

Pivotal Role for Endothelial Tetrahydrobiopterin in Pulmonary Hypertension

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Background—Pulmonary hypertension is a fatal disease characterized by vasoconstriction and vascular remodeling. Loss of endothelial nitric oxide bioavailability is implicated in pulmonary hypertension pathogenesis. Recent evidence suggests that the cofactor tetrahydrobiopterin (BH4) is an important regulator of nitric oxide synthase enzymatic function.

Methods and Results—In the *hph-1* mouse with deficient BH4 biosynthesis, BH4 deficiency caused pulmonary hypertension, even in normoxic conditions, and greatly increased susceptibility to hypoxia-induced pulmonary hypertension. In contrast, augmented BH4 synthesis in the endothelium, by targeted transgenic overexpression of GTP-cyclohydrolase I (GCH), prevented hypoxia-induced pulmonary hypertension. Furthermore, specific augmentation of endothelial BH4 in *hph-1* mice by crossing with GCH transgenic mice rescued pulmonary hypertension induced by systemic BH4 deficiency. Lung BH4 availability controlled pulmonary vascular tone, right ventricular hypertrophy, and vascular structural remodeling in a dose-dependent manner in both normoxia and hypoxia. Furthermore, BH4 availability had striking effects on the immediate vasoconstriction response to acute hypoxia. These effects of BH4 were mediated through the regulation of nitric oxide compared with superoxide synthesis by endothelial nitric oxide synthase.

Conclusions—Endothelial BH4 availability is essential for maintaining pulmonary vascular homeostasis, is a critical mediator in the pathogenesis of pulmonary hypertension, and is a novel therapeutic target. (*Circulation*. 2005;111:2126-2133.)

Key Words: hypertension, pulmonary ■ tetrahydrobiopterin ■ superoxide ■ endothelium ■ nitric oxide synthase

In a normal oxygen environment, the healthy adult pulmonary vascular bed is a low-pressure circuit. Pulmonary hypertension arises when vasoconstriction and structural remodeling of pulmonary arterioles lead to increased pulmonary vascular resistance. The consequent increase in pressure load causes right ventricular (RV) hypertrophy, which progresses to premature death from right-sided heart failure.¹ Pulmonary hypertension can develop in isolation (idiopathic pulmonary arterial hypertension) or in association with conditions such as connective tissue diseases, congenital heart disease, pulmonary thromboembolism, or chronic hypoxia.²

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Mutations in the *BMPR2* gene, encoding a receptor for bone morphogenetic protein, appear to underlie some cases of familial idiopathic pulmonary arterial hypertension. However, *BMPR2* mutations account for only 10% of sporadic idiopathic pulmonary arterial hypertension, and only 15% to 20% of individuals carrying a *BMPR2* mutation develop pulmonary hypertension, suggesting that additional disease-causing or disease-modifying genes remain critical to understanding pulmonary hypertension pathogenesis.³

The endothelium plays a key role in pulmonary vascular homeostasis. Endothelial dysfunction is clearly implicated in the pathogenesis of pulmonary hypertension, but the exact mechanisms remain poorly understood.⁴ Endothelium-derived nitric oxide (NO), through potent vasodilator and antiproliferative effects on vascular smooth muscle cells, is critical in maintaining normal pulmonary vascular tone and structure.⁵ Mice deficient in endothelial NO synthase (eNOS) are more sensitive to hypoxia-induced pulmonary hypertension,^{6,7} whereas pulmonary gene transfer of eNOS is partially protective.⁸ Patients with pulmonary hypertension have low NO levels in their exhaled breath,^{9,10} and both inhaled NO and phosphodiesterase type 5 inhibitors, which act to increase NO-mediated cGMP signaling, have emerged as therapeutic strategies in pulmonary hypertension. Paradoxically, mice with hypoxia-induced pulmonary hypertension have increased eNOS protein levels without a concomitant increase in NO bioactivity.^{11,12} Scavenging by superoxide radicals may account in part for reduced NO bioactivity.¹³ Increased superoxide production has been observed in experimental models of pulmonary hypertension,¹⁴ and markers of oxida-

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tive stress are increased in patients with pulmonary hypertension.¹⁵ Superoxide mediates pulmonary vasoconstriction¹⁶ and stimulates pulmonary smooth muscle cell proliferation.¹⁷ These observations suggest that perturbation of NO synthesis, together with increased superoxide production, may play a central role in the pathogenesis of pulmonary hypertension.

The cofactor tetrahydrobiopterin (BH4) is an important regulator of NOS enzymatic activity.¹⁸ Without BH4, NO production is reduced and superoxide production is increased as a result of the loss of enzymatic coupling between the reduction in molecular oxygen and oxidation of L-arginine.¹⁹ In the systemic vasculature, BH4-mediated eNOS regulation appears to be an important determinant of NO bioavailability, independent of total eNOS protein levels,^{19,20} but the role of BH4 in the pulmonary circulation remains unexplored. We hypothesized that endothelial BH4 availability is a critical determinant of both the acute and chronic responses to hypoxia and the development of pulmonary hypertension, mediated through the effect of BH4 on eNOS activity and enzymatic coupling. To test this hypothesis, we compared pulmonary hemodynamics and the development of hypoxic pulmonary hypertension in genetic mouse models with graded reductions in BH4 levels; then, we tested the ability of transgenic endothelium-targeted augmentation of BH4 to rescue the effects of BH4 deficiency and protect against hypoxia-induced pulmonary hypertension. Furthermore, we investigated the effect of these changes in pulmonary BH4 availability on local NO compared with superoxide production by eNOS and the functional consequences of BH4-mediated eNOS regulation on acute pulmonary vasomotor function.

Methods

Mice

The *hph-1* mouse, generated by ENU mutagenesis, was used as a model of BH4 deficiency. In these animals, tissue BH4 levels are low because of constitutively reduced expression of GTP cyclohydrolase I (GTPCH), the rate-limiting enzyme in BH4 biosynthesis.^{21–23} Wild-type (WT), *hph-1* heterozygous (+/–), and *hph-1* homozygous (*hph*) littermates on a C57BL/6 background were obtained by interbreeding *hph-1* heterozygotes.²³ In GCH-transgenic mice (GCH mice), overexpression of the human GTPCH under the control of the endothelial-specific Tie-2 promoter results in 2- to 3-fold elevation of endothelial BH4 levels.²⁴ GCH mice used in these studies were bred for 8 generations onto a C57BL/6 background. To generate *hph-1*/GCH transgenic mice (*hph*/GCH), ie, *hph-1* homozygotes carrying the Tie2-GCH transgene, *hph-1* homozygotes were crossed with GCH mice, and offspring were backcrossed with *hph-1*. Mice were genotyped as previously described for *hph-1* and GCH alleles.^{23,24} Mice were used at 15 weeks of age. Studies were conducted in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986.

Normoxia and Chronic Hypoxia Studies

Animals were either housed in normal air or placed in a specially constructed normobaric hypoxic chamber (FIO₂ 10%) for 1 week.²⁵ At 1 week, animals were weighed and anesthetized (Hypnorm 0.25 mL/kg; Midazolam 25 mg/kg IP), and RV systolic pressure (RVSP) was measured via direct cardiac puncture using a closed-chest technique in the spontaneously breathing, anesthetized animal.²⁵ The animals were then euthanized, the hearts were removed, and the individual ventricular chambers were weighed (septum with left ventricle). The right lungs were snap-frozen in liquid nitrogen and

stored at –80°C for biochemical measurements. The left lungs were fixed by inflation with 10% formalin, embedded in paraffin, and sectioned for histology.

BH4 Assay

Lung homogenates were oxidized with 1% iodine/2% KI in acid or base. Biopterins were determined by high-performance liquid chromatography (HPLC) with fluorescent detection, and BH4 was calculated by subtracting BH2+biopterin from total biopterins, as previously described.²⁴

Lung Histology

Histological assessment of vascular remodeling was performed as previously described.²⁵ Transverse lung sections were stained with van Gieson's elastic method or smooth muscle α -actin antibody (Clone 1A, Sigma) using an alkaline phosphate-conjugated avidin-biotin complex method and Vector Red substrate (Vector Laboratories), with methyl green counterstain (Vector Laboratories). "Distal muscularization" was defined as the proportion of vessels (<50 μ m diameter) with immunoreactivity for smooth muscle α -actin (as evidence for muscularization) over the total number of vessels stained with elastin. Three separate sections from each animal were quantified, and counting was performed by investigators blinded to genotype or treatment group.

eNOS Western Blotting

Lung homogenates were subjected to SDS-PAGE and transferred to nitrocellulose membranes. After transfer, membranes were incubated with mouse eNOS monoclonal antibodies (Transduction Labs), followed by horseradish peroxidase-conjugated secondary antibody. Proteins were visualized by chemiluminescence (SuperSignal, Pierce). Blot intensity was quantified with Scion Image software (release beta 4.0.2).

NOS Activity Assay

NOS activity was determined by ¹⁴C-arginine to ¹⁴C-citrulline conversion with HPLC quantification as described.²⁴ Lung homogenates (400 μ g protein) were incubated for 30 minutes at 37°C in 500 μ L Krebs-HEPES buffer containing ¹⁴C-arginine (2 μ L of 50 μ Ci/mL), FAD (10 μ mol/L), FMN (10 μ mol/L), and NADPH (1 mmol/L). Samples were run on a SCX 300 cation-exchange HPLC column (Sigma) with online scintillation detection. Background signals were corrected from samples with ¹⁴C-arginine alone without homogenates. The integrated ¹⁴C-citrulline elution peak was expressed as a percentage of total ¹⁴C counts.

Lucigenin-Enhanced Chemiluminometry

Total lung superoxide production was measured by lucigenin-enhanced chemiluminescence as previously described.²⁴ Lungs were homogenized in 400 μ L Krebs-HEPES buffer. Samples (100 μ L) of homogenate corrected for total protein content were added to 2 mL Krebs-HEPES buffer containing lucigenin (5 μ mol/L) in a scintillation vial at 37°C, and chemiluminescence was quantified over 20 minutes.

Dihydroethidium Fluorescent Microtopography

Endothelial superoxide production in tissue sections of pulmonary arteries was detected by use of the fluorescent probe dihydroethidium, adapting a method previously described for mouse aortas.²⁴ Fresh pulmonary artery cryosections (30 μ m) were incubated in Krebs-HEPES buffer containing acetylcholine (10 μ mol/L) for 30 minutes at 37°C with or without the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; 1 mmol/L; Calbiochem) and dihydroethidium (2 μ mol/L; Molecular Probes) for 5 more minutes at 37°C in darkness. Images were obtained with a laser confocal microscope (Bio-Rad MRC 1024) at identical acquisition settings. Endothelial superoxide production was quantified with Image-Pro Plus software (MediaCybernetics) as the sum of mean red intensities

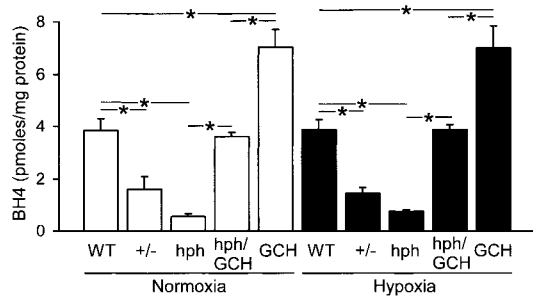


Figure 1. Lung BH4 levels in gene-modified mice. Mice were exposed to normoxia or hypoxia for 1 week. BH4 levels in lung homogenates, determined by HPLC, were progressively reduced in *hph-1^{+/-}* and *hph* mice, normalized in *hph/GCH* mice, and doubled in *GCH* mice. BH4 levels were unaffected by hypoxia (* $P < 0.001$; $n = 4$ to 15).

of red objects on the luminal surface of the pulmonary artery. For each ring, mean fluorescence was calculated from 4 separate fields.

Acute Hypoxia Studies

Lungs of anesthetized mice were ventilated with air at a constant end-expiratory pressure (15 cm H₂O) and perfused in situ in the open chest at a flow rate of 2 mL/min as described previously.²⁵ Lung preparations were precontracted with angiotensin II and allowed to equilibrate for 15 minutes. The increase in pulmonary artery pressure (hypoxic pulmonary vasoconstriction, or HPV) was then recorded after hypoxic challenges (2%O₂/5% CO₂/93%N₂) of 10 minutes in the absence or presence of L-NAME (1 mmol/L).

Statistical Analysis

Data are expressed as mean \pm SEM (n =number of animals). Data were analyzed by use of 1-way ANOVA with post-hoc Fisher test. Significance was set at $P < 0.05$.

Results

BH4 Deficiency Causes Pulmonary Hypertension

We first assessed the possible importance of BH4 in pulmonary vascular homeostasis using the *hph-1* mouse model of BH4 deficiency. In these animals, tissue BH4 levels in homozygotes are significantly reduced because of constitutively reduced expression of GTPCH, the rate-limiting enzyme in BH4 biosynthesis.^{21–23} Using breeding pairs of animals heterozygous for the *hph-1* allele, we obtained WT, *hph-1^{+/-}*, and *hph* littermates and observed a striking graded reduction in BH4 levels in lung tissue, with lung BH4 levels in *hph* animals reduced to only 15% of WT levels (Figure 1). RVSP in *hph* mice bred under normoxic conditions was significantly elevated; *hph-1^{+/-}* mice showed an intermediate elevation of RVSP compared with WT mice (Figure 2a). In keeping with the elevated RVSP, we found significant RV hypertrophy in *hph* mice (Figure 2b), whereas there were no differences in left ventricular mass or total body weight between WT, *hph-1^{+/-}*, and *hph* mice (Table).

We next evaluated the response to chronic hypoxia by exposure of mice to 10% oxygen for 1 week, which resulted in uniform elevation of the hematocrit in all animals (Table). Lung BH4 levels and the degree of BH4 deficiency in *hph* mice remained unchanged by hypoxia (Figure 1). As expected, WT mice developed pulmonary hypertension in response to hypoxia, evidenced by both elevated RVSP and RV hypertrophy (Figure 2). However, these responses to

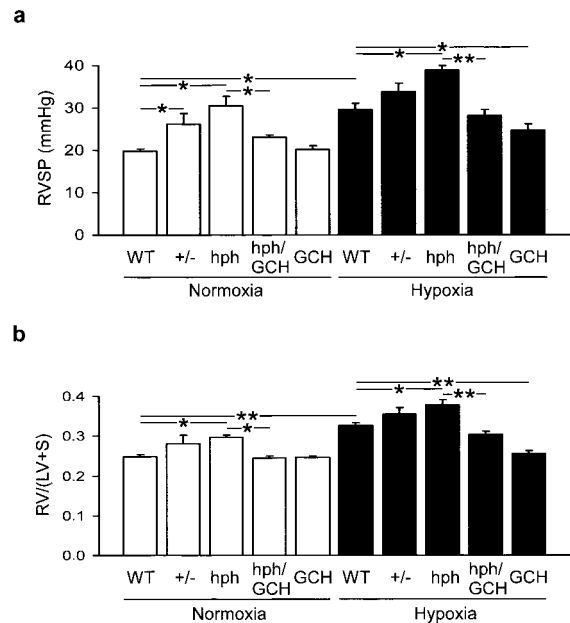


Figure 2. Endothelial BH4 determines susceptibility to pulmonary hypertension. a, RVSP in mice exposed to normoxia or hypoxia for 1 week (* $P < 0.01$, ** $P < 0.001$; $n = 4$ to 12). b, Ratio of RV to left ventricular and septal (LV+S) wet weights (* $P < 0.01$, ** $P < 0.001$; $n = 5$ to 18).

hypoxia were greatly exacerbated in *hph* mice (Figure 2), with an intermediate phenotype in *hph-1^{+/-}* littermates.

Endothelial BH4 Restoration Prevents Pulmonary Hypertension in *hph-1* Mice

To determine whether selective restoration of BH4 biosynthesis in the endothelium would be sufficient to rescue the adverse effects of systemic BH4 deficiency on the pulmonary vasculature, we crossed *hph* mice with *GCH*-transgenic mice (*GCH* mice) in which endothelium-targeted GTPCH overexpression selectively increases BH4 levels in the vascular endothelium 2- to 3-fold.²⁴ Lung BH4 levels in *hph-1/GCH*-transgenic (*hph/GCH*) mice were similar to WT in both normoxia and hypoxia, reflecting the significant contribution of endothelial cells to total lung BH4 on a background of systemic BH4 deficiency (Figure 1). Remarkably, the pulmonary hypertension and RV hypertrophy observed in *hph* mice under normoxic conditions were completely abolished in *hph/GCH* mice (Figure 2). Moreover, the further increases in both RVSP and RV hypertrophy in *hph* mice exposed to chronic hypoxia were strikingly reduced in *hph/GCH* mice (Figure 2). Thus, targeted transgenic restoration of endothelial BH4 synthesis is sufficient to rescue the effects of systemic BH4 deficiency in the *hph* mouse.

Endothelial BH4 Augmentation Attenuates Hypoxia-Induced Pulmonary Hypertension

We next investigated the effects of increasing endothelial BH4 above WT levels on the development of hypoxia-induced pulmonary hypertension by subjecting *GCH* mice to chronic hypoxia. Lung BH4 levels in *GCH* mice were doubled compared with WT mice, and this level of BH4 augmentation was maintained in chronic hypoxia (Figure 1).

Comparison of Body Weights, Left Ventricular Weights, and Hematocrits

	Normoxia					Hypoxia				
	WT	+/-	hph	hph/GCH	GCH	WT	+/-	hph	hph/GCH	GCH
Body weight, g	25.1±1.3	26.4±2.9	25.9±1.3	26.7±1.3	25.7±1.5	22.4±0.8	22.0±1.0	24.3±0.9	26.0±2.4	22.4±1.3
(LV+S)/BW, mg/g	3.4±0.1	3.4±0.1	3.3±0.1	3.3±0.1	3.5±0.1	3.5±0.1	3.5±0.1	3.4±0.1	3.2±0.1	3.6±0.1
Hematocrit, %	37±1	37±2	38±1	37±1	38±1	51±1*	51±1*	51±1*	50±1*	49±1*

(LV+S)/BW indicates ratio of weight of left ventricle and septum to body weight (n=5 to 18).

*P<0.001 vs normoxia groups (n=3 to 12).

In normoxia, RVSP and RV mass were no different in GCH mice compared with WT (Figure 2). However, endothelial BH4 augmentation resulted in striking protection against both hypoxia-induced pulmonary hypertension and RV hypertrophy in GCH mice (Figure 2).

Endothelial BH4 Controls Pulmonary Vascular Remodeling

Pulmonary vascular remodeling is a key pathological hallmark of pulmonary hypertension and an important pathogenic step in disease progression.¹ Quantitative analysis of distal muscularization in histological lung sections revealed striking evidence of vascular remodeling in both hph-1^{+/-} and hph lungs even in normoxic conditions, which was exacerbated by exposure to hypoxia. Endothelial BH4 restoration in hph/GCH lungs significantly reduced vascular remodeling in normoxia and abrogated the increased remodeling observed in hypoxic hph-1^{+/-} and hph mice compared with WT mice (Figure 3). More strikingly, GCH mice were completely protected against the development of hypoxia-induced pulmonary vascular remodeling compared with WT mice (Figure 3).

BH4 Determines Severity of Pulmonary Hypertension in a Dose-Dependent Manner

The overall importance of BH4 availability in determining the development and severity of pulmonary hypertension was further supported by striking inverse correlations in individual animals from all 5 different genotypes (WT, hph-1^{+/-}, hph, hph/GCH, and GCH) between lung BH4 levels and RVSP, RV hypertrophy, and pulmonary vascular remodeling (Figure 4). These relationships were highly significant in both normoxia and hypoxia. The divergence between normoxic and hypoxic phenotypes increased as pulmonary BH4 levels decreased, whereas the phenotypic responses to hypoxia diminished progressively with increasing BH4 levels.

NOS Function Is Regulated by BH4 Bioavailability

Because BH4 is a required cofactor for eNOS activity and is hypothesized to regulate eNOS activity and enzymatic coupling, we quantified eNOS protein levels and activity in lung tissue. Total eNOS protein levels in lung tissue were not significantly different between WT, hph-1^{+/-}, hph, hph/GCH, and GCH mice (Figure 5a and 5b). As described previously,¹¹ hypoxia resulted in a moderate increase in eNOS protein levels (≈1.4-fold) that was similar in all groups (data not shown). Thus, the differences in BH4 levels between the respective mouse lines were not accompanied by any evidence of changes in eNOS protein levels. In contrast, eNOS

enzymatic activity, assessed by radiolabeled L-arginine to L-citrulline conversion in lung extracts, was significantly reduced in hph-1^{+/-} and hph mice compared with WT mice but was restored to WT levels in hph/GCH mice and doubled in GCH mice (Figure 5c). Thus, changes in lung BH4 levels were quantitatively associated with a parallel reduction, restoration, or augmentation of lung eNOS activity, independently of eNOS protein levels.

The effect of BH4 deficiency on oxidative stress was evaluated by measurement of superoxide production in lung homogenates by lucigenin-enhanced chemiluminescence. Superoxide production in lungs from hph mice was significantly elevated but was restored to WT levels by targeted endothelial BH4 replacement in hph/GCH mice (Figure 5d). To specifically assess the effect of BH4 levels on eNOS coupling, endothelial superoxide production was measured by dihydroethidium fluorescent microtopography in pulmonary artery rings incubated in the absence or presence of the NOS inhibitor L-NAME. In BH4-deficient hph-1^{+/-} and hph mice, endothelial superoxide production was increased compared with WT mice (Figure 6). Incubation with L-NAME increased superoxide generation in WT rings, suggesting a tonic scavenging effect of NO on superoxide. In contrast, L-NAME incubation decreased endothelial superoxide formation in hph-1^{+/-} and hph mice, implying a significant contribution from eNOS-derived superoxide (Figure 6). Furthermore, this increased endothelial superoxide production and response to NOS inhibition were reversed by endothelial restoration of BH4 in hph/GCH mice and GCH mice, both of which showed responses similar to WT mice (Figure 6).

NOS Regulation by BH4 Modulates Acute Hypoxic Pulmonary Vasoconstriction

The acute constrictor response of the pulmonary circulation to hypoxia (HPV) serves to optimize pulmonary gas exchange under physiological conditions but contributes to elevated pulmonary pressures in pathological states characterized by chronic hypoxia. Both NO and superoxide can modulate HPV.^{27,28} To evaluate the functional importance of NO compared with superoxide production by eNOS in relation to endothelial BH4 availability, we quantified HPV in perfused lung preparations from WT, hph-1^{+/-}, hph, hph/GCH, and GCH mice. In WT lungs, inhibition of NO production with L-NAME increased HPV, reflecting the vasodilatory effect of eNOS-derived NO (Figure 7).²⁷ Baseline HPV in hph lungs was significantly greater than in WT lungs. Furthermore, the response to NOS inhibition by L-NAME in hph lungs was qualitatively different from WT

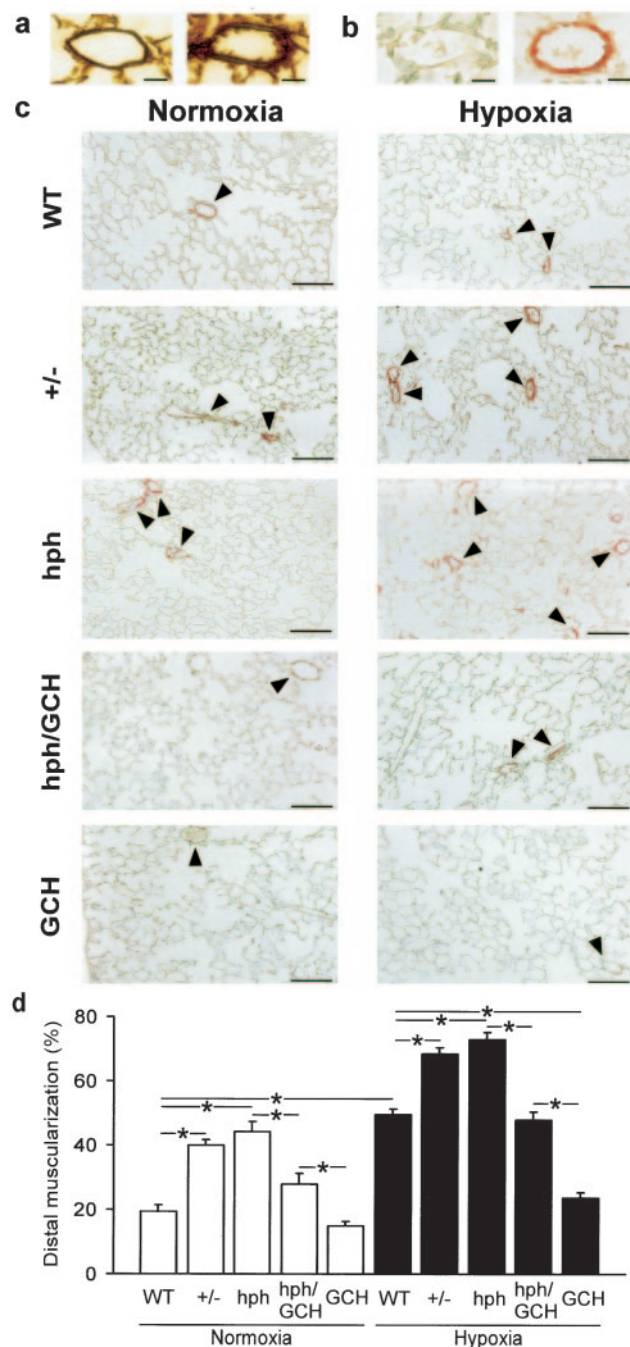


Figure 3. Endothelial BH4 controls pulmonary vascular remodeling. Mice were exposed to normoxia or hypoxia for 1 week. a, Transverse lung sections stained with van Gieson's elastic method showing normal distal pulmonary vessel (left) and remodeled distal vessel with double elastic lamina (right). b, Lung tissue sections immunostained for smooth muscle α -actin (red staining) and methyl green counterstain showing normal distal vessel (left) and a remodeled, muscularized vessel (right). c, Representative lung sections at low-power magnification from mice exposed to normoxia or hypoxia for 1 week. Arrows indicate vessels stained red with smooth muscle α -actin antibody. Scale bars represent 10 μ m (a, b) or 100 μ m (c). d, Vascular remodeling quantified as percentage of distal muscularized vessels per lung section at high-power magnification (* P <0.001; n=5 to 12).

lungs, causing a reduction in HPV to levels identical to those observed in WT mice. In *hph-1^{+/-}* mice, the HPV responses were intermediate between WT and *hph* mice. Strikingly, the magnitude of HPV and the effect of L-NAME were both restored to WT levels in *hph/GCH* mice, whereas HPV in *GCH* mice was further attenuated to levels below those observed in WT animals (Figure 7). These observations suggest that BH4-dependent eNOS regulation has a direct functional effect on acute HPV that is mediated through NOS coupling.

Discussion

We have identified a novel and important role for BH4 in the pulmonary circulation. Our observations, based on a number of complementary murine genetic models, provide clear evidence that endothelial BH4 availability in the pulmonary circulation regulates the pathophysiological response to hypoxia. Specifically, endothelial BH4 levels mediate susceptibility to or protection from pulmonary hypertension. We first demonstrate that BH4 deficiency results in the development of pulmonary hypertension and vascular remodeling under normoxic conditions and greatly exacerbates the response to hypoxia. Selective restoration of endothelial BH4 levels rescues the effects of systemic BH4 deficiency, whereas augmentation of endothelial BH4 biosynthesis protects against the development of hypoxia-induced pulmonary hypertension and vascular remodeling. The striking quantitative correlations between lung BH4 levels and the development of pulmonary hypertension across the 5 genetic mouse models evaluated in our studies reveal that BH4 bioavailability controls both pulmonary vascular tone and structural remodeling in a dose-dependent manner. Finally, we show that the effects of genetic alterations in BH4 availability on the pulmonary vasculature are mediated through the regulation of NO compared with superoxide synthesis by eNOS in response to both acute and chronic hypoxia.

The elevated RVSP in the BH4-deficient *hph-1* mice is at least comparable to that previously observed in mice deficient in eNOS (*eNOS^{-/-}*), with both strains showing exacerbated responses to chronic hypoxia.^{6,7,29} However, vascular remodeling, a hallmark of pulmonary hypertension, was not seen in lungs of *eNOS^{-/-}* mice in normoxia at sea-level altitudes,^{25,29} whereas vascular remodeling was clearly evident in both *hph-1* heterozygotes and homozygotes compared with WT littermates, even in normoxia. This discordance in the severity of the phenotype between genetic eNOS deficiency compared with eNOS dysfunction resulting from genetic BH4 deficiency suggests that loss of NO production alone is not the sole mediator of disease pathogenesis. Rather, our findings highlight the importance of increased eNOS-dependent superoxide production in playing a further pathogenic role in both the vasoconstrictor and remodeling processes. Indeed, vascular superoxide production has a number of potentially important effects in the vascular wall, including effects on NO signaling through scavenging and peroxynitrite generation and through modulation of redox-sensitive signaling pathways.¹³ Superoxide controls proliferation and apoptosis of pulmonary smooth muscle cells,¹⁷ and pulmonary hypertension in fetal lambs subjected to ligation of ductus

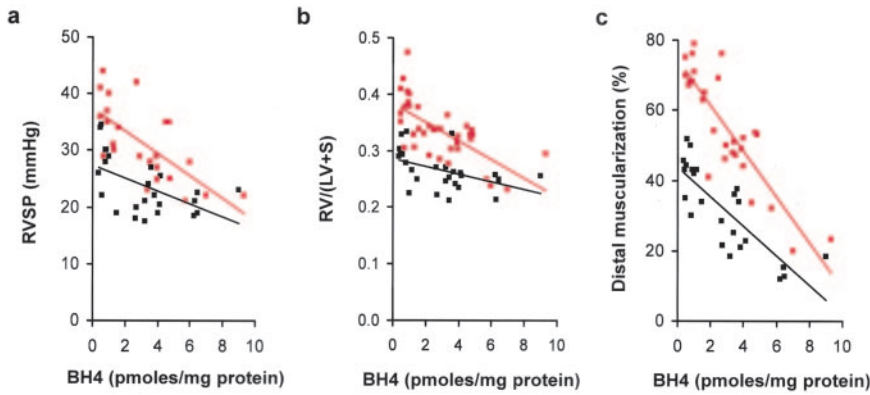


Figure 4. BH4 determines severity of pulmonary hypertension and vascular remodeling in dose-dependent manner. Shown are correlations of (a) RVSP with BH4 levels across all genotype groups in normoxia (black squares; $r=0.544$, $P<0.01$) and hypoxia (red squares; $r=0.695$, $P<0.001$), (b) RV/(LV+S) with BH4 levels in normoxia (black squares; $r=0.482$, $P<0.01$) and hypoxia (red squares; $r=0.668$, $P<0.001$), and (c) distal muscularization with BH4 levels in normoxia (black squares; $r=0.816$, $P<0.0001$) and hypoxia (red squares; $r=0.854$, $P<0.00001$).

arteriosus is associated with increased superoxide generation.¹⁴ Our findings highlight the importance of endothelial BH4 availability as a reciprocal modulator of both NO and superoxide production by eNOS and suggest a critical role for

BH4 availability in the pathogenesis of pulmonary hypertension. Thus, BH4 provides a mechanistic link between previous observations of increased NOS protein levels and reduced NO bioactivity.^{11,12} Targeting NOS regulation and enzymatic coupling, rather than eNOS protein levels or total enzymatic activity, may be a more promising therapeutic strategy in pulmonary hypertension.

The pulmonary phenotype in the eNOS^{-/-} mice may be further complicated by the fact that these mice have left ventricular hypertrophy secondary to systemic hypertension,³⁰ which is not seen in the BH4-deficient hph-1 mice. Thus, the effect of systemic BH4 deficiency, at least in hph-1 mice, appears to be more critical or specific to the pulmonary circulation as opposed to the systemic circulation. We note that this discordance was also seen in the transgenic mouse expressing a smooth muscle-specific dominant-negative BMPR-II, which developed pulmonary but not systemic hypertension, even though the SM22 smooth muscle-specific promoter directs systemic and pulmonary expression.³¹

The interplay between the various putative pathways implicated in pulmonary hypertension at both the cellular level (eg, endothelium, smooth muscle cells, platelets, macrophages, inflammatory cells, and progenitor cells) and the molecular level (eg, BMPR-II, endothelin, serine elastase, serotonin, potassium channels, angiotensin, NO, and superoxide) is complex and poorly defined.¹ It is unlikely that any single environmental factor or gene will explain all forms of pulmonary hypertension. Nevertheless, our findings underscore the central importance of endothelial dysfunction in pulmonary hypertension.⁴ The relative bioactivities of NO and reactive oxygen species are critical to maintaining pulmonary vascular homeostasis, and the balance may be shifted toward injury in pulmonary hypertension.⁵ Our studies show that BH4 has a pivotal effect on the pathogenesis of pulmonary hypertension mediated through eNOS enzymatic coupling that maintains the homeostatic balance between NO and superoxide bioactivity in the endothelium. The striking “concentration-response” relationship between lung BH4 levels and both functional and structural features of pulmonary hypertension suggests that alterations in BH4 availability modify susceptibility to or protection from pulmonary hypertension in both normoxia and hypoxia. Indeed, we observed a pulmonary hypertensive phenotype even in hph-1 heterozygotes in which the magnitude of BH4 deficiency is modest compared with hph-1 homozygotes yet is sufficient to alter

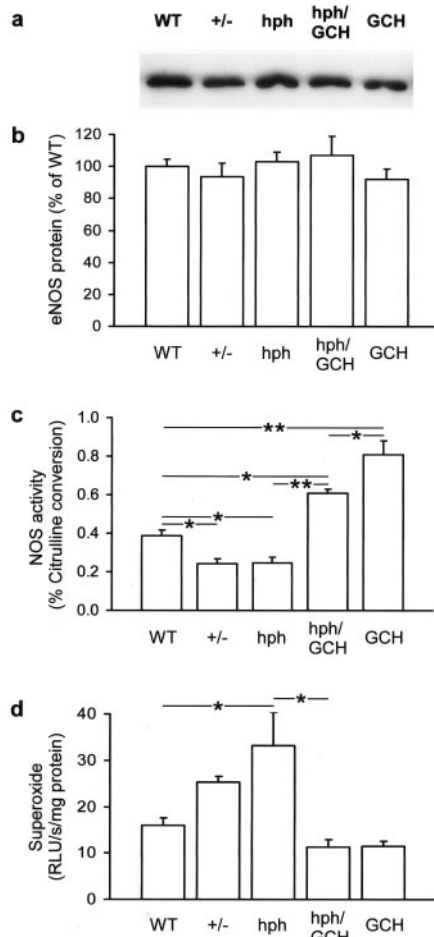


Figure 5. Effects of endothelial BH4 on pulmonary vasculature are mediated through regulation of NOS function and superoxide production. a, Western Blot showing total eNOS protein in lung homogenates from mice of each genotype. b, Total eNOS protein levels quantified as percentage of WT levels ($P=NS$ across all groups; $n=3$ to 8). c, NOS activity in lung homogenates measured by percentage of ¹⁴C-citrulline converted from ¹⁴C-arginine ($*P<0.01$, $**P<0.001$; $n=3$ to 9). d, Superoxide production in lung homogenates quantified by lucigenin-enhanced chemiluminescence and expressed as relative light units (RLU) per second per mg protein ($*P<0.01$; $n=6$ to 10).

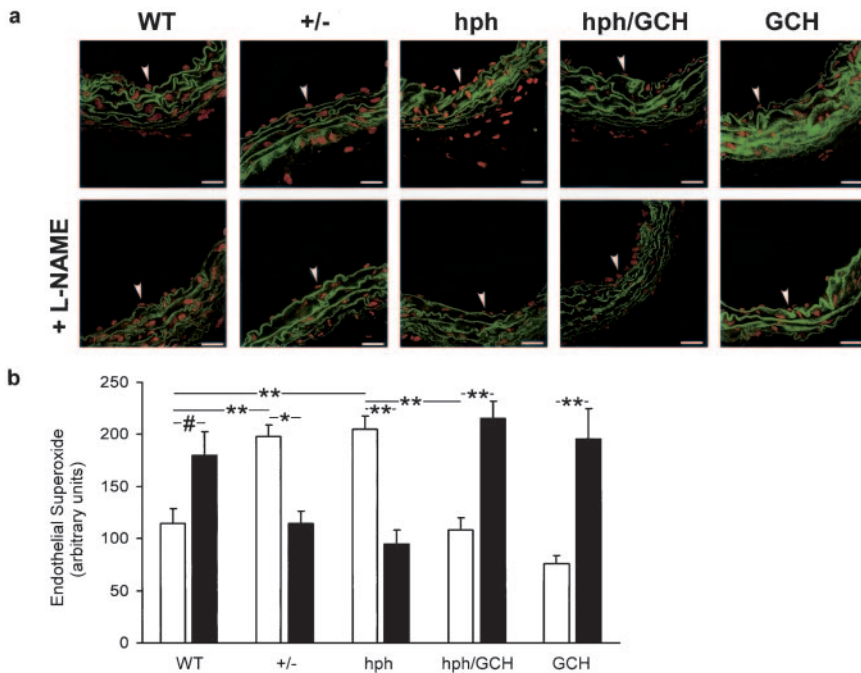


Figure 6. Endothelial BH4 regulates NOS-derived endothelial superoxide production. Pulmonary artery rings were imaged with dihydroethidium fluorescent microtopography. a, Representative sections of pulmonary artery rings incubated in absence or presence of NOS inhibitor L-NAME. Arrows show endothelial cells stained red after reaction of superoxide with dihydroethidium; elastic laminae exhibit green autofluorescence. Scale bar=20 μ m. b, Endothelial superoxide production quantified in arbitrary units as area of luminal red staining in absence or presence of L-NAME (# P <0.05, * P <0.01, ** P <0.001; n=4 to 5).

both pulmonary tone and structure. These observations compare similarly with eNOS^{+/-} (heterozygous) mice, which, like eNOS^{-/-} mice, have raised RVSP and hypersensitivity to hypoxia.⁷ Like the *eNOS* gene, our data suggest that loss of 1 allele of the *GCH1* gene encoding GTPCH, the rate-limiting enzyme in BH4 synthesis, is sufficient to produce a pulmonary vascular phenotype. Accordingly, we speculate that *GCH1* is a candidate modifier gene in pulmonary hypertension. Investigating possible associations between genetic variation in the *GCH1* gene and pulmonary hypertension may prove informative. Human *GCH1* mutations are rare but cause severe BH4 deficiency syndromes such as hyperphenylalaninemia and dopa-responsive dystonia, because BH4 is also a cofactor for enzymes involved in hepatic phenylalanine

metabolism and neuronal dopamine synthesis.²⁶ The effect of BH4 deficiency on the pulmonary circulation in these patients remains to be studied.

We note that BH4 deficiency in hph-1 mice did not lead to severe pulmonary hypertension with plexiform lesions as seen in humans,¹ even after exposure to hypoxia, and the mice do not suffer premature death. This may not be surprising, given that other recently published genetic mouse models of pulmonary hypertension do not recapitulate this extreme phenotype.^{31,32,33} Even in mice overexpressing S100A4/Mts1, only 5% of aging mice develop plexiform arteriopathy.³⁴ These observations imply that additional pathways need to be invoked for full pathogenic expression of the human disease.

A further limitation of this study is that we had not determined late changes to longer durations of hypoxia. Endothelial dysfunction may play a larger role in the initiation and early pathogenesis of pulmonary hypertension,⁴ and it is possible that endothelial BH4 augmentation alone may not fully protect against greater structural changes accompanying prolonged hypoxia, when additional molecular pathways may predominate.

Given the complex pathophysiology of pulmonary hypertension, targeting of multiple pathways with combination drug therapy may be necessary. The ability of BH4 to both augment NO synthesis and decrease superoxide production addresses 2 pathogenic mechanisms simultaneously and may be a key therapeutic target, as illustrated by the striking salutary effects of endothelial BH4 restoration or augmentation in the hph/GCH and GCH mice. Pharmacological or molecular strategies that target endothelial BH4 availability in patients with pulmonary hypertension need to be evaluated. It is also interesting to note that HMG-CoA inhibitors (statins), currently generating significant interest after their reversal of experimental pulmonary hypertension in rats,³⁵ upregulate GTPCH mRNA and BH4 levels in vascular endothelial cells.³⁶

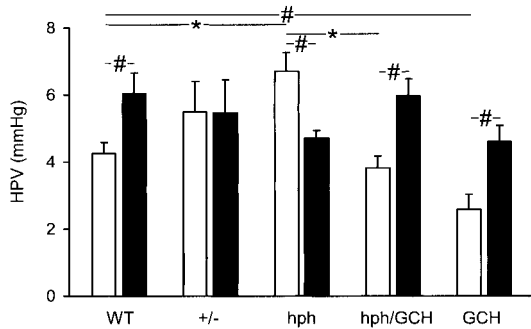


Figure 7. Regulation of NOS function by endothelial BH4 modulates acute pulmonary vasoconstriction. HPV, quantified as rise in pulmonary artery pressure after exposure to acute hypoxia (2% oxygen for 10 minutes), was assessed in intact perfused lungs in absence or presence of L-NAME. BH4 deficiency in hph mice resulted in greater hypoxic vasoconstriction because of NOS dysfunction as implied by qualitatively divergent responses to L-NAME. In addition, HPV responses were restored to WT levels by endothelial BH4 replacement in hph/GCH mice, and HPV was further reduced by endothelial BH4 augmentation in GCH mice (# P <0.05, * P <0.01; n=6 to 10).

In conclusion, we have used a range of complementary gene-modified murine models to provide compelling evidence that demonstrates a novel and important role for endothelial BH4 in the pulmonary circulation. Regulation of eNOS function by BH4, a critical mechanism in the pathogenesis of pulmonary hypertension, may offer new therapeutic opportunities in a disease that remains inadequately understood and treated.

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