Am J Physiol Regul Integr Comp Physiol 289: R299–R304, 2005.
First published March 17, 2005; doi:10.1152/ajpregu.00269.2004.

TRANSLATIONAL PHYSIOLOGY

Tetrahydrobiopterin deficiency exaggerates intimal hyperplasia after vascular injury

Chao-Hung Wang,[1,3] Shu-Hong Li,[1] Richard D. Weisel,[1] Paul W. M. Fedak,[1] Agnes Hung,[3] Ren-Ke Li,[1] Vivek Rao,[1] Keith Hyland,[2] Wen-Jin Cherng,[3] Lee Errett,[4] Yves Leclerc,[4] Daniel Bonneau,[4] David A. Latter,[4] and Subodh Verma[1,4]

[1]*Division of Cardiac Surgery, Toronto General Hospital, Toronto, Canada;* [2]*Institute of Metabolic Disease, Baylor University Medical Center, Dallas, Texas;* [3]*Cardiology Section, Department of Medicine, Chang Gung Medical College, Chang Gung Memorial Hospital, Keelung, Taiwan; and* [4]*Division of Cardiac Surgery, St. Michael's Hospital, Toronto, Canada*

Submitted 27 April 2004; accepted in final form 8 February 2005

Wang, Chao-Hung, Shu-Hong Li, Richard D. Weisel, Paul W. M. Fedak, Agnes Hung, Ren-Ke Li, Vivek Rao, Keith Hyland, Wen-Jin Cherng, Lee Errett, Yves Leclerc, Daniel Bonneau, David A. Latter, and Subodh Verma. Tetrahydrobiopterin deficiency exaggerates intimal hyperplasia after vascular injury. *Am J Physiol Regul Integr Comp Physiol* 289: R299–R304, 2005. First published March 17, 2005; doi:10.1152/ajpregu.00269.2004.—Decreased levels of tetrahydrobiopterin (BH4), an absolute cofactor for nitric oxide synthase (NOS), lead to uncoupling of NOS into a superoxide v. nitric oxide producing enzyme, and it is this uncoupling that links it to the development of vascular disease. However, the effects of in vivo deficiency of BH4 on neointimal formation after vascular injury have not been previously investigated. Hph-1 mice, which display 90% deficiency in guanine triphosphate cyclohydrolase I, the rate limiting enzyme in BH4 synthesis, were used. Hph-1 and wild-type mice, treated with either vehicle or BH4 ($n = 15$ per group), were subjected to wire-induced femoral artery injury, and NOS expression and activity, inflammation, cell proliferation, superoxide production, and neointimal formation were assessed. The major form of NOS expressed over vessel wall after vascular injury was endothelial NOS. Hph-1 mice exhibited lower NOS activity (2.8 ± 0.3 vs. 4.5 ± 0.4 pmol/min/mg protein, $P < 0.01$), and higher aortic superoxide content ($5.2 \pm 2.0 \times 10^5$ cpm vs. $1.6 \pm 0.7 \times 10^5$ cpm, $P < 0.01$) compared with wild-type controls, indicating uncoupling of NOS. Treatment of hph-1 mice with BH4 significantly increased NOS activity (from 2.8 ± 0.3 to 4.1 ± 0.4 pmol·min^{-1}·mg protein^{-1}, $P < 0.05$), and attenuated superoxide production (from $5.2 \pm 2.0 \times 10^5$ cpm to $0.8 \pm 0.7 \times 10^5$ cpm, $P < 0.05$). Hph-1 mice also had higher inflammatory reactions and more cell proliferation after vascular injury. Furthermore, hph-1 mice responded by a marked increase in neointimal formation at 4 wk after vascular injury, compared with wild-type controls (intima:media ratio: 4.5 ± 0.5 vs. wild-type 0.7 ± 0.1, $P < 0.001$). Treatment of hph-1 mice with BH4 prevented vascular injury-induced increase in neointimal formation (intima:media ratio: 1.4 ± 0.1 vs. hph-1, $P < 0.001$). Treatment had no effect on wild-type controls. In summary, we describe, for the first time, that in vivo BH4 deficiency facilitates neointimal formation after vascular injury. Modulation of BH4 bioavailability is an important therapeutic target for restenosis.

restenosis; superoxide; nitric oxide synthase

NEOINTIMAL FORMATION AND RESTENOSIS after vascular injury have been ascribed to a number of factors, including endothelial dysfunction, increased reactive oxygen species production, and accelerated smooth muscle cell migration and proliferation (5, 23). Nitric oxide (NO) produced by endothelium plays an important role in attenuating smooth muscle migration and proliferation, and decreasing neointimal hyperplasia by numerous mechanisms (25, 28). Tetrahydrobiopterin (BH4) is an absolute cofactor required for nitric oxide synthase (NOS) and maintains NOS as a NO vs. superoxide-producing enzyme (18). BH4 exerts this action through serving as an electron donor for the hydroxylation of L-arginine. Suboptimal concentrations of BH4, as observed in states of cardiovascular disease, led to an uncoupling of NOS with diminished NO and exaggerated superoxide anion production. NOS may become a source of oxygen-derived free radicals (9, 27), which may promote the development and progression of vascular disease. However, the effects of in vivo deficiency of BH4 on restenosis after vascular injury have not been examined.

The hph-1 mouse exhibits a genetic 90% deficiency in GTP-cyclohydrolase-1, the rate-limiting enzyme in BH4 synthesis (22), and is therefore associated with marked reduction in BH4 levels (1). We have previously used this model to investigate in vivo BH4 deficiency (15). In the present study, we adopted a femoral artery wire injury model to investigate the effects of in vivo BH4 deficiency on neointimal formation, in addition to the expression pattern of NOS and the inflammatory and proliferative responses to vascular injury. We also tested the effects of BH4 supplementation on the aforementioned outcomes.

METHODS

Animals and groups. Hph-1 and wild-type (C57BL × CBA) mice of the same strain (11 wk old) were obtained from Dr. Keith Hyland (Institute of Metabolic Disease, Baylor University Medical Center, Dallas). In accordance with guidelines of the Canadian Council on Animal Care, mice were housed in the Animal Care Facility of the Toronto General Hospital, Toronto, Canada. Animals were exposed to a 12:12-h light-dark cycle and fed chow and water ad libitum. The protocol was approved by the Toronto General Hospital Institutional Review Board.

Address for reprint requests and other correspondence: S. Verma, Division of Cardiac Surgery, St. Michael's Hospital, 30 Bond St., Queen Wing, Suite 8–003H, Toronto, ON, Canada, M5B 1W8 (E-mail: Subodh.Verma@sympatico.ca)

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Wild-type and Hph-1 mice were randomly divided into four groups (n = 15 per group): wild-type untreated, wild-type BH4-treated, hph-1 untreated, and hph-1 BH4 treated. BH4 treatment was administered at a dose of 10 $mg \cdot kg^{-1} \cdot day^{-1}$ ip (13). In each group, we investigated the vascular changes after 4, 7, 14, and 28 days after femoral artery angioplasty. BH4 was started 1 wk before surgery and administered for another 4 wk after surgery.

Mouse femoral artery injury model. Transluminal mechanical injury of bilateral femoral arteries was induced by inserting a straight spring wire (0.014 inch in diameter) as previously described (24). At different time points (4, 7, 14, and 28 days), femoral arteries were excised and fixed in 10% formalin. The paraffin-embedded sections were used for immunohistochemical staining.

Histological assessment of postangioplasty neointimal formation. Cross-sectional rings (4 μM) were stained with hematoxylin and eosin, Trichromes stain, Ki-67 (monoclonal rat anti-mouse antibody, DAKO, Carpinteria, CA), Mac-3 (rat anti-mouse monoclonal antibody for monocyte and macrophage, Pharmingin), endothelial constitutive NOS (eNOS, sc-8311), and inducible NOS (iNOS, sc-651) (polyclonal rabbit anti-human antibody with cross-reaction to mouse; Santa Cruz Biotechnology, Santa Cruz, CA). Formalin-fixed, paraffin-embedded sections were treated with pepsin, or HIER, if necessary, and stained with primary antibody (Ki-67, Mac-3, eNOS and iNOS). Species-specific biotinylated linking secondary antibodies (anti-rat, anti-rabbit, Vector) and horseradish peroxidase-conjugated ultrastreptavidin-labeling reagent (Signet) were used. Peroxidase activity was developed using NovaRed solutions (Vector), followed by counterstaining with Mayer's hematoxylin. Cell proliferation was measured by counting cells positive for Ki-67 staining. Cross-sectional area of the intima and media of the femoral arteries were measured with National Institutes of Health Image software, and the ratio of intimal area to medial area (I/M ratio) was calculated. The specificity of the primary antibodies, eNOS and iNOS, has been tested on mouse brain extract and LPS/IFN-g-stimulated macrophage (RAW 264.7), respectively, with Western blotting. Both antibodies have been widely applied for the NOS research (3, 4, 6, 7, 10, 11, 21, 26, 32).

Aortic superoxide production. Superoxide production was determined by a chemiluminescence method as previously described (16). Freshly harvested aortas (18 h after last BH4 injection in mice supplied with BH4) were opened lengthwise, divided into multiple segments, and equilibrated in Krebs-HEPES buffer (composition in mmol/l: 99 NaCl, 4.7 KCL, 1.2 $MgSO_4$, 1 KH_2PO_4, 1.9 $CaCl_2$, 25 $NaHCO_3$, 11.1 glucose, and sodium HEPES 20) gased with 95% O_2, 5% CO_2 for 30 min at 37°C in the presence of indomethacin (10–5 mol/l). Lucigenin-enhanced chemiluminescence was measured in 2 ml Krebs-HEPES buffer containing lucigenin (5 μmol/l) by use of a Berthold FB12 single-tube luminometer, modified to maintain a sample temperature of 37°C. Chemiluminescence was measured continuously for 10 min after allowing dark adaptation and was expressed as cpm/mg vessel dry weight. The specificity of the chemiluminescence method was tested with superoxide dismutase (an antioxidant) and N^G-nitro-L-arginine methyl ester (L-NAME) (an NOS inhibitor). Superoxide dismutase (400 U/L) significantly attenuated the production of superoxide from the aorta (from 1.1 ± 0.2 × 10^5 cpm to 0.2 ± 0.02 × 10^5 cpm). Seventy one percent of the aortic superoxide production was attenuated by L-NAME, which suggests that the majority of aortic superoxide produced originated by NOS.

Aortic NOS activity. Determination of NOS activity was carried out by using NOS*detect* Assay Kit (Stratagene, La Jolla, CA). Briefly, protein was extracted from tissue with homogenization buffer (25 mM Tris·HCL, pH 7.4, 1 mM EDTA, 1 mM EGTA). Standard reaction was performed by adding 40-μl reaction mixtures (1.25 μmol/l FAD, 1.25 μmol/L FMN, 1.25 mmol/l NADPH, 0.75 mM $CaCl_2$ and 60,000 to 80,000 cpm purified L-[2,3,4,5–3H]arginine; Amersham Life Sciences) to 10 μl of tissue extract in a final volume of 50 μl. After incubation at 37°C for 30 min, the reaction was stopped by the addition of 400 μl of stop buffer (50 mM HEPES, pH 5.5, 5 mM

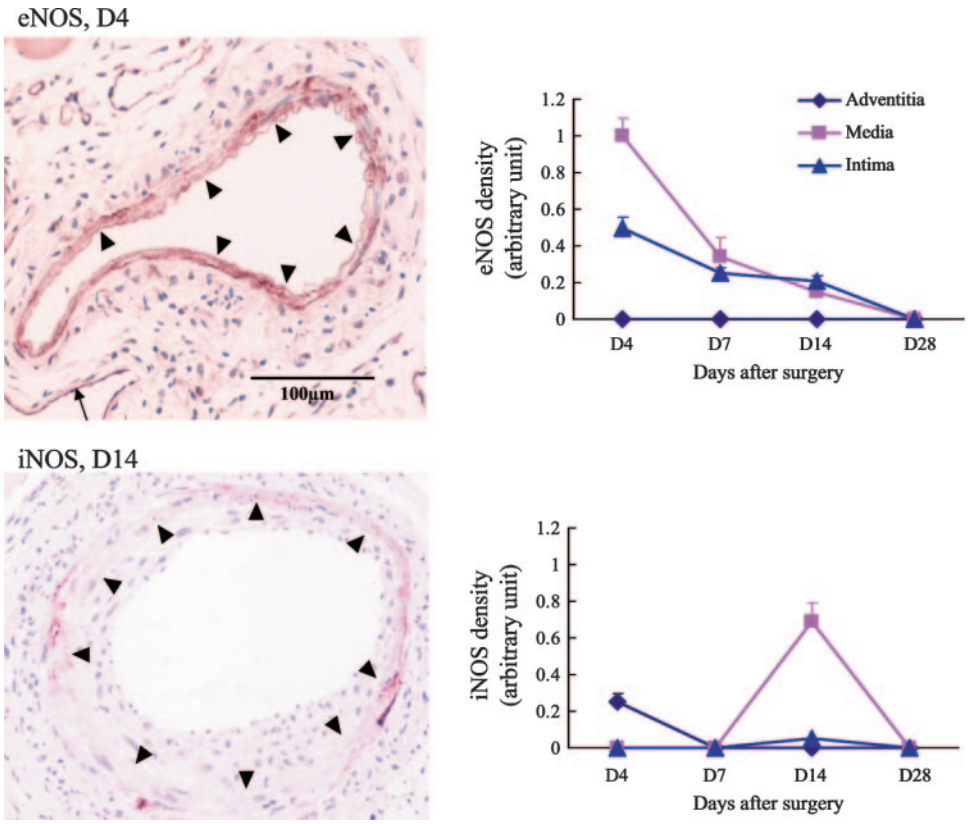

Fig. 1. Expression of eNOS and iNOS after vascular injury. After vascular injury, femoral arteries were harvested at different time points (4, 7, 14, and 28 days). The strength of eNOS expression at media layer on *day 4* was used as an arbitrary unit (=1) to semiquantitate the expression of eNOS and iNOS at other time points. The expression pattern and strength of eNOS and iNOS were similar in either wild-type or hph-1 mice and were analyzed in each high power field (HPF) over the intima, media, and adventitia (analyzed vessel number = 6–8). Arrows indicate the eNOS expression on the endothelial cells in venous structure. Arrowheads indicate internal elastic laminae (magnification ×100).

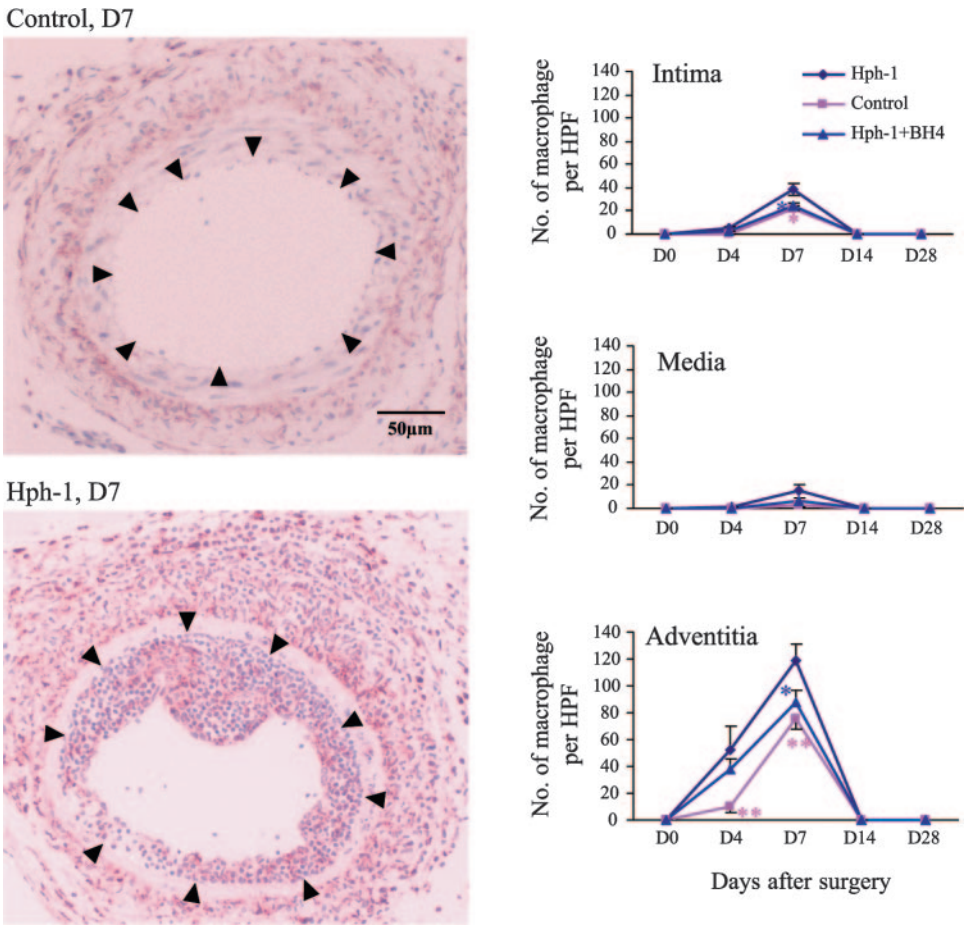

Fig. 2. Increased macrophage accumulation in tetrahydrobiopterin (BH4)-deficient mice after vascular injury: Animal preparation was mentioned as before. Macrophage accumulation was assessed in control and hph-1 mice treated with and without BH4 by immunostaining arterial sections with Mac-3 antibody (shown as brown). Compared to control, hph-1 mice exhibited increased macrophage accumulation in the neointima at *day 7*, and adventia at *day 4* and *7*; this was reversed with BH4 treatment. BH4 treatment did not affect macrophage accumulation in control wild-type mice (not shown). The number of Mac-3-stained cells was counted in each high power field (HPF) over the intima, media, and adventitia (analyzed vessel number = 6–8). Arrowheads indicate internal elastic laminae. *$P < 0.05$ different from control and hph-1+BH4 (magnification ×100).

EDTA). [^{3}H]Citrulline was quantified after separation from [^{3}H]arginine by cation exchange columns.

Statistics. Data were analyzed by one-way ANOVA. When statistically significant effects were found, Tukey's test was performed to isolate the differences between the groups. Student's *t*-test and chi-square were used when appropriate. A *P* value of less than 0.05 was considered significant. All data in the text and figures are presented as means ± SE.

RESULTS

Expression of eNOS and iNOS after femoral artery injury. After vascular injury, endothelial cells on the vessel wall were denuded and femoral artery was dilated. Neointima started to develop ~7 days after vascular injury, with profound neointimal formation evident at 4 wk after vascular injury. In our model of femoral angioplasty, eNOS was the major NOS expressed on the injured vessel wall. Although the medial layer was the main site that expressed eNOS, the adventitia also expressed small amounts of eNOS. The peak expression of eNOS was observed on *day 4* after vascular injury (Fig. 1). Throughout the 28-day period after vascular injury, there was only a minimal expression of iNOS, which peaked *day 14* postinjury.

Increased inflammation and cell proliferation in hph-1 mice: reversal with BH4. Sections were stained with Mac-3, an antibody directed against monocytes and macrophages, as a surrogate marker of inflammation after vascular injury. Additionally, Ki-67 staining was used as a marker to estimate cell proliferation. Four days after vascular injury, there was a significant increase of macrophage number within the adventitia, while at this time point, there was minimal macrophage accumulation in the media and no neointimal formation (Fig. 2). On *day 7* after vascular injury, macrophage number peaked in the adventitia accompanied with an increased flux of macrophages noted within the neointima. After this time point, macrophage number decreased in both the adventitia and neointima, although there was a substantial increase in neointimal formation. Macrophage accumulation was significantly enhanced after vascular injury in hph-1 mice in the intima on *day 7* and adventitia on both *day 4* and *7*; a response that was blunted by BH4 treatment ($P < 0.01$, Fig. 2).

Cell proliferation peaked on *day 4* in media and adventitia, and on *day 7* in neointima. Femoral arteries from hph-1 mice depicted a marked fourfold increase in cell proliferation in the media and adventitia compared with wild-type controls (Fig. 3, $P < 0.01$); this response was blunted by BH4 treatment of hph-1 mice ($P < 0.01$). Treatment of wild-type controls with BH4 neither altered macrophage accumulation nor cell proliferation.

Increased neointimal formation in hph-1 mice: reversal with BH4. In this study, mice were used at 11 wk of age. Since the guide wire was relatively smaller for the lumen size of the vessel, the degree of neointimal formation elicited in the control wild-type mice was modest (average I/M ratio was 0.7 in wild-type mice at 4 wk after injury). Nevertheless, I/M ratio

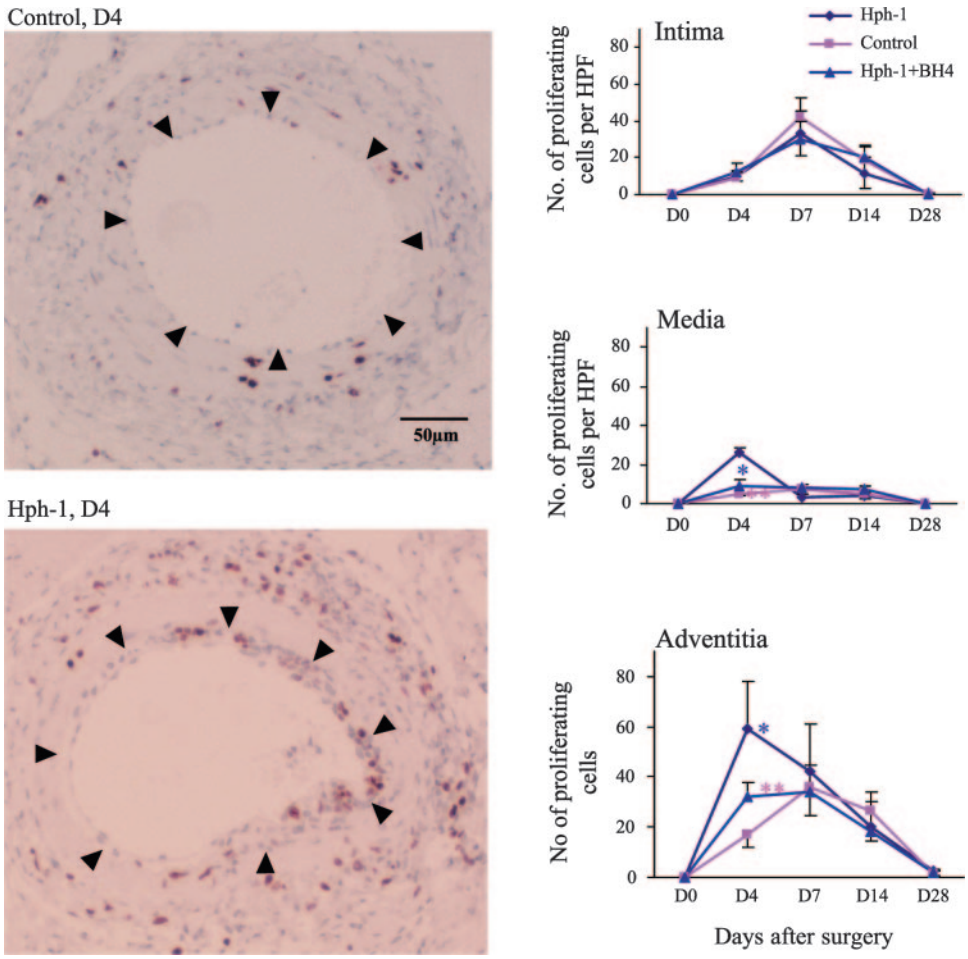

Fig. 3. Increased cell proliferation in BH4-deficient mice after vascular injury: Animal preparation was mentioned as previously. Cell proliferation was assessed in control and hph-1 mice treated with and without BH4 by immunostaining arterial sections with Ki-67 (shown as brown). Compared to control, hph-1 mice exhibited increased cellular proliferation in the media and adventitia, which peaked at 4 days; this was reversed with BH4 treatment. BH4 treatment did not affect cell proliferation in control wild-type mice (not shown). The number of Ki-67-stained cells was counted in each HPF over the intima, media, and adventitia (analyzed vessel number = 6–8). Arrowheads indicate internal elastic laminae. *$P < 0.05$ different from control and hph-1 + BH4 (magnification ×100).

in hph-1 mice was almost seven-fold higher than wild-type controls (4.5 ± 0.5 vs. 0.7 ± 0.1, $P < 0.001$, Fig. 4, *A* and *B*), subjected to similar vascular injury. BH4 supplementation attenuated neointimal formation in hph-1 mice (1.4 ± 0.1 vs. hph-1 untreated, $P < 0.001$, Fig. 4, *A* and 4*B*). BH4 treatment had no effect on neointimal formation in wild-type control mice.

Uncoupling of NOS in hph-1 mice: Increased superoxide and decreased eNOS activity. Aortae from hph-1 mice exhibited functional uncoupling of NOS. As such, superoxide production in hph-1 mice aorta was significantly higher than wild-type controls (5.2 ± 2.0 × 10^5 cpm vs. 1.6 ± 0.7 × 10^5 cpm, $P < 0.05$, Fig. 4*C*) and NOS activity was lower in hph-1 mice (2.8 ± 0.3 vs. 4.5 ± 0.4 pmol·min^{-1}·mg protein^{-1}, $P < 0.01$) (Fig. 4*D*). BH4 supplementation decreased superoxide production (0.8 ± 0.7 × 10^5 cpm vs. hph-1 5.2 ± 2.0 × 10^5 cpm, $P < 0.05$), and restored NOS activity in hph-1 mice (from 2.8 ± 0.3 to 4.1 ± 0.4 pmol·min^{-1}·mg protein^{-1}, $P < 0.05$), without an effect in wild-type controls.

DISCUSSION

This is the first demonstration that in vivo deficiency of BH4 exaggerates neointimal formation following vascular injury. The hph-1 mouse is a well-established model of BH4 deficiency, with 90% genetic deficiency of GTP-cyclohydrolase-1, the rate limiting enzyme in the synthesis of BH4 (1, 22). After femoral artery injury, hph-1 mice exhibited a marked seven-fold increase in neointimal formation, which was attenuated by BH4 treatment. Hph-1 mice revealed an increase in superoxide content with decreases in NOS activity consistent with the hypothesis of NOS uncoupling; these biochemical parameters were restored after BH4 treatment. These data underscore the importance of BH4 as a cellular target for restenosis after vascular injury.

The healthy endothelium is a net producer of NO (15, 28). NO is believed to act as a key regulator of vascular homeostasis and exerts an important braking effect on inflammation, atherosclerosis, and restenosis. In the endothelial cell, BH_4 is a critical cofactor for NOS activation, and hence, NO production is dependent upon the presence of adequate amounts of BH_4 (19). BH_4 exerts this action through serving as an electron donor for the hydroxylation of L-arginine. Diminished levels of BH_4, as observed in states of cardiovascular disease, lead to an uncoupling of NOS with diminished NO and exaggerated superoxide anion production. Although the role of BH4 treatment is still debated (14, 20), the majority of studies have demonstrated the beneficial effects of BH_4 supplementation on vascular function in vitro and in vivo (17, 29–31) and suggest that BH_4 is an emerging target for pharmacological manipulation in atherosclerosis. Given the importance of NO in the development of restenosis, we sought to determine whether in

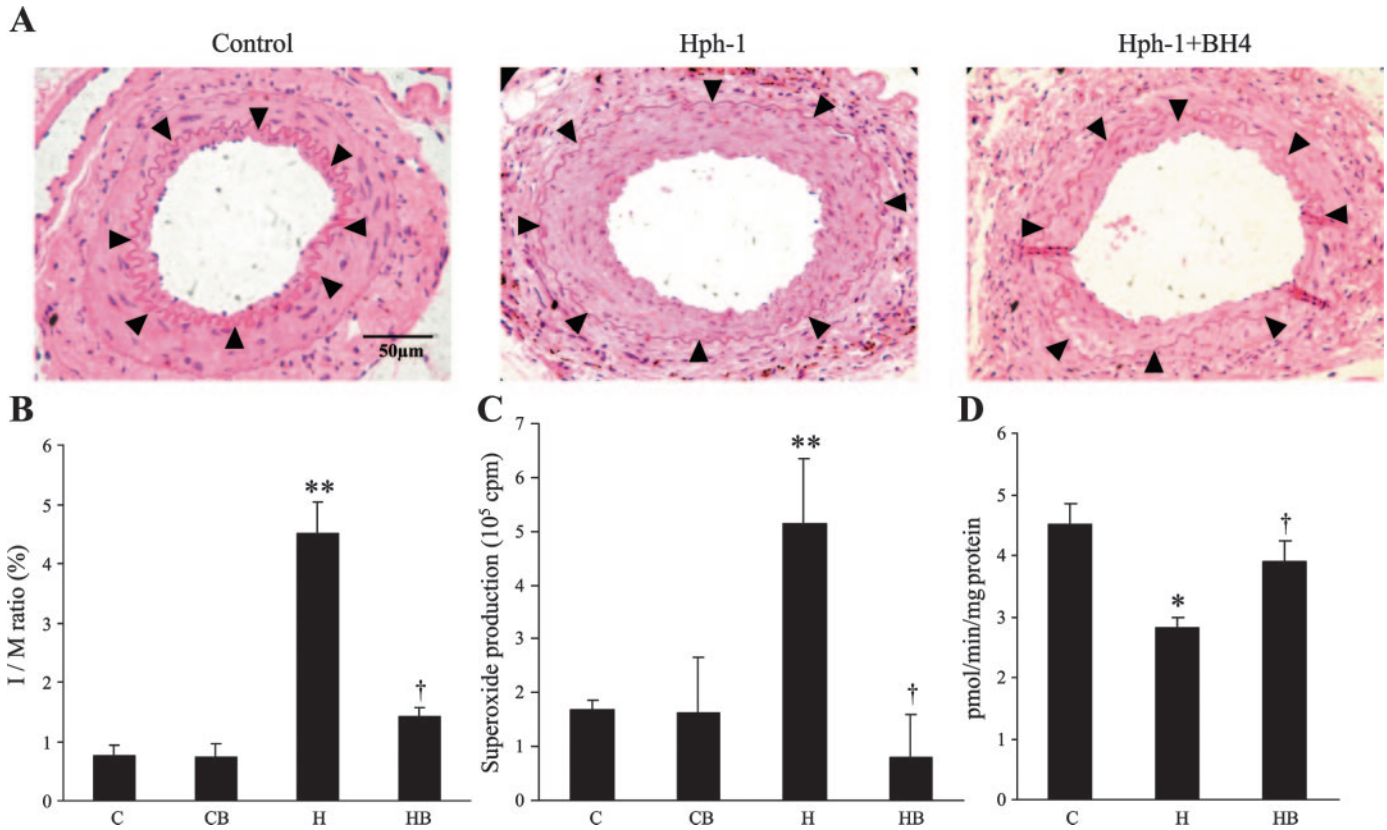

Fig. 4. Neointimal formation 28 days after angioplasty, aortic superoxide production, and NOS activity in control and hph-1 mice. *A*: hematoxylin and eosin-stained arterial sections, demonstrating increased neointimal formation in hph-1 mice; an effect that is reversed by BH4 treatment. Arteries from hph-1 mice depict increased neointimal formation; this effect is reversed with BH4 treatment (*B*). likewise, arteries from hph-1 mice exhibit increased superoxide production and decreased NOS activity; this effect is reversed with BH4 treatment (*C* and *D*). Arrowheads indicate internal elastic laminae. Data are presented as means ± SE. C, control; CB, control + BH4; H, hph-1; HB, hph-1 + BH4. $*P < 0.05$ different from C, $**P < 0.01$ different from all groups, respectively. $\dagger P < 0.05$ different from H (magnification ×100).

vivo BH4 deficiency would promote neointimal formation after vascular injury.

In line with previous findings by Cosentino et al. (8), our data support the notion that aorta from hph-1 mice exhibit an increased superoxide content and decreased NOS activity, suggesting in vivo uncoupling of NOS in these mice. eNOS was found to be the major form of NOS expressed on injured vessel walls. Even though endothelial cells were denuded by intraluminal injury, eNOS can be expressed by the residual cells in vessel medial layer, which most likely represent smooth muscle cells (2). We believe that this upregulated expression of dysfunctional and uncoupled eNOS is the source of increased superoxide production after vascular injury in hph-1 deficient mice. Femoral artery angioplasty resulted in exaggerated macrophage infiltration and cell proliferation, and marked increase in intimal hyperplasia in hph-1 mice. Treatment with BH4 attenuated superoxide production restored NOS activity and prevented neointimal formation after vascular injury.

There are a few study limitations. First, superoxide and NOS activity could not be assessed in the injured artery; however, it was assessed in the thoracic aorta instead. This was done primarily for technical reasons, as the miniature quantities of femoral tissue would require that a number of animals be killed to obtain an adequate amount of pooled sample. Nevertheless, as shown above, eNOS was also expressed by stressed smooth muscle cells in media (2). We believe that the information gauged from the aorta with respect to uncoupling of NOS can be applied to the systemic arterial circulation in these mice. Secondly, because the NADPH oxidase activity was not measured, increased aortic superoxide generation cannot be totally attributed to NOS uncoupling. Future studies should focus on this to clarify the underlying mechanisms. Finally, in this study, we did not measure blood pressure, which also plays a causal role in neointimal formation. However, previously, Cosentino et al. (8) had demonstrated elevated blood pressure in hph-1 mice, and Hong et al. (13) had found that BH4 supplementation significantly lowered blood pressure in spontaneous hypertensive rats.

In summary, in vivo BH4 deficiency promotes marked neointimal formation following vascular injury, which suggests that increasing BH4 bioavailability may represent a new target for interventions geared toward preventing the development of adverse vascular remodeling in states of chronic atherosclerosis and restenosis.

ACKNOWLEDGMENTS

We would like to thank Mei-Yun Wu and Shin-Yi Wang for performing analysis on histological images.

GRANTS

C. H. Wang is a Career Investigator of Cardiovascular Research Foundation of Chang Gung Memorial Hospital in Taiwan. C. H. Wang, W. J. Cherng, and A. Hung were supported by National Science Council of Taiwan (NSC 93–2134-B-182A-133 and NSC 93–2134-B-182A-134). S. Verma and R. D. Weisel were supported by the Heart and Stroke Foundation of Canada.

REFERENCES

1. **Brand MP, Heales SJ, Land JM, and Clark JB.** Tetrahydrobiopterin deficiency and brain nitric oxide synthase in the hph1 mouse. *J Inherit Metab Dis* 18: 33–39, 1995.
2. **Buchwalow IB, Podzuweit T, Bocker W, Samoilova VE, Thomas S, Wellner M, Baba HA, Robenek H, Schnekenburger J, and Lerch MM.** Vascular smooth muscle and nitric oxide synthase. *FASEB J* 16: 500–508, 2002.
3. **Buchwalow IB, Podzuweit T, Samoilova VE, Wellner M, Haller H, Grote S, Aleth S, Boecker W, Schmitz W, and Neumann J.** An in situ evidence for autocrine function of NO in the vasculature. *Nitric Oxide* 10: 203–212, 2004.
4. **Buchwalow IB, Schulze W, Karczewski P, Kostic MM, Wallukat G, Morwinski R, Krause EG, Muller J, Paul M, Slezak J, Luft FC, and Haller H.** Inducible nitric oxide synthase in the myocard. *Mol Cell Biochem* 217: 73–82, 2001.
5. **Cannon RO, III.** Restenosis after angioplasty. *N Engl J Med* 346: 1182–1183, 2002.
6. **Carpenter L, Cordery D, and Biden TJ.** Protein kinase Cδ activation by interleukin-1β stabilizes inducible nitric-oxide synthase mRNA in pancreatic β-cells. *J Biol Chem* 276: 5368–5374, 2001.
7. **Cernuda-Morollon E, Rodriguez-Pascual F, Klatt P, Lamas S, and Perez-Sala D.** PPAR agonists amplify iNOS expression while inhibiting NF-κB: implications for mesangial cell activation by cytokines. *J Am Soc Nephrol* 13: 2223–2231, 2002.
8. **Cosentino F, Barker JE, Brand MP, Heales SJ, Werner ER, Tippins JR, West N, Channon KM, Volpe M, and Luscher TF.** Reactive oxygen species mediate endothelium-dependent relaxations in tetrahydrobiopterin-deficient mice. *Arterioscler Thromb Vasc Biol* 21: 496–502, 2001.
9. **Dixon LJ, Morgan DR, Hughes SM, McGrath LT, El-Sherbeeny NA, Plumb RD, Devine A, Leahey W, Johnston GD, and McVeigh GE.** Functional consequences of endothelial nitric oxide synthase uncoupling in congestive cardiac failure. *Circulation* 107: 1725–1728, 2003.
10. **Donovan M, Carmody RJ, and Cotter TG.** Light-induced photoreceptor apoptosis in vivo requires neuronal nitric-oxide synthase and guanylate cyclase activity and is caspase-3-independent. *J Biol Chem* 276: 23000–23008, 2001.
11. **Gutmann DH, Winkeler E, Kabbarah O, Hedrick N, Dudley S, Goodfellow PJ, and Liskay RM.** Mlh1 deficiency accelerates myeloid leukemogenesis in neurofibromatosis 1 (Nf1) heterozygous mice. *Oncogene* 22: 4581–4585, 2003.
12. **Hayden MR and Tyagi SC.** Is type 2 diabetes mellitus a vascular disease (atherosclerosis) with hyperglycemia a late manifestation? The role of NOS, NO, and redox stress. *Cardiovasc Diabetol* 2: 2, 2003.
13. **Hong HJ, Hsiao G, Cheng TH, and Yen MH.** Supplemention with tetrahydrobiopterin suppresses the development of hypertension in spontaneously hypertensive rats. *Hypertension* 38: 1044–1048, 2001.
14. **Hurshman AR, Krebs C, Edmondson DE, and Marletta MA.** Ability of tetrahydrobiopterin analogs to support catalysis by inducible nitric oxide synthase: formation of a pterin radical is required for enzyme activity. *Biochemistry* 42: 13287–13303, 2003.
15. **Hyland K, Gunasekara RS, Munk-Martin TL, Arnold LA, and Engle T.** The hph-1 mouse: a model for dominantly inherited GTP-cyclohydrolase deficiency. *Ann Neurol* 54 Suppl 6: S46–S48, 2003.
16. **Hyndman ME, Verma S, Rosenfeld RJ, Anderson TJ, and Parsons HG.** Interaction of 5-methyltetrahydrofolate and tetrahydrobiopterin on endothelial function. *Am J Physiol Heart Circ Physiol* 282: H2167–H2172, 2002.
17. **Katusic ZS.** Vascular endothelial dysfunction: does tetrahydrobiopterin play a role? *Am J Physiol Heart Circ Physiol* 281: H981–H986, 2001.
18. **Kaufman S.** New tetrahydrobiopterin-dependent systems. *Annu Rev Nutr* 13: 261–286, 1993.
19. **Kinoshita H, Milstien S, Wambi C, and Katusic ZS.** Inhibition of tetrahydrobiopterin biosynthesis impairs endothelium-dependent relaxations in canine basilar artery. *Am J Physiol Heart Circ Physiol* 273: H718–H724, 1997.
20. **Kirsch M, Korth HG, Stenert V, Sustmann R, and de Groot, H.** The autoxidation of tetrahydrobiopterin revisited. Proof of superoxide formation from reaction of tetrahydrobiopterin with molecular oxygen. *J Biol Chem* 278: 24481–24490, 2003.
21. **Koliatsos VE, Dawson TM, Kecojevic A, Zhou Y, Wang YF, and Huang KX.** Cortical interneurons become activated by deafferentation and instruct the apoptosis of pyramidal neurons. *Proc Natl Acad Sci USA* 101: 14264–14269, 2004.
22. **McDonald JD, Cotton RG, Jennings I, Ledley FD, Woo SL, and Bode VC.** Biochemical defect of the hph-1 mouse mutant is a deficiency in GTP-cyclohydrolase activity. *J Neurochem* 50: 655–657, 1988.
23. **Ross R.** Atherosclerosis–an inflammatory disease. *N Engl J Med* 340: 115–126, 1999.
24. **Sata M, Maejima Y, Adachi F, Fukino K, Saiura A, Sugiura S, Aoyagi T, Imai Y, Kurihara H, Kimura K, Omata M, Makuuchi M, Hirata Y, and Nagai R.** A mouse model of vascular injury that induces rapid onset of medial cell apoptosis followed by reproducible neointimal hyperplasia. *J Mol Cell Cardiol* 32: 2097–2104, 2000.
25. **Schwarzacher SP, Lim TT, Wang B, Kernoff RS, Niebauer J, Cooke JP, and Yeung AC.** Local intramural delivery of L-arginine enhances nitric oxide generation and inhibits lesion formation after balloon angioplasty. *Circulation* 95: 1863–1869, 1997.
26. **Thienel T, Chwalisz K, and Winterhager E.** Expression of MAPkinases (Erk1/2) during decidualization in the rat: regulation by progesterone and nitric oxide. *Mol Hum Reprod* 8: 465–474, 2002.
27. **Vasquez-Vivar J, Kalyanaraman B, Martasek P, Hogg N, Masters BS, Karoui H, Tordo P, and Pritchard KA Jr.** Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. *Proc Natl Acad Sci USA* 95: 9220–9225, 1998.
28. **Verma S and Anderson TJ.** Fundamentals of endothelial function for the clinical cardiologist. *Circulation* 105: 546–549, 2002.
29. **Verma S, Dumont AS, and Maitland A.** Tetrahydrobiopterin attenuates cholesterol induced coronary hyperreactivity to endothelin. *Heart* 86: 706–708, 2001.
30. **Verma S, Lovren F, Dumont AS, Mather KJ, Maitland A, Kieser TM, Triggle CR, and Anderson TJ.** Tetrahydrobiopterin improves endothelial function in human saphenous veins. *J Thorac Cardiovasc Surg* 120: 668–671, 2000.
31. **Verma S, Maitland A, Weisel RD, Fedak PW, Pomroy NC, Li SH, Mickle DA, Li RK, and Rao V.** Novel cardioprotective effects of tetrahydrobiopterin after anoxia and reoxygenation: Identifying cellular targets for pharmacologic manipulation. *J Thorac Cardiovasc Surg* 123: 1074–1083, 2002.
32. **Yu JS, Tsai HC, Wu CC, Weng LP, Li HP, Chung PJ, and Chang YS.** Induction of inducible nitric oxide synthase by Epstein-Barr virus B95–8-derived LMP1 in Balb/3T3 cells promotes stress-induced cell death and impairs LMP1-mediated transformation. *Oncogene* 21: 8047–8061, 2002.