

Tetrahydrobiopterin in Pulmonary Hypertension Pulmonary Hypertension in Guanosine Triphosphate-Cyclohydrolase- Deficient Mice

Kirkwood A. Pritchard, Jr, PhD; Yang Shi, PhD; G. Ganesh Konduri

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^{1,2} 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 circula-

tion 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.^{3,4} 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.⁵ Expression and activity of eNOS are developmentally regulated, with large increases in both during late gestation.⁴ 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.^{6,7}

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.⁸⁻¹⁰ 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.¹¹ 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.¹² 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.^{13,14}

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

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From the Department of Surgery, Division of Pediatric Surgery (K.A.P., Y.S.), Children's Research Institute (K.A.P., Y.S., G.G.K.), Cardiovascular Center (K.A.P., Y.S., G.G.K.), Department of Pediatrics, Division of Neonatology (G.G.K.), Medical College of Wisconsin and Children's Hospital of Wisconsin, Milwaukee, Wis.

Correspondence to Kirkwood A. Pritchard, Jr, PhD, Medical College of Wisconsin, 8701 Watertown Plank Rd, Milwaukee, WI 53226. E-mail kpritch@mcw.edu

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supported by data from morphological characterization of different organs (lung, heart, and kidney), measurement of BH₄ levels in these organs, and NO_x 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 NOS-dependent O₂⁻ generation (based on hydroethidine staining attenuated by N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NOS 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 NOS 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⁻, the question remains whether the O₂⁻ comes from an enzymatic source other than NOS 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*-nitrosoourea-derived murine models is specificity because *N*-ethyl-*N*-nitrosoourea 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 altered gene expression actually occurs in patients with pulmonary hypertension. This work will likely focus new attention on how 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 Khoo 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

1. Khoo JP, Zhao L, Alp NJ, Bendall JK, Nicoli T, Rockett K, Wilkins MR, Channon KM. Pivotal role for endothelial tetrahydrobiopterin in pulmonary hypertension. *Circulation*. 2005;111:2126–2133.
2. Nandi M, Miller A, Stidwill R, Jacques TS, Lam AAJ, Haworth S, Heales S, Vallance P. Pulmonary hypertension in a GTP-cyclohydrolase 1-deficient mouse. *Circulation*. 2005;111:2086–2090.
3. Rairigh RL, Le Cras TD, Ivy DD, Kinsella JP, Richter G, Horan MP, Fan ID, Abman SH. Role of inducible nitric oxide synthase in regulation of pulmonary vascular tone in the late gestation ovine fetus. *J Clin Invest*. 1998;101:15–21.
4. North AJ, Star RA, Brannon TS, Ujji K, Wells LB, Lowenstein CJ, Snyder SH, Shaul PW. Nitric oxide synthase type I and type III gene expression are developmentally regulated in rat lung. *Am J Physiol*. 1994;266:L635–L641.
5. Han RN, Babaei S, Robb M, Lee T, Ridsdale R, Ackerley C, Post M, Stewart DJ. Defective lung vascular development and fatal respiratory distress in endothelial NO synthase-deficient mice: a model of alveolar capillary dysplasia? *Circ Res*. 2004;94:1115–1123.
6. Fagan KA, Fouty BW, Tyler RC, Morris KG Jr, Hepler LK, Sato K, LeCras TD, Abman SH, Weinberger HD, Huang PL, McMurtry IF, Rodman DM. The pulmonary circulation of homozygous or heterozygous eNOS-null mice is hyperresponsive to mild hypoxia. *J Clin Invest*. 1999;103:291–299.
7. Storme L, Rairigh RL, Parker TA, Kinsella JP, Abman SH. Acute intra-uterine pulmonary hypertension impairs endothelium-dependent vasodilation in the ovine fetus. *Pediatr Res*. 1999;45:575–581.
8. Sessa WC. eNOS at a glance. *J Cell Sci*. 2004;117:2427–2429.
9. Gallis B, Corthals GL, Goodlett DR, Ueba H, Kim F, Presnell SR, Figeys D, Harrison DG, Berk BC, Aebersold R, Corson MA. Identification of flow-dependent endothelial nitric-oxide synthase phosphorylation sites by mass spectrometry and regulation of phosphorylation and nitric oxide production by the phosphatidylinositol 3-kinase inhibitor LY294002. *J Biol Chem*. 1999;274:30101–30108.
10. Garcia-Cardena G, Fan R, Shah V, Sorrentino R, Cirino G, Papapetropoulos A, Sessa WC. Dynamic activation of endothelial nitric oxide synthase by Hsp90. *Nature*. 1998;392:821–824.
11. McCabe TJ, Fulton D, Roman LJ, Sessa WC. Enhanced electron flux and reduced calmodulin dissociation may explain “calcium-independent” eNOS activation by phosphorylation. *J Biol Chem*. 2000;275:6123–6128.
12. Mata-Greenwood E, Jenkins C, Russell JA, Konduri GG, Farrow KN, Black SM, Steinhorn RH. Endothelial nitric oxide synthase function is developmentally regulated: uncoupling of eNOS occurs postnatally. *Am J Physiol Lung Cell Mol Physiol*. 2005. In press.
13. Matoba T, Shimokawa H, Nakashima M, Hirakawa Y, Mukai Y, Hirano K, Kanaide H, Takeshita A. Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in mice. *J Clin Invest*. 2000;106:1521–1530.
14. Thomas SR, Chen K, Keaney JF Jr. Hydrogen peroxide activates endothelial nitric-oxide synthase through coordinated phosphorylation and dephosphorylation via a phosphoinositide 3-kinase-dependent signaling pathway. *J Biol Chem*. 2002;277:6017–6024.
15. Cosentino F, Barker JE, Brand MP, Heales SJ, Werner ER, Tippins JR, West N, Channon KM, Volpe M, Luscher TF. Reactive oxygen species mediate endothelium-dependent relaxations in tetrahydrobiopterin-deficient mice. *Arterioscler Thromb Vasc Biol*. 2001;21:496–502.
16. Su Y, Block ER. Role of calpain in hypoxic inhibition of nitric oxide synthase activity in pulmonary endothelial cells. *Am J Physiol Lung Cell Mol Physiol*. 2000;278:L1204–L1212.
17. Konduri GG, Ou J, Shi Y, Pritchard KA Jr. Decreased association of Hsp90 impairs endothelial nitric oxide synthase in fetal lambs with persistent pulmonary hypertension. *Am J Physiol Heart Circ Physiol*. 2003;285:H204–H211.
18. Konduri GG, Eis AL, Bakhtushvili I. Oxidant stress from uncoupled nitric oxide synthase impairs vasodilation in persistent pulmonary hypertension of newborn. *Pediatr Res*. 2005. In press.
19. Lin MI, Fulton D, Babbitt R, Fleming I, Busse R, Pritchard KA Jr, Sessa WC. Phosphorylation of threonine 497 in endothelial nitric-oxide synthase coordinates the coupling of L-arginine metabolism to efficient nitric oxide production. *J Biol Chem*. 2003;278:44719–44726.
20. Bauer PM, Fulton D, Boo YC, Sorescu GP, Kemp BE, Jo H, Sessa WC. Compensatory phosphorylation and protein-protein interactions revealed by loss of function and gain of function mutants of multiple serine phosphorylation sites in endothelial nitric-oxide synthase. *J Biol Chem*. 2003;278:14841–14849.
21. Vasquez-Vivar J, Hogg N, Pritchard KA Jr, Martasek P, Kalyanaraman B. Superoxide anion formation from lucigenin: an electron spin resonance spin-trapping study. *FEBS Lett*. 1996;403:127–130.
22. Li Y, Zhu H, Kuppusamy P, Roubaud V, Zweier JL, Trush MA. Validation of lucigenin (bis-N-methylacridinium) as a chemiluminescent probe for detecting superoxide anion radical production by enzymatic and cellular systems. *J Biol Chem*. 1998;273:2015–2023.
23. Stepp DW, Ou J, Ackerman AW, Welak S, Klick D, Pritchard KA Jr. Native LDL and minimally oxidized LDL differentially regulate superoxide anion in vascular endothelium in situ. *Am J Physiol Heart Circ Physiol*. 2002;283:H750–H759.

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