

Pulmonary Hypertension in a GTP-Cyclohydrolase 1-Deficient Mouse

Manasi Nandi, PhD; Alyson Miller, PhD; Raymond Stidwill; Thomas S. Jacques, PhD, MRCP;
Amanda A.J. Lam, PhD; Sheila Haworth, FRCP;
Simon Heales, PhD, FRCPath; Patrick Vallance, FRCP, FMed Sci

Background—GTP-cyclohydrolase 1 (GTP-CH1) catalyzes the first step for the de novo production of tetrahydrobiopterin (BH₄), a cofactor for nitric oxide synthase (NOS). The hyperphenylalaninemic mutant mouse (hph-1) displays a 90% reduction in GTP-CH1 activity. Reduced BH₄ decreases NOS activity and may lead to endothelial dysfunction, and there is increasing evidence that a dysfunction of the NOS pathway may be implicated in pulmonary hypertension. The aim of the study was to investigate whether reduced BH₄ in the hph-1 mouse results in a pulmonary hypertensive phenotype.

Methods and Results—Morphological characterization of the heart, lung, and kidney and measurements of systemic and right ventricular blood pressures were performed in both hph-1 and wild-type mice. BH₄ and NO_x levels were also measured. Hph-1 mice had significantly lower NO_x and BH₄ levels, consistent with previous findings. Both morphological and in vivo data were indicative of a pulmonary but not systemic hypertensive phenotype. We observed increased right ventricle–left ventricle plus septum ratios attributable only to an increase in right ventricular mass, increased smooth muscle medial area in pulmonary resistance vessels, and significantly higher right ventricular pressures in vivo. There were no significant differences between left ventricular masses and systemic pressures, and there was no observed evidence of systemic hypertension in kidney sections between wild-type and hph-1.

Conclusions—This study demonstrates that mice deficient in GTP-CH1/BH₄ display a pulmonary hypertensive but not systemic hypertensive phenotype. (*Circulation*. 2005;111:2086-2090.)

Key Words: hypertension, pulmonary ■ nitric oxide synthase ■ remodelling

The enzyme GTP-cyclohydrolase 1 (GTP-CH1) catalyzes the first step for the de novo production of tetrahydrobiopterin (BH₄), a cofactor for nitric oxide synthase (NOS).^{1,2} Supplementation of BH₄ restores endothelial function in a number of human and animal disease models,^{3–5} but less is known about the functional consequences of BH₄ deficiency in blood vessels in vivo.

See p 2022

Recently, we found that GTP-CH1 is developmentally regulated in the pulmonary vasculature of the pig and that in the first few weeks of life, endothelium-dependent relaxation of pulmonary artery is enhanced by addition of BH₄ (M.N., unpublished data, 2001). These observations suggested that BH₄ might affect pulmonary vascular function and that loss of BH₄ would impair NO responses. Because endothelial NOS (eNOS) protein is decreased in certain patients with primary pulmonary hypertension,⁶ and eNOS-knockout mice display significantly elevated right ventricular (RV)/pulmonary pressures under normoxia,^{7,8} increased RV pressures and remod-

eling after hypoxia,⁸ and a reduction in pulmonary pressures after adenoviral transfection of eNOS,⁷ we hypothesized that BH₄ deficiency would result in a pulmonary hypertensive phenotype. To test this, we have studied the hyperphenylalaninemic mutant mouse (hph-1), a model that has a 90% reduction in GTP-CH1 activity.⁹

Methods

Tissue Collection for Morphological Characterization

Experiments were performed under a Home Office License and conducted according to the Animals Scientific Procedures Act 1986, United Kingdom. Twelve- to 17-week-old (≈ 25 g), sex-matched wild-type mice (C57BL/6 \times CBA [WT]) and homozygous hph-1 mice were studied.

Perfusion and Fixing of Tissues for Paraffin Sectioning

Airway perfusion with 10% buffered formol saline via a cannula at a perfusion pressure of 20 cm H₂O was performed, and fixed lungs were cut into transverse sections and dehydrated in 70% ethanol before being processed in a Hypercenter XP enclosed tissue proces-

Received June 15, 2004; revision received January 14, 2005; accepted January 20, 2005.

From the Centre for Clinical Pharmacology (M.N., A.M., R.S., S. Haworth, P.V.), British Heart Foundation Laboratories, The Rayne Institute, University College London, and the Division of Neuropathology (T.S.J.), Neurometabolic Unit and Division of Neurochemistry (A.A.J.L., S. Heales), Institute of Neurology and National Hospital for Neurology and Neurosurgery, London, United Kingdom.

Correspondence to Manasi Nandi, Centre for Clinical Pharmacology, British Heart Foundation Laboratories, The Rayne Institute, University College London, 5 University St, London WC1E 6JJ, United Kingdom. E-mail m.nandi@ucl.ac.uk

© 2005 American Heart Association, Inc.

Circulation is available at <http://www.circulationaha.org>

DOI: 10.1161/01.CIR.0000163268.32638.F4

sor. Kidneys were immersion fixed in formol saline and cut into 3 to 4 transverse and longitudinal sections before dehydration and processing. All subsequent histological analysis was performed by an individual blind to the mouse type.

Measurement of Pulmonary Arterial Medial Area

Standard immunohistochemical staining of 4- μm paraffin wax sections was performed with a mouse α -smooth muscle actin primary antibody 1:3000 dilution (DAKO). Light microscopy (Zeiss Axioskop2) was used to anatomically identify ≈ 10 to 15 arteries per animal at the level of the alveolar ducts, respiratory bronchioles, and terminal bronchioles, and the arterial smooth muscle medial area was measured with Open Laboratory Software version 3.04 (Improvision). The medial smooth muscle area was expressed as a percentage of the total external area of the vessel. Extension of muscle into normally nonmuscularized arteries (<25 μm) was assessed as described previously into nonmuscular, partially muscular, and fully muscular arteries.⁸

Histological Examination of Pulmonary Sections

Standard hematoxylin-and-eosin staining and elastic van Gieson staining were performed on 4- μm lung sections and analyzed for evidence of any structural abnormalities, such as fibrosis and thickening of the alveolar septa, or for any evidence of edema that may have occurred secondary to left ventricular (LV) dysfunction.

Histological Examination of Kidneys

Kidney sections (4 μm) were stained with hematoxylin and eosin and elastic van Gieson and analyzed for evidence of renal damage caused by sustained systemic hypertension.

Measurement of Ventricular Weight

The RV was dissected from the LV plus septum (LV+S) and each component weighed separately. The RV:LV+S ratio was calculated. Additionally, RV and LV+S were indexed to body weight.

Hemodynamic Measurements

hph-1 and WT mice were anesthetized with 5% halothane (Abbot Laboratories) and placed supine on a thermostatically controlled heating blanket (37°C). Anesthesia was maintained with 2% to 3% halothane inhalation through a small mask. To measure systemic blood pressure, the right carotid artery was isolated and a fluid-filled (heparin; 100 U/mL diluted in 0.9% saline), 0.28-mm internal-diameter cannula (Critchley Electrical Products Pty Ltd) introduced into the artery. To measure RV pressure, the right jugular vein was isolated and a fluid-filled cannula introduced into the heart and advanced into the RV. After a 5-minute stabilization period, mean systemic and RV pressures were measured and analyzed with MacLab version 3.4/e and Chart 4 Windows software.

BH₄ Measurements

In a separate set of mice from the same generation as those used in the present study, BH₄ levels were measured in the brain, liver, and kidney with high-performance liquid chromatography and electrochemical detection, as described previously.⁹

NO_x Measurements

Serum samples were analyzed for NO_x levels. Briefly, whole blood was obtained by cardiac puncture and centrifuged at 12 000g at 4°C and the serum (supernatant) removed. Serum samples were centrifuged in 0.5- μm centrifuge filters (Millipore) at 12 000g for 1 hour. Plasma NO_x was measured by a modified Griess reaction wherein plasma NO₃⁻ is first converted to NO₂⁻ by nitrate reductase (1 hour; 37°C), and the NO₂⁻ generated was quantified in terms of the diazonium product formed after reaction with sulfanilamide and *N*-(1-naphthyl)ethylenediamine.¹⁰

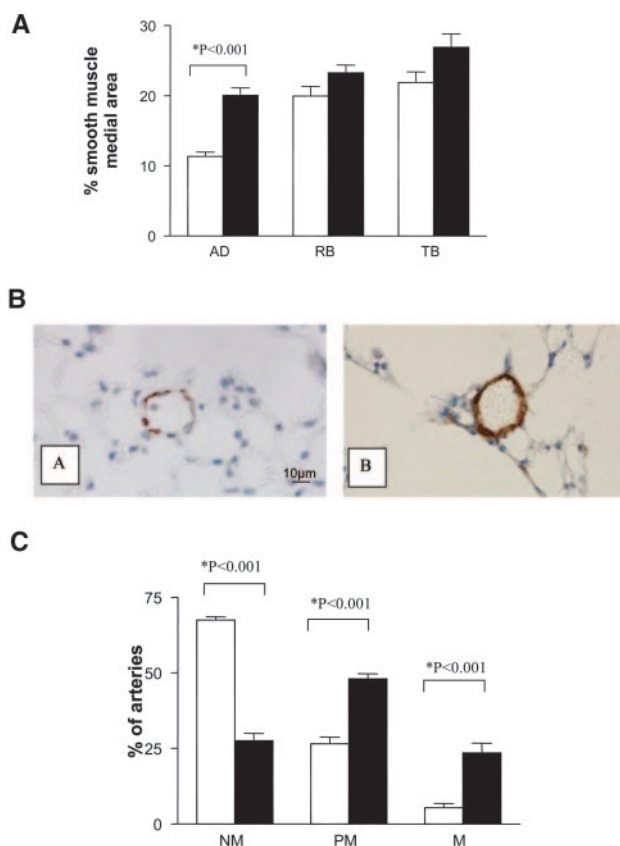


Figure 1. a, Percentage smooth muscle medial area of resistance arteries at level of alveolar duct (AD), respiratory bronchioles (RB), and terminal bronchioles (TB) in hph-1 (solid bars) and WT (open bars) mice. n=6 for each group (equal male-female ratio). *One-way ANOVA with Tukey-Kramer post hoc test. b, α -Actin smooth muscle staining on artery associated with AD, from WT (A) and hph-1 (B) mice (original magnification $\times 20$). c, Percentage of arteries at level of alveoli that are non-muscular (NM), partially muscular (PM), or fully muscular (M) in WT (open bars) and hph-1 (solid bars) mice. *One-way ANOVA with Tukey-Kramer post hoc test; n=6 for each group.

Data Analysis

Data are expressed as mean \pm SEM, and statistical significance was tested with either ANOVA with Tukey-Kramer post hoc test or Student *t* test, where appropriate.

Results

Histological Examination of Pulmonary Vasculature

The mean percentage of smooth muscle medial area in all arteries was greater in hph-1 mice (Figure 1a), with a significant difference seen in the arteries associated with the alveolar ducts (Figures 1a and 1b). The proportions of precapillary arteries at the level of the alveoli that were either nonmuscular (no actin staining), partially muscular (<75% circumferential actin staining), or fully muscular (>75% actin circumferential staining) differed between hph-1 and WT mice (Figure 1c), with fewer nonmuscular vessels and more partially or fully muscular vessels in hph-1 mice.

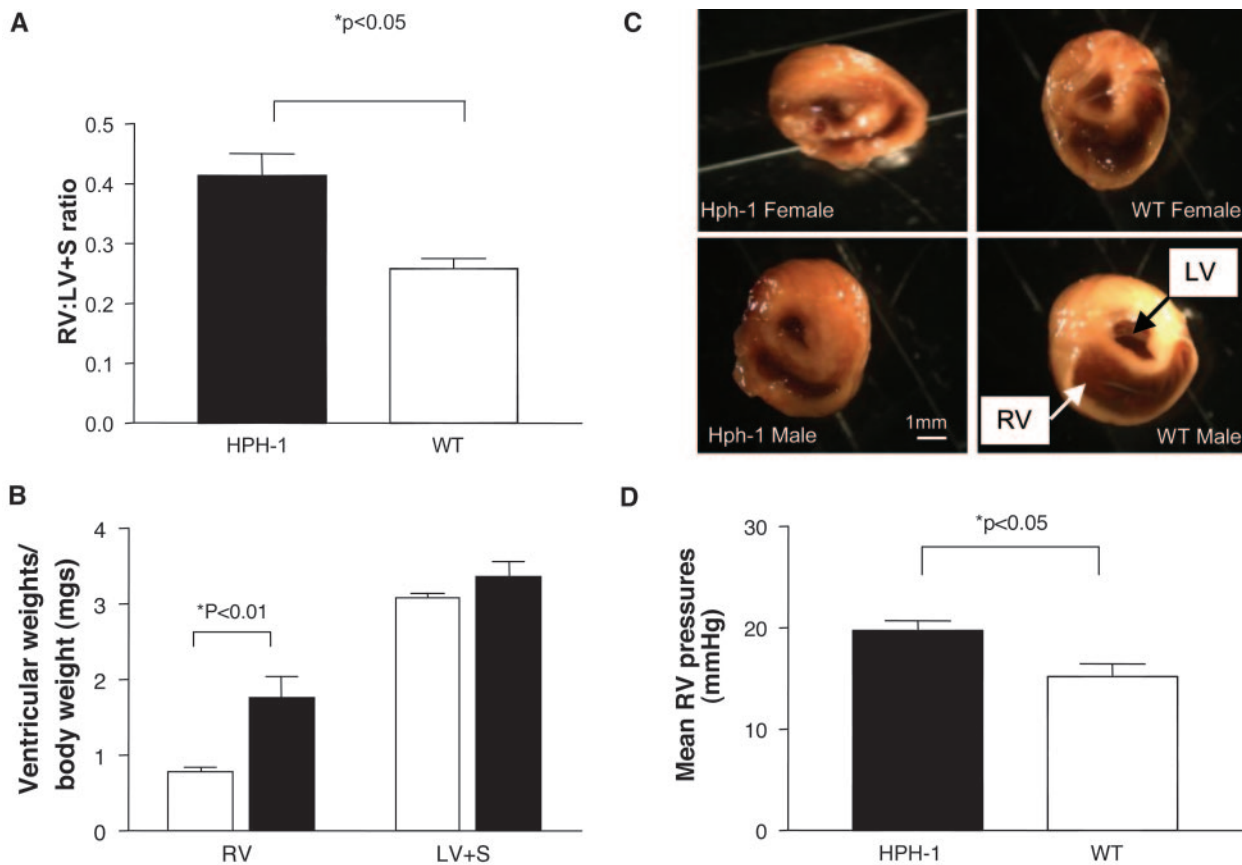


Figure 2. A, RV:LV+S ratio in *hph-1* and WT mice. *hph-1* n=13 (8 female; 5 male), WT n=10 (6 female, 4 male). *P<0.05, Student *t* test. B, RV and LV as indexed to body weight. *hph-1* n=13 (8 female; 5 male), WT n=10 (6 female, 4 male). *P<0.05, Student *t* test. C, Transverse sections of RV and LV cut at atrial-ventricular junction in *hph-1* and WT mice. D, Mean RV pressures in *hph-1* mice (n=11; 5 female, 6 male) and WT mice (n=11; 5 female, 6 male). *Student *t* test.

Ventricular Weights

The mean RV:LV+S ratio was higher in *hph-1* mice than in WT mice, and this was entirely due to the RV component, as shown when indexed to body weight (Figures 2A, 2B, and 2C).

Lung Staining

Detailed examination of hematoxylin-and-eosin-stained lung sections revealed no abnormalities such as edema, fibrosis, hypercellularity, or thickening of the alveolar septa.

Hemodynamic Measurements

There was a significant increase in mean RV pressure in *hph-1* mice (Figure 2D), with no significant difference in mean systemic blood pressure (*hph-1* 86.7±2.56 mm Hg, WT 93.6±3.0 mm Hg).

Kidney Staining

No features of hypertension were identified in the kidneys. Specifically, features indicative of hypertensive nephropathy



Figure 3. Sections from WT (A, B, C) and mutant *hph-1* mice (D, E, F) showed no abnormalities of glomerular (A, D) or arteriolar (B, C, E, F) histology. A, B, D, E: Hematoxylin and eosin; C and F: elastic van Gieson.

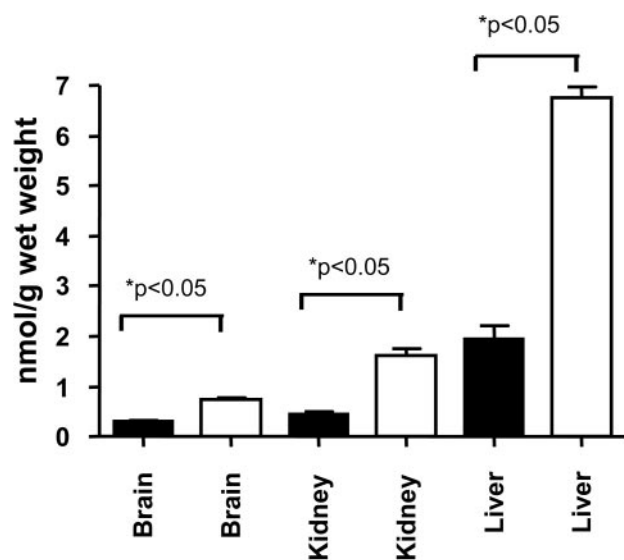


Figure 4. BH₄ levels in brain, kidney, liver from hph-1 (solid bars) and WT (open bars) mice (n=6). *Student *t* test.

(eg, intimal fibroelastosis, hyaline arteriosclerosis, and fibrinoid necrosis) were not seen (Figure 3).

BH₄ Measurements

BH₄ levels were significantly lower in hph-1 mice than in WT in all tissues studied (Figure 4). BH₄ measurements were technically difficult to obtain in lung tissue, but given that there was at least a 50% reduction in the other tissues studied, it is reasonable to conclude that this represents a global effect in this animal model.

NO_x Measurements

Serum NO_x levels were significantly lower in hph-1 mice than in WT mice (Figure 5).

Discussion

The results from this study demonstrate that mice deficient in GTP-CH1/BH₄ display the structural and hemodynamic fea-

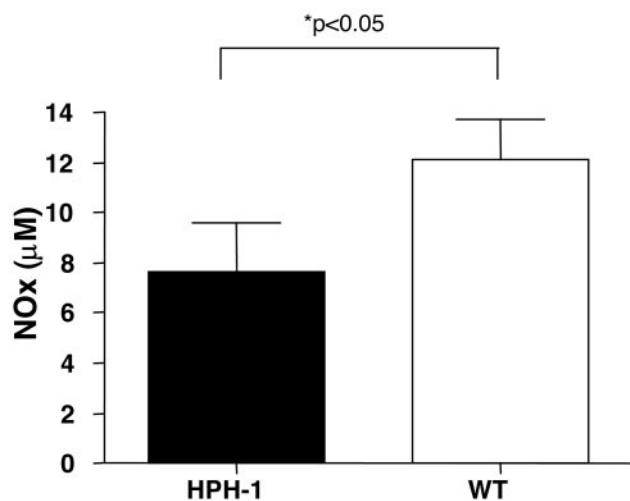


Figure 5. Serum NO_x levels in hph-1 (5 male, 3 female) and WT (4 male, 4 female) mice. *Student *t* test.

tures of pulmonary hypertension. All 3 structural characteristics of pulmonary hypertension (RV hypertrophy, increased smooth muscle wall area of resistance arteries, and extension of muscle into normally nonmuscular arteries) were present in hph-1 mice, and RV pressures were elevated. This identifies the critical importance of GTPCH-1 in the lung.

GTP-CH1 is a key enzyme controlling the synthesis of BH₄ and is expressed in pulmonary vascular endothelium (M.N., unpublished observations, 2001). Although there are no genetic knockouts of GTP-CH1, possibly because of embryonic lethality, the hph-1 mouse is a chemical mutant that shows a 90% reduction in GTP-CH1 activity and a reduction of BH₄, and is a useful model to study BH₄ deficiency. The neurological phenotype of hph-1 mice has been studied extensively,^{9,11,12} and in addition to BH₄ deficiency, these mice have reduced NOS activity that can be restored *ex vivo* by supplementation with BH₄.^{9,13,14} Morphological examination of the pulmonary vasculature of hph-1 mice showed increased smooth muscle medial area in the smallest resistance arteries, with no change in lumen area. There was also extension of muscle into normally nonmuscularized precapillary arteries. These structural changes are characteristic of pulmonary hypertension, and the increase in wall-to-lumen ratio in the small pulmonary resistance vessels would be expected to enhance responses to contractile stimuli¹⁵ and increase resting pulmonary vascular resistance. Consistent with these findings, mean RV pressures were elevated *in vivo*, which confirms the presence of elevated pulmonary pressures. These findings were seen in both male and female mice in studies undertaken by individuals blind to the mouse type. Although a pulmonary hypertensive phenotype was evident in both sexes, it appeared more profound in the female mice.

Pulmonary hypertension can occur secondary to LV dysfunction, but the absence of edema in lung sections, the preservation of normal systemic blood pressure, and the lack of hypertrophy of the LV suggest that it is more likely that the pulmonary hypertension observed in the hph-1 mice resulted from reduced NO availability and subsequent pulmonary vascular changes. Mean RV pressures were significantly elevated in the hph-1 mice, and it would now be interesting to undertake a more detailed evaluation of the pulmonary hemodynamics, because this would facilitate a direct comparison with other models.

We were not able to measure BH₄ accurately in the lungs and therefore could not determine whether BH₄ levels were reduced in the relevant cells in the lungs; however, overall, the hph-1 mice displayed a 90% reduction in GTP-CH1 activity, and there was a >50% reduction in BH₄ in all tissues in which it was measured. Because the defect in GTP-CH1 is thought to be global in hph-1 mice, it appears likely that BH₄ was indeed reduced in the lung, as in other tissues. Therefore, the simplest explanation of the present data are that GTP-CH1/BH₄ deficiency in hph-1 mice reduces NO generation, and this is supported by the observation of a reduction in the serum NO_x levels in the hph-1 mice. An alternative explanation is that the deficiency of BH₄ alters levels of aromatic amino acid derivatives (ie, biogenic amines), because BH₄ is a cofactor for the aromatic amino acid hydroxylases. Indeed,

the concentration of BH₄ required to activate the aromatic amino acid hydroxylases is 20 orders of magnitude greater than that required to activate NOS.¹⁶ Therefore, it may be predicted that deficiency of BH₄ would preferentially affect the levels of biogenic amines, including 5-hydroxytryptamine (5HT) and noradrenaline, an effect seen in the brains of hph-1 mice.¹⁷ However, in contrast to the effects we observed, deficiency of 5HT would be expected to render the animals resistant to pulmonary hypertension.¹⁸ It would now be interesting to determine to what extent biogenic amines may have been reduced in the lung in this model and why pulmonary hypertension predominates. One possibility is that deficiency of BH₄ affects all isoforms of NOS; alternatively, an increase in superoxide production from BH₄-deficient NOS may be important in mediating the effect.¹⁴ It is also possible that reduced β -noradrenergic receptor stimulation or noradrenaline-stimulated NO release may contribute to the phenotype.¹⁹

Hph-1 mice showed pulmonary but not systemic hypertension. This contrasts with a previous study in which an increase in systemic blood pressure was measured by the tail-cuff method in hph-1 mice.¹⁴ The reasons for this difference are not known but may relate to the different methods of assessing blood pressure or the effects of anesthetic on systemic blood pressure; however, the pulmonary vasculature appears to be particularly sensitive to changes in NOS activity,^{20,21} and inhibition of NOS results in attenuation of endothelium-dependent vasodilation in the pulmonary vasculature.²² The present study identifies pulmonary hypertension in the hph-1 mouse and suggests a critical role of GTP-CH1 in the regulation of pulmonary vascular tone.

Acknowledgments

This study was sponsored by the Medical Research Council and The British Heart Foundation.

References

- Nichol CA, Smith GK, Duch DS. Biosynthesis and metabolism of tetrahydrobiopterin and molybdopterin. *Annu Rev Biochem.* 1985;54:729–764.
- Tayeh MA, Marletta MA. Macrophage oxidation of L-arginine to nitric oxide, nitrite, and nitrate: tetrahydrobiopterin is required as a cofactor. *J Biol Chem.* 1989;264:19654–19658.
- Stroes E, Kastelein J, Cosentino F, Erkelens W, Wever R, Koomans H, Luscher T, Rabelink T. Tetrahydrobiopterin restores endothelial function in hypercholesterolemia. *J Clin Invest.* 1997;99:41–46.
- Tiefenbacher CP, Chilian WM, Mitchell M, DeFily DV. Restoration of endothelium-dependent vasodilation after reperfusion injury by tetrahydrobiopterin. *Circulation.* 1996;94:1423–1429.
- Tiefenbacher CP, Bleeke T, Vahl C, Amann K, Vogt A, Kubler W. Endothelial dysfunction of coronary resistance arteries is improved by tetrahydrobiopterin in atherosclerosis. *Circulation.* 2000;102:2172–2179.
- Giaid A, Saleh D. Reduced expression of endothelial nitric oxide synthase in the lungs of patients with pulmonary hypertension. *N Engl J Med.* 1995;333:214–221.
- Champion HC, Bivalacqua TJ, Greenberg SS, Giles TD, Hyman AL, Kadowitz PJ. Adenoviral gene transfer of endothelial nitric-oxide synthase (eNOS) partially restores normal pulmonary arterial pressure in eNOS-deficient mice. *Proc Natl Acad Sci U S A.* 2002;99:13248–13253.
- 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.
- Canevari L, Land JM, Clark JB, Heales SJ. Stimulation of the brain NO/cyclic GMP pathway by peripheral administration of tetrahydrobiopterin in the hph-1 mouse. *J Neurochem.* 1999;73:2563–2568.
- Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Anal Biochem.* 1982;126:131–138.
- Hyland K, Gunasekara RS, Munk-Martin TL, Arnold LA, Engle T. The hph-1 mouse: a model for dominantly inherited GTP-cyclohydrolase deficiency. *Ann Neurol.* 2003;54(suppl 6):S46–S48.
- Brand MP, Briddon A, Land JM, Clark JB, Heales SJ. Impairment of the nitric oxide/cyclic GMP pathway in cerebellar slices prepared from the hph-1 mouse. *Brain Res.* 1996;735:169–172.
- Brand MP, Heales SJ, Land JM, Clark JB. Tetrahydrobiopterin deficiency and brain nitric oxide synthase in the hph1 mouse. *J Inherit Metab Dis.* 1995;18:33–39.
- 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.
- Folkow B. The haemodynamic consequences of adaptive structural changes of the resistance vessels in hypertension. *Clin Sci.* 1971;41:1–12.
- Katusic ZS. Vascular endothelial dysfunction: does tetrahydrobiopterin play a role? *Am J Physiol Heart Circ Physiol.* 2001;281:H981–H986.
- Hyland K, Gunasekera RS, Engle T, Arnold LA. Tetrahydrobiopterin and biogenic amine metabolism in the hph-1 mouse. *J Neurochem.* 1996;67:752–759.
- Eddahibi S, Hanoun N, Lanfumey L, Lesch KP, Raffestin B, Hamon M, Adnot S. Attenuated hypoxic pulmonary hypertension in mice lacking the 5-hydroxytryptamine transporter gene. *J Clin Invest.* 2000;105:1555–1562.
- Priest RM, Hucks D, Ward JP. Noradrenaline, beta-adrenoceptor mediated vasorelaxation and nitric oxide in large and small pulmonary arteries of the rat. *Br J Pharmacol.* 1997;122:1375–1384.
- Cases A, Haas J, Burnett JC, Romero JC. Hemodynamic and renal effects of acute and progressive nitric oxide synthesis inhibition in anesthetized dogs. *Am J Physiol Regul Integr Comp Physiol.* 2001;280:R143–R148.
- Licker M, Boussairi H, Hohn L, Morel DR. Role of nitric oxide in the regulation of regional blood flow and metabolism in anaesthetized pigs. *Acta Physiol Scand.* 1998;163:339–348.
- Fineman JR, Heymann MA, Soifer SJ. N^{omega}-nitro-L-arginine attenuates endothelium-dependent pulmonary vasodilation in lambs. *Am J Physiol.* 1991;260(part 2):H1299–H1306.