological characterization of different organs (lung, heart, and kidney), measurement of BH4 levels in these organs, and NOx in the serum. Lungs of hph-1 mice, which become deficient in BH4 because of the decreased activity of GTP-cyclohydrolase-1 (GTPCH-1), develop pulmonary disease consistent with histological evidence seen in humans, namely distal muscularization and smooth muscle hypertrophy. Graded responses in the severity of pulmonary hypertension in transgenic inbred strains of the hph-1 mouse show a direct correlation between BH4 in the lung and the degree of pulmonary hypertension. Khoo et al1 showed that cell-specific overexpression of GTPCH-1 restores BH4 in the vascular endothelium and that this was sufficient to protect mice from pulmonary hypertension. Thus, BH4 plays an important role in maintaining the balance of NO and O2− generation by eNOS, and in so doing, maintaining pulmonary function. One intriguing observation in both studies is the ability of BH4 deficiency to induce pulmonary but not systemic hypertension. These data suggest BH4, as an antioxidant, is more important to the lung, which is continuously exposed to oxygen or other pro-oxidants in the atmosphere (eg, ozone, nitrogen oxides) than to systemic vascular beds, which have no such direct contact. These observations are in contrast to an earlier report that used the same mutant mouse, in which decreased BH4 availability impaired vasodilation and increased blood pressure.15 Another question that requires further study is whether the hph-1 mouse has normal lung development and pulmonary circulation at birth, so that the role of BH4-dependent NO release in fetal lung development may be addressed.

Although ample evidence exists in these articles showing that a decrease in BH4 availability exaggerates pulmonary vasoconstriction and NO-dependent O2− generation (based on hydroethidine staining attenuated by Nε-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO that blocks both NO and O2− generation), these findings do not account for the pulmonary vasoconstriction seen in normal, wild-type animals during hypoxia. They also do not reveal whether O2− generation is increased when normal animals are subjected to hypoxia. Measurements of O2− production from hypoxic mice would have provided stronger proof that increased O2− is involved in hypoxic pulmonary vasoconstriction. Measurements of BH4 in the study by Khoo et al1 indicate that this cofactor does not change during hypoxia, yet the mice still develop increased pulmonary vascular resistance. This raises the question of whether other mechanisms exist in hypoxia-induced increases in pulmonary vascular resistance.

One mechanism of eNOS dysfunction that may be relevant to pulmonary hypertension in normal animals involves altered hsp90-dependent signaling. Su and Block16 previously reported that hypoxia uncouples eNOS activity in cultured endothelial cells by a calpain-dependent decrease in hsp90 interactions with eNOS. We demonstrated a decrease in hsp90 association with eNOS-impaired NO production in an ovine ductal ligation model of fetal pulmonary hypertension.17 Interestingly, the mechanisms by which hsp90 dissociates from eNOS do not appear to involve BH4 availability in that BH4 is unchanged in pulmonary endothelial cells isolated from fetal lambs12 and sepiapterin does not correct impaired pulmonary vasodilation in pulmonary vessels isolated from ductal ligation lambs.18 This is in contrast to the effects of both L-NAME and the SOD mimetic Tiron, which restored pulmonary vasodilator responses to adenosine triphosphate, a well-recognized eNOS agonist.12,18 Confounding the role of eNOS dysfunction in vasodilation is the observation that O2− may mediate pulmonary physiology via dismutation to H2O2, which increases vasodilation.13,14 This mechanism was shown to increase pulmonary vasodilation in response to NO agonists in postnatal pulmonary circulation.12 Thus, relative levels of SOD in pulmonary vessels also may be important in regulating vascular tone in response to physiological agonists when O2− generation is increased. Finally, as eNOS is phosphorylated and dephosphorylated at numerous sites, kinase/phosphatase-dependent signaling also may influence eNOS.19,20

The observations of increased O2− production in wild-type animals and in hph-1/GTPCH-1 transgenic animals treated with L-NAME are intriguing. These data suggest that a loss in NO production may increase O2− production. Because eNOS is fully capable of generating both NO and O2− (as evidenced by endogenous lucigenin), the question remains whether the O2− comes from an enzymatic source other than NO because L-NAME inhibits both NO and O2− generation. Even though data in the articles point toward uncoupled eNOS activity, the analytical assays used to quantify O2− fall short of providing definitive proof. It is widely recognized that lucigenin can be reduced by reductases, and when this occurs the reduced lucigenin is fully capable of generating its own O2−.21 Although low concentrations of lucigenin (5 μmol/L) are used to minimize non-O2−-dependent reduction in cultured cells or intact small vessels,22 this is not the case with homogenates in which lucigenin has full access to cellular reductases. With regard to the hydroethidine assay, marked increases in nonspecific fluorescence are known to occur at the edge of the cut tissues.23 With this as background, it is unclear why frozen sections continue to be used for qualitative changes in vascular O2− generation. Our experience is that the nonspecific increases in hydroethidine fluorescence do not occur if intact vascular beds are perfused with physiological buffers containing hydroethidine before microdissection. Although relative changes in fluorescence between control and experimental vessels suggest that one vessel generates more O2− than the other, the use of frozen sections cannot exclude the possibility that the increase in fluorescence is the result of varying degrees of tissue damage caused by freeze-thawing. Many of these concerns could have been alleviated with studies using hph-1 endothelial cell cultures to examine uncoupled eNOS activity under more controlled conditions with more definitive assays. Clearly, additional studies will be required in this area. Even in the context of these limitations and concerns, it is still important that tissues from the hph-1 mice generated greater increases in luminescence and fluorescence than did controls.

One concern with N-ethyl-N-nitrosoare-derived murine models is specificity because N-ethyl-N-nitrosoare induces random gene mutations, resulting in a phenotype that may manifest under some conditions but not others. To address this concern, Khoo et al1 developed related strains that encompassed a wide range of GTPCH-1 expression and BH4 synthesis. The studies were performed with transgenic mice that overexpressed GTPCH-1 in the endothelium, with littermates used as genetic controls. This rigorous approach
provided convincing proof that BH4 bioavailability plays a direct role in modulating eNOS function and thereby pulmonary hypertension. The questions addressed by these articles are important because they suggest eNOS function as defined by BH4 availability plays a central role in pulmonary function and the pathogenesis of pulmonary hypertension.

These articles should open up new clinical research studies aimed at determining whether a loss in GTPCH-1 activity or alterations in GTPCH-1 activity affect pulmonary function. Before restoring pulmonary vascular function with BH4, it is important to perform studies aimed at determining whether GTPCH-1 defects occur in pulmonary hypertension. Clearly, the questions raised by the studies of Kho et al and Nandi et al are intriguing and offer a tempting, although unproven, explanation for why individuals react differently to environmental stresses (eg, why some people develop pulmonary hypertension at high altitudes whereas others remain symptom free).

References


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