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Tetrahydrobiopterin and the regulation of hypoxic pulmonary vasoconstriction

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ABSTRACT: Tetrahydrobiopterin (BH_4) is an essential cofactor for nitric oxide synthases (NOS). This study investigated the effect of increasing BH_4 levels on hypoxia-induced pulmonary vasoconstriction (HPV).

Sprague Dawley rats and hph-1 (BH_4 deficient) mice were given BH_4 before and during HPV in an isolated perfused lung preparation. BH_4 inhibited HPV in a concentration-dependent manner and increased NO metabolites in the perfusate. Bradykinin-induced reductions in HPV were blunted in hph-1 mice and pre-administration of BH_4 restored the response. The effect of BH_4 was attenuated by L-NAME (NOS inhibitor), PTIO (NO scavenger), and catalase (H_2O_2 catalyser) administered prior to HPV but enhanced by MnTMPyP (superoxide dismutase mimetic). The effect of BH_4 on HPV was partially recapitulated by NH_4, a stereoisomer that shares antioxidant properties with BH_4 but is not a NOS cofactor.

The bioavailability of BH_4 is an important determinant of the pulmonary vascular response to hypoxia. Its effects are mediated *via* nitric oxide, hydrogen peroxide and its antioxidant properties, and are attenuated by oxidant stress. Pharmacological administration of BH_4 may have therapeutic potential in pulmonary hypertension.

KEYWORDS: Animal models, hypoxia, pulmonary vasoconstriction, pulmonary hypertension, nitric oxide, superoxide, tetrahydrobiopterin

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Nitric oxide synthases (NOS), particularly endothelial NOS (eNOS), play a major role in maintaining normal pulmonary vascular tone and structure through the production of nitric oxide (NO) from L-arginine [1–3]. Critical to NO synthesis by NOS is the bioavailability of tetrahydrobiopterin (BH_4). When BH_4 is deficient through inadequate synthesis or oxidation, NOS becomes uncoupled and generates superoxide instead of NO [4].

The hph-1 mouse, generated by *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis, has low tissue BH_4 levels because of constitutively reduced expression of GTP cyclohydrolase-1, the rate-limiting enzyme in BH_4 biosynthesis [5]. Congenital deficiency of BH_4 in the hph-1 mouse leads to pulmonary hypertension, distal pulmonary vessel muscularisation and right ventricular hypertrophy [6, 7]. The pulmonary vascular pathology in hph-1 mice is more marked than that reported in NOS-deficient mice and suggests that loss of NO production alone is not the sole reason for the vascular pathology [1, 8]. In addition to reduced NO synthesis, and consistent with uncoupling of NOS, biochemical analysis of tissue homogenates from hph-1 mice shows increased local pulmonary vascular superoxide production [7]. Vascular superoxide production has a number of important actions on vascular tissue, such as scavenging of NO, peroxynitrite formation and modulation of redox-sensitive signalling pathways [9], which may adversely affect vascular structure.

There is increasing evidence of oxidative stress in pulmonary hypertension [10, 11], including reports of nitrotyrosine formation (a marker of peroxynitrite production) in the lungs of patients with the disease [12]. Superoxide and peroxynitrite oxidise BH_4, and enhanced oxidative degradation is thought to be a major cause of reduced BH_4 bioavailability and eNOS uncoupling in endothelial dysfunction states [13–15]. As a result, there is interest in using exogenous BH_4 therapeutically in pulmonary hypertension to restore tissue levels and promote NO production from NOS. Recent studies suggest that BH_4 may also act independently of NO, generating hydrogen peroxide, another molecule with vasorelaxant properties [16, 17].

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The purpose of this study was to investigate the pharmacological effects of BH_4 on pulmonary vascular tone, specifically hypoxia-induced vasoconstriction (HPV) in an *in situ* isolated perfused lung preparation. Employing the BH_4 deficient hph-1 mouse, we explored further the underlying mechanism of BH_4 in balancing NO and superoxide production in pulmonary vasculature.

METHODS

Animals

Male Sprague-Dawley rats (250–360 g) from Charles River (Margate, UK) and hph-1 mice aged 3–5 months generated by ENU mutagenesis were used for experiments. Wild-type (WT) animals, hph-1 heterozygous (+/-) and hph-1 homozygous (hph-1) littermates on a C57BL/6 background were obtained by interbreeding hph-1 heterozygotes [5]. All studies were conducted in accordance with UK Home Office Animals (Scientific Procedures) Act 1986.

Reagents

6R-BH_4 (sapropterin dihydochloride or BH_4) was supplied by BioMarin Pharmaceuticals, Inc. (Novato, CA, USA). U46619 (9, 11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$), L-NAME (N_ω-nitro-L-arginine methyl ester hydrochloride), Catalase, MnTMPyP (Mn(III) tetrakis(1-methyl-4-pyridyl) porphyrin pentachloride), PTIO (2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide), bradykinin (BK) and chemical components of the perfused solutions were obtained from Sigma-Aldrich (Poole, UK). NH_4 (6R, S-5,6,7,8-tetrahydro-D-neopterin) was from Schircks Laboratories (Jona, Switzerland). All drugs were prepared fresh every day before experiments. 10 $\mu g \cdot uL^{-1}$ BH_4 or NH_4 were dissolved in HEPES buffered saline (composition: NaCl 137 mM, KCl 4.0 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 1 mM, HEPES 10 mM, glucose 10 mM; pH adjusted to 7.4 with NaOH).

Isolated perfused rat lung preparation

The pulmonary vascular response to BH_4 was studied using the isolated rat lung preparation previously described [18]. Briefly rats were anaesthetised with Hypnorm (fentany-fluanisone). The lungs were left *in situ* after the trachea had been cannulated, ventilated with air (21% O_2, 5% CO_2 balanced N_2) at constant end expiration pressure of 4 mmHg with 60 breaths·min^{-1}; and perfused with a mixture (1:1) of DMEM (Dubelcco's Modified Eagles Medium) with 4% Ficoll, and blood withdrawn from the experimental animal (pH 7.4) at a flow rate of 18 mL·min^{-1} using a non-pulsatile pump (Masterflex model 7519; Cole-Parmer Instrument Co. Ltd, London, UK). Pulmonary artery pressure (P_{pa}) was measured using a pressure transducer connected to PowerLab Data Acquisition system (ADInstruments Limited, Chalgrove, UK). HPV was produced by ventilating the lung with 2% O_2, 5% CO_2 and 93% N_2 for 15 min. The ventilation was then returned to air for 10 min to resume the PAP baseline before the next hypoxic challenge. Successive hypoxic challenges (usually 3) were repeated until the HPV response was consistent before vehicle or BH_4 treatment was given to examine the effect on HPV.

BH_4

BH_4 (final perfusate concentration 0.3 $\mu g \cdot mL^{-1}$) was added to the perfusate ~4 min before the hypoxic challenge. Once the pressor response had returned to baseline, the hypoxic challenge was repeated with or without a second aliquot of BH_4 (calculated to produce perfusate concentrations of 0.3 or 1 $\mu g \cdot mL^{-1}$) added to the perfusate (fig. 1). Perfusate samples (100 μL) were collected after passage through the lungs 1 min prior to and 4 min after BH_4 administration and before the hypoxic challenge for measurement of nitrate/nitrite (NO_x) concentration. The effect of BH_4 was calculated as the per cent change in HPV response and NO_x levels after BH_4 administration compared to the HPV response and NO_x levels before BH_4 administration in the same animal.

Isolated perfused mouse lung preparation

Animals were anaesthetised and the lungs ventilated with air (volume controlled ventilation, 230 μL tidal volume, 80 breaths·min^{-1} and 2 cmH_2O positive end-expiratory pressure) *via* the trachea. The lungs were perfused through the main pulmonary artery accessed *via* the right ventricle and perfusate collected from the left atrium for recycling in a closed circuit (type 839; Hugo Sachs Electronik-Harvard Apparatus GmbH, March-Huggstetten, Germany). Using a nonperistaltic pump, the perfusate (DMEM with 4% Ficol, HEPES-buffered, pH 7.4) was delivered at an initial flow of 0.2 mL·min^{-1}, increasing in increments after 10 min to 2 mL·min^{-1}. The reservoir volume was set at 10 mL and pH monitored by the pH sensor placed in the reservoir. P_{pa} was measured using a pressure transducer connected to the inflow catheter. Left atrial pressure was

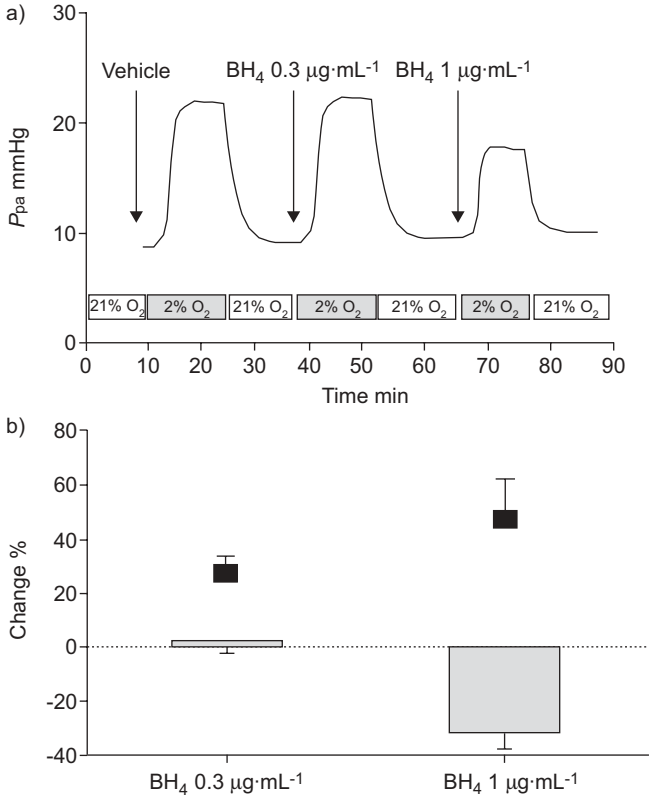

FIGURE 1. a) Representative pulmonary arterial pressure (P_{pa}) trace illustrating the protocol for tetrahydrobiopterin (BH_4) administration prior to induction of hypoxia-induced pulmonary vasoconstriction (HPV) in the rat isolated perfused lung. b) The effect of BH_4 on HPV response (reduction expressed as a percentage of HPV before BH_4 administration in the same animal, ■) and percentage increase in perfusate NO_x levels (■) following BH_4 administration (n=5).

measured using a pressure transducer connected to the outflow catheter. Pressure tracings were collected and analysed using PowerLab Data Acquisition system. U46619 (thromboxane analogue; 5 μM) was added to reservoir to induce basal pulmonary vasoconstriction. 2 min after U46619 administration, a stable P_{pa} was achieved and recording was continued for a further 6 min before HPV was induced by ventilating the lung with 2% O_2, 5% CO_2 and 93% N_2. Ventilation was returned to air until P_{pa} returned to baseline (fig. 2a).

BH_4 and NH_4

Vehicle (fig. 2a), BH_4 (1, 10 and 100 μg·mL^{-1}; fig. 2b) or NH_4 (100 μg·mL^{-1}) was added to the perfusate during stable HPV. The effect of BH_4 and NH_4 was calculated as the P_{pa} reduction (ΔP_{pa}) 3 min after drug administration expressed as a percentage of the maximum HPV response (HPV_{max}) in the same hypoxia challenge.

Bradykinin

BK (10 ng·mL^{-1}) was administered during stable HPV in WT, +/- and hph-1 mice (fig. 3). Futhermore, in separate sets of hph-1 mice, BH_4 (100 μg·mL^{-1}) was added to reservoir 3 min before 10 ng·mL^{-1} of BK. The response to BK was calculated as the maximum reduction in P_{pa} (usually within 1 min of BK administration) expressed as a percentage of the HPV_{max} in the same hypoxia challenge.

Inhibition of NO and superoxide

In separate experiments, L-NAME (100 μM), catalase (200 U·mL^{-1}), MnTMPyP (1 μM) and PTIO (1 μM) was added to the perfusate 3 min before inducing HPV, followed by BH_4 (100 μg·mL^{-1}) administration during stable HPV (fig. 4). BH_4 effect was calculated as P_{pa} reduction (ΔP_{pa}) 3 min after BH_4 administration expressed as a percentage of the HPV_{max} in the same hypoxia challenge.

Measurements of nitrate/nitrite (NO_x)

Perfusate samples were centrifuged at 1000 × *g* for 4 min. The supernatant was immediately removed and stored at -20°C until analysis. NO_x concentration was measured in duplicate using an ozone-based chemiluminescence method [19]. Briefly, prior to analysis the samples were treated with a 1:1 volume of cold ethanol and centrifuged at 14,000 × *g* for 5 min. NO_x levels were measured by injecting 50 μL of the supernatant in a glass purge vessel containing vanadium(III) in 1 N hydrochloric acid at 90°C, which reduced NO_x to NO gas. A nitrogen stream was bubbled through the purge vessel containing vanadium(III), first through 1 N NaOH, and then into a NO analyser (Sievers Model 280 NO Analyser; Sievers Instruments, Boulder, CO, USA), which detected NO released from NO_x by chemiluminescent detection. Results are presented as the percentage of the NO_x levels after BH_4 administration in the perfusate compared with the levels prior to administration.

Statistical analysis

In all experiments, results are expressed as mean ± SEM; n equals the number of mice or rats per experiment. Statistical analysis of the data was performed by using unpaired t-test when appropriate, nonparametric test with Man–Whitney modification, or one-way ANOVA when applied to multiple group comparisons. A value of $p<0.05$ was considered statistically significant.

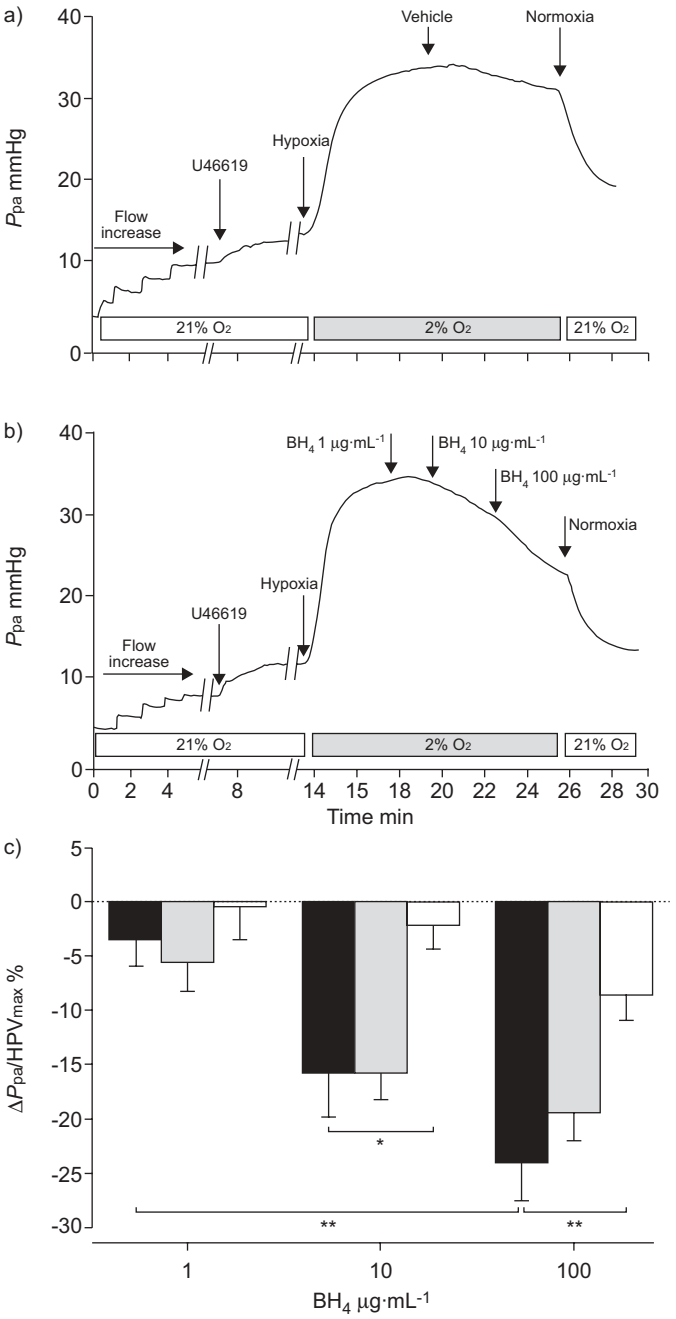

FIGURE 2. Representative traces demonstrating the protocol in isolated mouse lung and showing the effects of a) vehicle and b) tetrahydrobiopterin (BH_4) administration on pulmonary arterial pressure (P_{pa}) during hypoxic pulmonary vasoconstriction (HPV) in wild-type (WT) mice. c) Vasorelaxant effect of BH_4 administration (1, 10 and 100 μg·mL^{-1} perfusate) on P_{pa} during HPV in WT (■), heterozygous (▩) and hph-1 (□) mice. The effect of BH_4 was calculated as P_{pa} reduction (ΔP_{pa}) 3 min after BH_4 administration expressed as a % of the maximum HPV response (HPV_{max}) in the same hypoxia challenge. *: $p<0.05$; **: $p<0.01$. n=3–9.

RESULTS

BH_4 attenuates HPV in the isolated perfused rat lung

The effect of increasing concentration of BH_4 on HPV and NO_x levels was first examined in the isolated perfused rat lung.

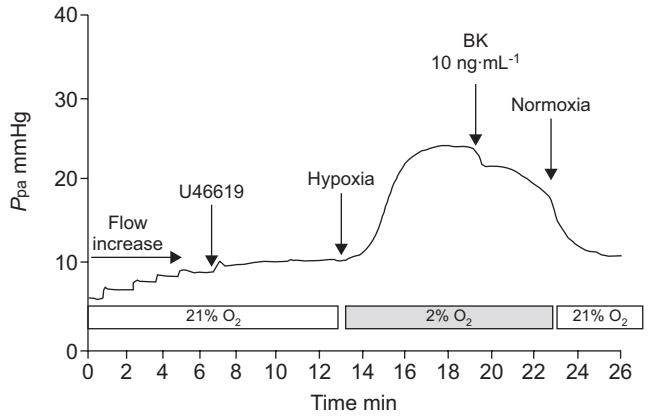

FIGURE 3. Representative trace of bradykinin (BK) effect on pulmonary artery pressure (P_{pa}) during hypoxic pulmonary vasoconstriction (HPV) in the isolated perfused mouse lung. BK administration induces an abrupt reduction followed by a gradual decline in P_{pa}. The effect of BK was calculated as the reduction (at 1 min) in P_{pa} expressed as a percentage of the maximum HPV response in the same hypoxia challenge.

Consistent HPV responses were established (11.6±2.8 mmHg, n=15) in each lung preparation before exposure to exogenous BH_4. BH_4 at 1 μg·mL^{-1}, but not 0.3 μg·mL^{-1}, attenuated the pressor response to hypoxia (-31.9%, HPV 8.5±2.7 mmHg *versus* 12.4±2.8 mmHg with or without BH_4 1 μg·mL^{-1}, respectively; p<0.01, n=5; fig. 1). Perfusate NO_x levels were increased at both BH_4 concentrations (27±6% and 47±15%, respectively; fig. 1b).

BH_4 attenuates HPV in the isolated perfused mouse lung

To explore the effect of restoring BH_4 in the context of impaired endogenous synthesis of the cofactor, studies were conducted in WT, hph-1 heterozygous (+/-) and hph-1 homozygous (hph-1) mice. Hypoxia induced similar maximum rises in P_{pa} in each of the mouse genotypes: 17.1±1.3 mmHg in WT, 14.7±1.1 mmHg in heterozygous and 16.1±1.0 mmHg in hph-1 (n=12–15). Compared to vehicle (fig. 2a), BH_4 1, 10 and 100 μg·mL^{-1} administered during stable HPV induced concentration-dependent reductions in P_{pa} (fig. 2b). Data from all three genotypes are summarised in figure 2c. BH_4 produced greater falls in WT than in hph-1 mice at doses of 10 and 100 μg·mL^{-1} (fig. 2c).

BH_4 restores the response to BK in hph-1 mouse

In order to examine endothelial function in hph-1 mice, the vasorelaxant response to BK was investigated in the isolated perfused lung (fig. 3). BK administration to achieve a perfusate concentration of 10 ng·mL^{-1} during stable HPV reduced P_{pa} in WT (-12.1±1.8%) and heterozygous (-9.7±1.2%) mice, but had no detectable effect in hph-1 mice (0±1.7%) (table 1). BH_4 (100 μg·mL^{-1}) given prior to BK restored the response to BK in hph-1 mice (-11.7±2.4%).

The effect of L-NAME, catalase, MnTMPyP and PTIO on the response to BH_4 in mice

To further dissect the mechanism by which BH_4 regulates pulmonary vascular tone, the roles of NO and reactive oxygen species were investigated. In this set of experiments, the maximum HPV response before treatment was in the range of 8–10 mmHg in all the three genotypes (see initial HPV, table 2). L-NAME, catalase, MnTMPyP and PTIO had minor effects on the baseline normoxic P_{pa} (-0.5 to 1.1±0.5 mmHg) and these were similar among the three genotypes (see table 1, online supplementary material).

L-NAME and BH_4

NOS inhibitor L-NAME (100 μM) administration enhanced the HPV response in all three genotypes (table 2), and reduced the effect of BH_4 (100 μg·mL^{-1}) in WT (-20±3% *versus* -11±1.7%, in the absence and presence of L-NAME, respectively; p<0.05; fig. 4a).

Catalase and BH_4

Pretreatment with catalase increased the HPV response significantly in heterozygous and hph-1 mice, but not in WT mice (table 2). It attenuated the effect of BH_4 in WT mice significantly (-20±3% *versus* -7.8±2%; p<0.01; fig. 4b), and to a lesser degree in heterozygous and hph-1 mice.

MnTMPyP and BH_4

The intracellular superoxide dismutase mimetic MnTMPyP did not affect HPV (table 2). It enhanced the vasodepressor effect of BH_4 significantly in all the genotypes and almost returned P_{pa} to baseline levels. The net change was greater in hph-1 animals (-32.5±3% *versus* -7.9±2.9%, in the presence and absence of MnTMPyP, respectively; p<0.01) than in WT mice (-34.8±2.8% with MnTMPyP *versus* -20±3% without; p<0.01; fig. 4c).

PTIO and BH_4

Pretreatment with an NO scavenger, PTIO, increased HPV significantly in WT mice (table 2) and attenuated the subsequent BH_4-induced reduction in P_{pa} (-20.5±3% *versus* -7.3±0.9%; p<0.01; fig. 4d).

The effect of NH_4 on HPV response

To evaluate the antioxidant action of BH_4, its stereoisomer NH_4 was used for comparison in the isolated perfused lung WT mouse lung. NH_4 100 μg·mL^{-1} produced a smaller fall in P_{pa} (-6.4±2.3%) than BH_4 100 μg·mL^{-1} (-31%; p<0.01; fig. 4e).

DISCUSSION

In the present study, exogenous BH_4 attenuated the acute pulmonary vascular pressor response to hypoxia in a concentration-dependent manner. There appear to be three mediators of the effect: increased NO production, a direct antioxidant action and H_2O_2. Evidence that the response to BH_4 is mediated in part by coupling NOS to increase NO production and improve pulmonary endothelial function comes from: 1) the associated increase in NO (NO_x levels) in the perfusate; 2) the observation that BH_4 restored the response to BK, an endothelium-dependent vasodilator, in the hph-1 mouse; and 3) that the effect was inhibited by pretreatment of L-NAME, a NOS inhibitor, and PTIO, a NO scavenger. A direct antioxidant effect from BH_4 is inferred from the response to NH_4, a BH_4 stereoisomer that has antioxidant properties but which is not a NOS cofactor. A role for H_2O_2 is supported by the observation that catalase, which promotes the decomposition of H_2O_2 to oxygen and water, attenuated the vasorelaxant effect of BH_4. Conversely, the effect of BH_4 is reduced by increased superoxide production, as seen in the hph-1 mouse.

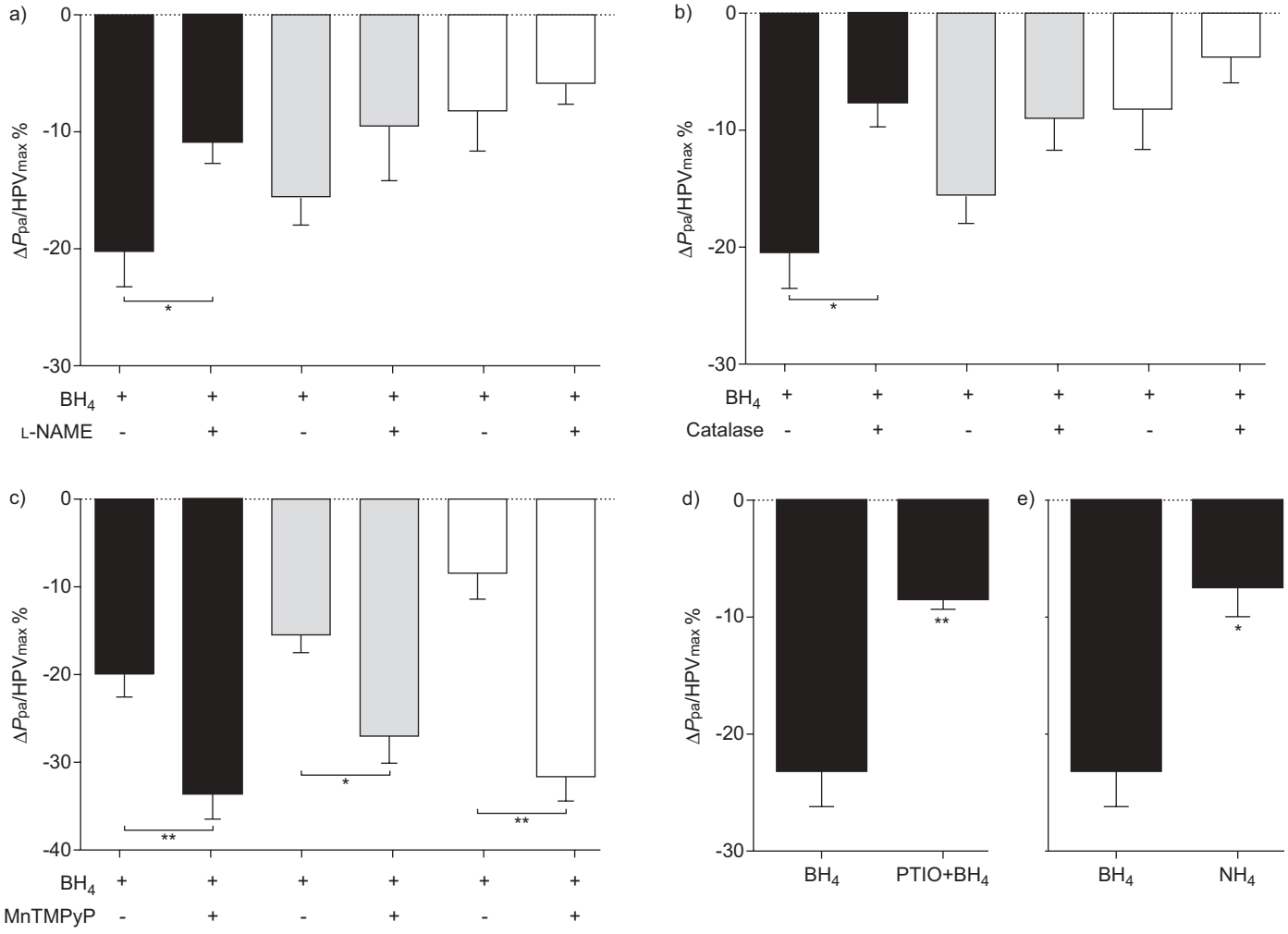

FIGURE 4. The vasorelaxant effect of tetrahydrobiopterin (BH_4) on pulmonary artery pressure (P_{pa}) during hypoxic pulmonary vasoconstriction (HPV) in the isolated perfused mouse lung administered before and after a) L-NAME (N_ω-nitro-L-arginine methyl ester hydrochloride; 100 μM; n=3–10), b) catalase (200 U·mL^{-1}; n=3–10) and c) MnTMPyP (Mn(III) tetrakis(1-methyl-4-pyridyl) porphyrin pentachloride; 1 μM; n=3–10) in wild-type (WT; ■), heterozygous (▩) and hph-1 (□) mice, and d) PTIO (2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide; 10 μM, n=4–7) in WT mice. *: $p<0.05$; **: $p<0.01$. The effect of BH_4 is calculated as the P_{pa} reduction (ΔP_{pa}) 3 min after BH_4 administration expressed as a percentage of the maximum HPV response (HPV_{max}) in the same hypoxia challenge. e) The effect of BH_4 and NH_4 (6R, S-5,6,7,8-tetrahydro-D-neopterin; 100 μg·mL^{-1}) on P_{pa} administered during HPV in WT mice. *: $p<0.05$ compared with BH_4 effect (n=3–7 each group).

Prior treatment with MnTMPyP, an intracellular superoxide dismutase mimetic, restored the response to BH_4 in hph-1 mice and reversed HPV to near baseline P_{pa} levels.

HPV is a characteristic of the pulmonary circulation, in which resistance vessels constrict in response to alveolar hypoxia in order to maintain perfusion/ventilation matching [20]. NO synthesis contributes to this vasoregulatory mechanism [21]. For example, reduced NO bioavailability through inhibition of NOS with L-NAME or NO scavenging with PTIO augments HPV. Conversely, increased NO synthesis attenuates HPV. Attenuation of HPV with BH_4 in the isolated perfused rat lung is consistent with the known biochemistry of BH_4 as an essential cofactor for NOS dimerisation, stability and electron transfer during arginine oxidation [22]. Interestingly, in a previous experiment we showed that hph-1 mice, which are relatively deficient in BH_4, were more sensitive to hypoxia [7]. In this study, we sought to maximally stress the pulmonary vasculature with the vasoconstrictor U46619 as well as hypoxia, and achieved similar maximal responses to HPV in all three genotypes (WT, and heterozygous and homozygous hph-1 mice).

L-NAME and PTIO, at doses described previously to cause full inhibition of the NO system [23, 24], only partially blocked the effects of BH_4 on P_{pa} during the stable phase of HPV in WT mice, indicating that ''NOS coupling'' is not the sole mechanism by which BH_4 modulates HPV. In addition to uncoupled NOS, the generation of superoxide and free radicals from NADPH oxidase and the mitochondrial electron transport system has been implicated in HPV. BH_4 is a potent reducing agent [25] and has been shown to prevent ischaemia reperfusion injury through its antioxidant properties rather than acting as a NOS cofactor [26]. To address this contribution to its effect on HPV, we examined the effect of NH_4, an equipotent antioxidant analogue of BH_4 with no cofactor activity. At the same

TABLE 1 Effect of bradykinin (BK) on pulmonary artery pressure (P_{pa}) during hypoxia-induced pulmonary vasoconstriction (HPV) in wild-type (WT), heterozygous and homozygous hph-1 mice[#]

	ΔP_{pa} mmHg (HPV response)	Decrease in ΔP_{pa}/HPV % (BK effect)
WT	17.8±2.3	12.1±1.8
Heterozygous	15.6±0.6	9.7±1.2
Hph-1 without BH_4	15.8±0.8	0.0±1.7
Hph-1 with BH_4	17.9±2.0	11.7±2.4

[#]: n=3–4 each group. The effect of BK (10 ng·mL^{-1}) on HPV in hph-1 mice was measured before and after tetrahydrobiopterin (BH_4) (100 μg·mL^{-1}). The maximum HPV response (initial ΔP_{pa}) was not significantly different among the three genotypes.

concentration, the reduction in P_{pa} produced by NH_4 was ~30% of that produced by BH_4. Whether the antioxidant effect of BH_4 is related mainly to reduction of superoxide or the nitrosyl radical (peroxynitrite), and how these reactions might influence the total effect of BH_4 on pulmonary pressure, is unknown and needs further investigation.

H_2O_2 is also a mediator of the effect of BH_4 on P_{pa}. The NOS family is a major source of vascular H_2O_2, derived from superoxide by the action of various superoxide dismutases. H_2O_2 has been proposed to be an endothelium-derived hyperpolarising factor (EDHF). Recent data suggest that H_2O_2 may be an important regulator of vascular homeostasis in small vessels [27]. EDHF-mediated relaxation and hyperpolarisation of mesenteric arteries in response to acetylcholine is reduced progressively in eNOS, n/eNOS, and n/i/eNOS deficient mice as the number of disrupted NOS genes increases [27]. The vascular effects of H_2O_2 are unmasked by BH_4 deficiency, where superoxide production is increased and NO synthesis reduced [7]. Catalase inhibits endothelium-dependent relaxation in the aorta of hph-1, but not WT mice [28] and significantly augmented HPV in heterozygous and hph-1, but not in WT mice in our study.

TABLE 2 Effect of inhibiting nitric oxide, superoxide and hydrogen peroxide bioavailability on hypoxic pulmonary vasoconstriction (HPV) in isolated perfused mouse lung

Experimental protocol	ΔP_{pa} mmHg (HPV response)		
	WT	Heterozygous	Hph-1
Maximum HPV	7.2±2.1	8.0±2.1	7.5±0.9
HPV + L-NAME (100 μM)	13.4±4.2[#]	11.1±0.9[¶]	12.8±0.7*
HPV + PTIO (10 μM)	9.8±0.7[#]		
HPV + catalase (200 U·mL^{-1})	10.3±2.4	10.7±3.2[¶]	11.7±3.1*
HPV + MnTMPyP (1 μM)	9.1±1.8	9.1±2.1	8.8±0.5

ΔP_{pa}: reduction in pulmonary arterial pressure; WT: wild type; L-NAME: N_ω-nitro-L-arginine methyl ester hydrochloride; PTIO: 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide; MnTMPyP: Mn(III) tetrakis(1-methyl-4-pyridyl) porphyrin pentachloride; [#]: $p<0.05$ compared with WT controls; [¶]: $p<0.05$ compared with heterozygous controls; *: $p<0.05$ compared with hph-1 controls.

Paradoxically, increasing BH_4 levels also increases H_2O_2. It is now accepted that the NOS family produce superoxide under normal conditions, not just when uncoupled. BH_4 enables both superoxide and NO production from NOS and increases H_2O_2 production [24]. It may also generate H_2O_2 directly following oxidation by molecular oxygen in aqueous solution [17]. Catalase inhibited BH_4 induced vasorelaxation in mouse cerebral arteries [24] and rabbit iliac arteries, and inhibited the vasodilatory effect of BH_4 on HPV in WT mice in our study. The mechanism by which H_2O_2 produces its vasorelaxant effects includes activation of soluble guanylate cyclase [29], stimulating the production of prostanoids [30] and sarcoplasmic endoplasmic reticulum Ca^{2+} ATPase [16].

Conversely, oxidation of BH_4 limits its bioavailability and so efficacy. Peroxynitrite, the product of superoxide and NO, oxidises BH_4 to BH_2 with 10 times greater potency than it reacts with other cellular oxidants, such as ascorbic acid and

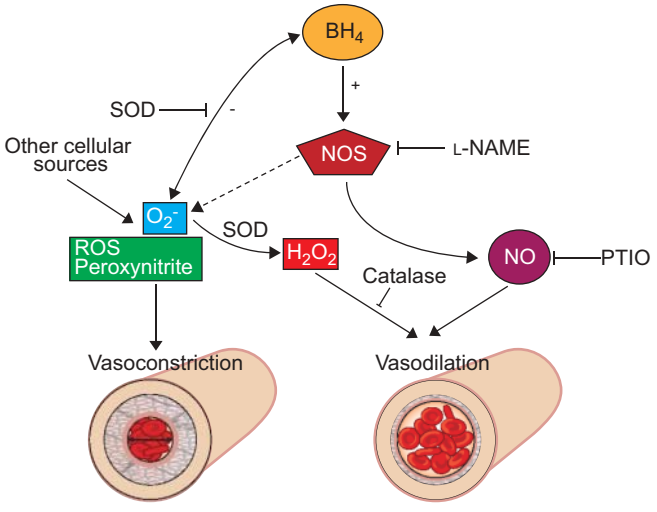

FIGURE 5. Schematic representation of the proposed mechanisms by which tetrahydrobiopterin (BH_4) regulates pulmonary vascular tone. BH_4 mediates its effects *via* nitric oxide synthase (NOS) coupling to balance the generation of nitric oxide and superoxide, and consequently the formation of hydrogen peroxide and peroxynitrite. BH_4 also has antioxidant activity. The effect of BH_4 is attenuated in the presence of oxidative stress and antioxidant agents, such as superoxide dismutase (SOD), augment its efficacy. The overall net effect of BH_4 is vasorelaxation. L-NAME: N_ω-nitro-arginine methyl ester hydrochloride; ROS: reactive oxygen species; PTIO: 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide.

glutathione [31]. Oxidised BH_2 competes with BH_4 for NOS but does not act as a cofactor, thereby fostering the uncoupling of NOS [32] and further exacerbating NOS dysfunction. Excess superoxide production previously reported in hph-1 mice [7] and oxidation of BH_4 likely impaired its effect on HPV in heterozygous and hph-1 mice. The addition of MnTMPyP to the perfusate prior to the hypoxic challenge enhanced the vasodilatory effect of BH_4 in all WT, heterozygous and hph-1 mice. These data are consistent with the observations of FARROW *et al.* [33], that recombinant human superoxide dismutase treatment increased eNOS activity and expression, elevated BH_4 levels, decreased oxidative stress and induced pulmonary vasodilation in the persistent pulmonary hypertension model of neonatal lambs.

A limitation of this study is the lack of direct biochemical measurement of superoxide and H_2O_2 levels coincident with the biochemical manipulations. There are sufficient data from published work to support the interpretations made [24, 28, 33–35]. The absence of these measurements, however, does not permit comment on the extent to which pharmacological manipulation of each contributes to the effects observed.

In summary, BH_4 participates in the regulation of the pulmonary vascular response to hypoxia through the generation of NO and superoxide, and consequently the formation of hydrogen peroxide and peroxynitrite (fig. 5). The net effect of pharmacological supplementation with BH_4 is to inhibit the pressor response. The effect is attenuated in the presence of oxidative stress and combining BH_4 with an antioxidant strategy (for example, a superoxide dismutase mimetic) augments the efficacy of BH_4 treatment. Pharmacological administration of BH_4 may have therapeutic potential in pulmonary hypertension and warrants further investigation.

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STATEMENT OF INTEREST

A statement of interest for M.R. Wilkins and for the study itself can be found at www.erj.ersjournals.com/misc/statements.dtl

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