Differential effects of eNOS uncoupling on conduit and small arteries in GTP-cyclohydrolase I-deficient hph-1 mice

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System in blood vessel wall, NO is mainly synthesized in endothelial nitric oxide synthase (eNOS; Refs. 31, 32). Endothelium-derived nitric oxide (NO) plays a key role in control of the cardiovascular system. In blood vessel wall, NO is mainly synthesized in endothelial nitric oxide synthase (eNOS; Refs. 31, 32). Endothelium-derived NO in turn activates soluble guanylate cyclase in underlying tetrahydrobiopterin; hydrogen peroxide; vasculature

TRANSLATIONAL PHYSIOLOGY

Differential effects of eNOS uncoupling on conduit and small arteries in GTP-cyclohydrolase I-deficient hph-1 mice

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Differential effects of eNOS uncoupling on conduit and small arteries in GTP-cyclohydrolase I-deficient hph-1 mice. Am J Physiol Heart Circ Physiol 301: H2227–H2234, 2011. First published September 30, 2011; doi:10.1152/ajpheart.00588.2011.—In the present study, we used the hph-1 mouse, which displays GTP-cyclohydrolase I (GTPCH I) deficiency, to test the hypothesis that loss of tetrahydrobiopterin (BH4) in conduit and small arteries activates compensatory mechanisms designed to protect vascular wall from oxidative stress induced by uncoupling of endothelial nitric oxide synthase (eNOS). Both GTPCH I activity and BH4 levels were reduced in the aortas and small mesenteric arteries of hph-1 mice. However, the BH4-to-7,8-dihydrobiopterin ratio was significantly reduced only in hph-1 aortas. Furthermore, superoxide anion and 3-nitrotyrosine production were significantly enhanced in aortas but not in small mesenteric arteries of hph-1 mice. In contrast to the aorta, protein expression of copper- and zinc-containing superoxide dismutase (CuZnSOD) was significantly increased in small mesenteric arteries of hph-1 mice. Protein expression of catalase was increased in both aortas and small mesenteric arteries of hph-1 mice. Further analysis of endothelial nitric oxide synthase (eNOS)/cyclic guanosine monophosphate (cGMP) signaling demonstrated that protein expression of phosphorylated Ser1177-eNOS as well as basal cGMP levels and hydrogen peroxide was increased in hph-1 aortas. Increased production of hydrogen peroxide in hph-1 mice aortas appears to be the most likely mechanism responsible for phosphorylation of eNOS and elevation of cGMP. In contrast, up-regulation of CuZnSOD and catalase in resistance arteries is sufficient to protect vascular tissue from increased production of reactive oxygen species generated by uncoupling of eNOS. The results of our study suggest that anatomical origin determines the ability of vessel wall to cope with oxidative stress induced by uncoupling of eNOS.

Nitrergic Oxide (NO) plays a key role in control of the cardiovascular system. In blood vessel wall, NO is mainly synthesized in endothelial nitric oxide synthase (eNOS; Refs. 31, 32). Endothelium-derived NO in turn activates soluble guanylate cyclase in underlying vascular smooth muscle cells leading to increased levels of cyclic guanosine monophosphate (cGMP) and vasodilatation (34). It is well established that tetrahydrobiopterin (BH4) is an essential cofactor for allosteric and redox activation of eNOS (33). BH4 is synthesized via the de novo pathway from guanosine 5′-triphosphate (GTP) by activation of the rate-limiting enzyme GTP-cyclohydrolase I (GTPCH I; Ref. 29). We (10) have previously shown that under physiological conditions, the vascular endothelium of wild-type mouse aorta has a high rate of BH4 production, while vascular smooth muscle cells do not contain detectable quantities of BH4 (10, 14).

A number of previous studies (1, 9, 21, 35) have suggested that in intact arteries increased oxidation of BH4 is an important mechanism responsible for the pathogenesis of endothelial dysfunction. These findings support the concept that BH4 is a molecular target for oxidative stress and that increased oxidation of BH4 to 7,8-dihydrobiopterin (7,8-BH2) may cause uncoupling of eNOS (17). On the other hand, several studies (43, 46) have reported that reduced bioavailability of BH4 could be also caused by decreased BH4 biosynthesis via GTPCH I. Under this condition eNOS also becomes uncoupled and causes increased production of superoxide anion (26, 45). Reduced NO bioavailability is a major mechanism responsible for initiation and progression of endothelial dysfunction in vascular disease (8, 16).

The hyperphenylalaninemic mutant (hph-1) mice are currently the best experimental model of genetically induced GTPCH I deficiency. The mutation does not alter the sequence within the reading frame of GTPCH I but reduces the steady-state levels of GCH mRNA (24). Both GTPCH I activity and BH4 biosynthesis are significantly reduced during their lifetime (15). The hph-1 mice develop endothelial dysfunction (6), which can be reversed by normalization of intracellular BH4 levels (19, 28). However, the exact molecular mechanisms underlying the control of the vascular function in conduit and resistance arteries of hph-1 mice have not been defined. In the present study, we tested the hypothesis that long-term loss of BH4 in aorta and small mesenteric arteries activates compensatory mechanisms designed to protect vascular wall from oxidative stress induced by uncoupling of eNOS.

MATERIALS AND METHODS

Experimental animals. Housing facilities and all experimental protocols were approved by the Institutional Animal Care and Use Committee of the Mayo Clinic and complied with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Breeder pairs of homozygous hph-1 mutant mice on B6CBA background or heterozygous hph-1 mutant mice on C57BL/6 background were provided by Dr. Alex Chen (Michigan State University, East Lansing, MI) and by Dr. Keith Channon (University of Oxford, Oxford, UK; Ref. 18), respectively. Experiments were performed in male wild-type and homozygous hph-1 mice with a B6CBA or a C57BL/6 background. All mice were maintained on standard chow with free access to drinking water. At the age of 12–16 wk, mice were

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anesthetized (pentobarbital, 60 mg/kg body wt ip) and killed. Aortas and the whole bowel were carefully removed and placed in cold (4°C) modified Krebs-Ringer bicarbonate solution (in mmol/l: 118.6 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25.1 NaHCO₃, 10.1 glucose, and 0.026 EDTA). Aortas and all branches of the small mesenteric arteries were dissected free from connective tissue in cold Krebs solution, and the blood was flushed out.

**Systolic blood pressure.** Mice were trained for blood pressure measurement as described (11) and systolic blood pressure was recorded in nonanesthetized quiescent mice by a tail-cuff method (Harvard Apparatus, Kent, England).

**Blood cell count.** Mice were anesthetized in a bell jar containing 1% isoflurane, and 150 μl blood were quickly drawn from the orbital venous sinus and transferred immediately to EDTA containing tubes (Microtainer; Becton Dickinson, Franklin Lakes, NJ). Blood cell counts were performed with ABAXIS VetScan HM2 hematology system (Union City, CA).

**Glucose and cholesterol profile.** Blood samples obtained through puncture of the right ventricle were immediately transferred to a tube containing EDTA. Glucose levels were measured in whole blood with Glucose (Accu Check (Roche Diagnostics, Indianapolis, IN). After centrifugation at 2,000 rpm (4°C, 10 min), supernatants were stored at −80°C until assayed. Cholesterol levels were measured on the Hitachi 912 chemistry analyzer using cholesterol CHOD-PAP reagents (Roche Diagnostics).

**Measurements of BH₄ and 7,8-BH₂ levels.** Aortas and mesenteric arteries were homogenized in buffer containing 50 mmol/l Tris (pH 7.4), 1 mmol/l dithiothreitol, and 1 mmol/l EDTA at 4°C and were centrifuged at 10,000 rpm. Biopterin levels were determined after differential oxidation in acid (which converts both BH₄ and 7,8-BH₂ to biopterin) and base (which converts only 7,8-BH₂ to biopterin) conditions by reversed-phase HPLC as described previously (9). Data were collected and analyzed by 32 Karat chromatography software (Beckman Coulter) and normalized against tissue protein levels. BH₄ content was calculated from the difference in biopterin levels after acid and base oxidations.

**Measurement of GTPCH I enzyme activity.** Tissue supernatant homogenates were filtered using a Sephadex G25M column (Amersham, Piscataway, NJ) to remove endogenous neopterin, BH₄, and phenylalanine. An incubation solution of 0.1 mol/l Tris·HCl (pH 7.4), 100 mmol/l PMSF, 10 mg/ml BSA, and 10 μmol/l GTP was added to 100 μl of lysate, and samples were incubated for 2 h at 37°C. After incubation, samples were placed on ice, and the reaction was terminated by the addition of 1 mol/l HCl. An iodine solution [1% I₂/2% KI (1:1, wt/vol)] was added and the samples were incubated for 1 h at room temperature in the dark. Subsequently, 10 μl of 20% ascorbic acid (wt/vol) was added followed by 20 μl of 1 mol/l NaOH. Neopterin triphosphate was then dephosphorylated by incubation with 10 μl of calf intestinal alkaline phosphatase (250 mU/μl, Fisher) for one h at 37°C. Samples were centrifuged at 12,000 rpm for 10 min. Neopterin was measured by reversed-phase HPLC with fluorescence detection. The results were expressed as picomoles per milligrams of protein per hour.

**Table 1. Effect of mouse strain background on enzymatic activity of GTPCH I and biopterin levels in the aorta.**

<table>
<thead>
<tr>
<th>Background</th>
<th>GTPCH I, pmol·mg⁻¹·h⁻¹</th>
<th>BH₄, pmol/mg</th>
<th>BH₂, pmol/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6CBA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>0.32 ± 0.05*</td>
<td>5.85 ± 0.58</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td>hph-1</td>
<td>0.15 ± 0.03*</td>
<td>3.51 ± 0.66</td>
<td>0.26 ± 0.07*</td>
</tr>
<tr>
<td>C57BL/6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>0.44 ± 0.02*</td>
<td>5.07 ± 0.34</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>hph-1</td>
<td>0.29 ± 0.04*</td>
<td>1.22 ± 0.15</td>
<td>0.17 ± 0.02*</td>
</tr>
</tbody>
</table>

Data are means ± SE (n = 6–10). GTPCH I, GTP-cyclohydrolase I; BH₄, tetrahydrobiopterin; BH₂, dihydrobiopterin. *P < 0.05 vs. wild-type mice (unpaired Student’s t-test).

**Table 2. Characteristics of wild-type and hph-1 mice on B6CBA background.**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Wild Type</th>
<th>hph-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>117 ± 3</td>
<td>122 ± 2</td>
</tr>
<tr>
<td>Mean blood pressure, mmHg</td>
<td>101 ± 2</td>
<td>101 ± 2</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>92 ± 2</td>
<td>91 ± 2</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>33.2 ± 0.8</td>
<td>30.7 ± 1.5</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>207 ± 15</td>
<td>201 ± 27</td>
</tr>
<tr>
<td>Cholesterol, mg/dl</td>
<td>86 ± 3</td>
<td>91 ± 4</td>
</tr>
<tr>
<td>White blood cells, 10⁶/mm³</td>
<td>9.0 ± 0.6</td>
<td>11.2 ± 0.8</td>
</tr>
<tr>
<td>Lymphocytes, 10⁶/mm³</td>
<td>8.4 ± 0.6</td>
<td>9.7 ± 0.9</td>
</tr>
<tr>
<td>Monocytes, 10⁶/mm³</td>
<td>0.2 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Granulocytes, 10⁶/mm³</td>
<td>0.5 ± 0.1</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>Red blood cells, 10⁹/mm³</td>
<td>9.7 ± 0.1</td>
<td>9.6 ± 0.1</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>42.8 ± 1.3</td>
<td>42.4 ± 0.4</td>
</tr>
<tr>
<td>Hemoglobin, g/dl</td>
<td>15.0 ± 0.2</td>
<td>15.1 ± 0.3</td>
</tr>
<tr>
<td>Platelets, 10⁹/mm³</td>
<td>868 ± 28</td>
<td>875 ± 73</td>
</tr>
</tbody>
</table>

Data are means ± SE (n = 5–10). Note that no significant difference was found between wild-type and hph-1 mice as determined by unpaired Student’s t-test analyses.

**Western blot analysis.** Whole aortas and small mesenteric arteries were homogenized in lysis buffer containing the following (in mmol/l): 50 NaCl, 50 NaF, 50 sodium pyrophosphate, 5 EDTA, 5 EGTA, 0.1 NaVO₄, 10 HEPES, 0.5 PMSF, and 10 μg/ml leupeptin, and 1% Triton X-100, pH 7.4. Equal amounts of protein (50 μg) were separated by SDS-PAGE and transferred to nitrocellulose membrane (Amersham), after which the membranes were probed using primary antibodies against constitutive copper- and zinc-containing superoxide dismutase (CuZnSOD), manganese-containing SOD (MnSOD), extracellular SOD (ecSOD; StressGen), GTPCH I (10), eNOS, Ser¹⁷⁷-phosphorylated eNOS (Transduction Labs), catalase, and β-actin (Sigma). The bands were visualized using a commercially available chemiluminescent substrate.
available ECL kit (Amersham). Densitometry was carried out using NIH-Image (Scion-Image, Scion, Frederick, MD).

Detection of superoxide anion. Intracellular superoxide anion production was quantified using recently described HPLC/fluorescence assay that employs dihydroethidium as a probe (12). A stable fluorescent product 2-hydroxyethidium is formed from the reaction between dihydroethidium and superoxide anion. Aortas and mesenteric arteries were equilibrated for 15 min in Krebs-HEPES buffer at 37°C in CO2 incubator. Some of the aortas and mesenteric arteries were incubated in the absence or presence of NOS inhibitor N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME, 30 μM) for 15 min or sodium diethyldithiocarbamate (DDC, 10 mM) for 30 min, respectively. Dihydroethidium (50 μM; Molecular Probes) was added, and blood vessels were incubated for additional 15 min. The samples were then washed of dihydroethidium and incubated in Krebs-HEPES buffer for additional 1 h at 37°C. The arteries were then homogenized in 4°C cold methanol and centrifuged at 10,000 rpm. The supernatant was analyzed by HPLC/fluorescence (Beckman Coulter) in 37% acetonitrile in 0.1% trifluoroacetic acid aqueous solution. Data were quantified using 2-hydroxyethidium standard from the reaction between dihydroethidium and Fremy’s salt as described previously (49) and normalized against tissue protein levels.

Measurement of hydrogen peroxide. An Amplex red hydrogen peroxide (H\textsubscript{2}O\textsubscript{2})/peroxidase assay kit (Invitrogen) was used to per-

![Fig. 2. Quantitative analysis of tetrahydrobiopterin (BH\textsubscript{4}) metabolism in aorta and in mesenteric arteries of WT and hph-1 mice on B6CBA background. Bar graphs showing levels of tetrahydrobiopterin (A), 7,8-dihydrobiopterin levels (B), and tetrahydrobiopterin-to-7,8-dihydrobiopterin ratio (C) in the aortas and the mesenteric arteries of WT and hph-1 mice. Data are means ± SE (n = 9–10). *P < 0.05 vs. WT mice (unpaired Student’s t-test).](http://ajpheart.physiology.org/)

![Fig. 3. A: quantitative analysis of intracellular superoxide anion production in the aorta of WT (B6CBA) and hph-1 mice in the presence or absence of nitric oxide synthase (NOS) inhibitor N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME, 30 μM). Results are means ± SE (n = 8–9) and are expressed in nmol/mg protein. *P < 0.05 vs. WT mice; †P < 0.05 vs. hph-1 mice without L-NAME (two-way ANOVA). B: Superoxide anion levels in the mesenteric arteries of WT and hph-1 mice. Results are means ± SE (n = 7) and are expressed in nmol/mg protein. Please note that no difference was found between WT and hph-1 mice. C: effect of SOD inhibition with diethyldithiocarbamate (DDC, 10 mM) on superoxide anion production in mesenteric arteries of hph-1 mice. Data are means ± SE (n = 4) and are expressed in nmol/mg protein. *P < 0.05 vs. without DDC treatment (unpaired Student’s t-test). D: quantitative analysis of 3-nitrotyrosine-positive proteins in the aortas and the mesenteric arteries of WT and hph-1 mice. Bar graph indicates the results of the optical densitometry (O.D.) from Western blots as quantified by UN-SCAN-IT (Silk Scientific). Data are shown as means ± SE (n = 8 for aorta and n = 4 for mesenteric arteries). *P < 0.05 vs. WT mice (unpaired Student’s t-test).](http://ajpheart.physiology.org/)
form the measurements of $H_2O_2$ release from mouse aorta as described previously (12). One whole aorta (from the aortic arch to femoral artery branch) is needed for $n = 1$ experiment. Freshly dissected aortas were opened longitudinally and incubated in buffer for 2 h at 37°C in CO$_2$ incubator. H$_2$O$_2$ concentrations were measured in the medium according the manufacturer’s instructions. After incubation, aortas were homogenized in 2% SDS, and protein concentrations were determined. The results were expressed as nanomoles per milligrams of protein per hour.

**Measurements of intracellular cGMP levels.** Ten to fifteen millimeters of aorta were equilibrated for 15 min in MEM (containing 0.1% BSA, 100 U/ml penicillin, and 100 µg/ml streptomycin) at 37°C in CO$_2$ incubator and were then incubated for 30 min with 3-isobutyl-1-methylxanthine (100 µmol/l) to inhibit the degradation of cyclic nucleotides by phosphodiesterases. During the last 2 min of the 30-min incubation, certain rings were stimulated with acetylcholine (10 µmol/l, Sigma, St. Louis, MO). Aortas were then removed and quickly frozen in liquid N2 and stored at −80°C until assayed. cGMP radioimmunoassay kit (Amersham) was used to perform the measurements, and the results were normalized against tissue protein levels.

**Calculations and statistical analysis.** All results are expressed as means ± SE and “p” indicates the number of animals from which tissues were harvested. Wild-type and hph-1 mice groups were compared by unpaired Student’s t-test for simple comparisons. For multiple comparisons between wild-type and hph-1 mice, a two-way ANOVA was used where appropriate. A value of $P < 0.05$ was considered significant.

**RESULTS**

**Characteristics of hph-1 mutant mice.** To determine the effect of genetic background of hph-1 mice on metabolism of BH$_4$, we first measured GTPCH I activity and vascular levels of BH$_4$ and BH$_2$ in hph-1 mice bred on B6CBA background. In addition, we also examined biopterin metabolism in hph-1 mice and their wild-type littermates bred on C57BL/6 background. As shown on Table 1, BH$_4$ metabolism was significantly impaired in aortas of both strains of mice. Together with previous findings (19, 28) demonstrating identical alterations in vascular phenotype (pulmonary hypertension) in hph-1 mice compared with wild-type mice (Fig. 1A). Enzymatic activity of GTPCH I was significantly reduced by >50% in both the aorta and the mesenteric arteries of hph-1 mutant mice ($P < 0.05$; Fig. 1, B and C, respectively).

BH$_4$ and 7,8-BH$_2$ levels. Measurements of biopterins indicated that BH$_4$ levels were significantly reduced in both the aortas and the mesenteric arteries of hph-1 mice ($P < 0.05$; Fig. 2A). In contrast, oxidative products of BH$_4$, 7,8-BH$_2$, were increased in hph-1 mouse aortas while levels of 7,8-BH$_2$ remained unchanged in small mesenteric arteries (Fig. 2B). Consequently, the BH$_4$-to-7,8-BH$_2$ ratio was significantly reduced in the aortas of hph-1 mice ($P < 0.05$; Fig. 2C) but not in small mesenteric arteries.

**Production of superoxide anion.** In agreement with previous reports (6, 19), BH$_4$ deficiency was associated with increased production of superoxide anion in the aorta ($P < 0.05$; Fig. 3A). To confirm that eNOS was the source of superoxide anion production, we treated aortas with NOS inhibitor L-NAME. Indeed, L-NAME significantly reduced superoxide anion levels in hph-1 mice aortas to the level comparable to wild-type mice (Fig. 3A). In contrast, we could not detect any increase in superoxide anion production in the small mesenteric arteries of hph-1 mice (Fig. 3B). However, in vitro treatment with the CuZnSOD-inhibitor DDC significantly increased basal levels of superoxide anion in hph-1 mesenteric arteries (Fig. 3C).

Moreover, the level of 3-nitrotyrosine, a marker for peroxynitrite formation, was increased in the aorta but not in the small mesenteric arteries of hph-1 mice ($P < 0.05$; Fig. 3D).

**Protein expression of antioxidants.** To investigate further the mechanisms for increased oxidative stress, we performed Western blot analyses of antioxidant proteins. Protein expressions of CuZnSOD, MnSOD, and ecSOD were unaltered in the Hph-1 mice were normotensive, and body wt did not differ between wild-type and hph-1 mice (Table 2). Plasma cholesterol and blood glucose levels were unchanged in hph-1 mice. Furthermore, GTPCH I deficiency did not significantly alter the numbers of white and red blood cells as well as platelets (Table 2).

**Expression and activity of GTPCH I.** In wild-type mice, expression and activity of the BH$_4$-generating enzyme were much higher in the mesenteric arteries than in the aorta (Fig. 1). Western blot showed that protein expression of GTPCH I was downregulated in both the aorta and the mesenteric arteries of hph-1 mice compared with wild-type mice (Fig. 1A). Enzymatic activity of GTPCH I was significantly reduced by >50% in both the aorta and the mesenteric arteries of hph-1 mutant mice ($P < 0.05$; Fig. 1, B and C, respectively).

Fig. 4. Representative Western blot analysis of copper- and zinc-containing superoxide dismutase (CuZnSOD; A), manganese-containing SOD (MnSOD; B), extracellular SOD (ecSOD; C) protein expressions in aortas of WT and hph-1 mice on B6CBA background. Bar graphs indicate the results of the relative densitometry compared with β-actin protein. Results are means ± SE ($n = 6–8$).
aortas of hph-1 mice compared with wild-type mice (Fig. 4). Interestingly, CuZnSOD expression was significantly increased in the mesenteric arteries of hph-1 mice \((P < 0.05)\), while MnSOD expression was unchanged (Fig. 5). Protein expression of ecSOD was very low in mesenteric arteries and did not differ between wild-type and hph-1 mice (Fig. 5).

Protein expression of hydrogen peroxide detoxifying enzyme catalase was significantly enhanced in hph-1 mice aortas and mesenteric arteries \((P < 0.05;\) Fig. 6).

**Expression and phosphorylation of eNOS.** Western blot analysis showed a significant increase in protein expressions of phosphorylated eNOS at Ser\(^{1177}\) in hph-1 mice aortas but not in mesenteric arteries compared with wild-type mice \((P < 0.05;\) Fig. 7). Total eNOS proteins did not differ between wild-type and hph-1 mice (Fig. 7). In contrast, inducible NOS protein expressions in wild-type and hph-1 mice blood vessels were weak and did not differ between both groups (data not shown).

**cGMP levels.** GTPCH I deficiency was associated with higher basal levels of cGMP in hph-1 mice aortas \((P < 0.05;\) Fig. 8). Furthermore, in vitro stimulation of isolated aortas with 10 \(\mu\)M acetylcholine significantly enhanced cGMP production in wild-type and hph-1 mice. However, the acetylcholine-induced increase in cGMP levels was significantly reduced in hph-1 mice aortas \((P < 0.05;\) Fig. 8).

**Expression and phosphorylation of eNOS.** Western blot analysis showed a significant increase in protein expressions of phosphorylated eNOS at Ser\(^{1177}\) in hph-1 mice aortas but not in mesenteric arteries compared with wild-type mice \((P < 0.05;\) Fig. 7). Total eNOS proteins did not differ between wild-type and hph-1 mice (Fig. 7). In contrast, inducible NOS protein expressions in wild-type and hph-1 mice blood vessels were weak and did not differ between both groups (data not shown).

**GTPCH I deficiency was associated with higher basal levels of cGMP in hph-1 mice aortas \((P < 0.05;\) Fig. 8). Furthermore, in vitro stimulation of isolated aortas with 10 \(\mu\)M acetylcholine significantly enhanced cGMP production in wild-type and hph-1 mice. However, the acetylcholine-induced increase in cGMP levels was significantly reduced in hph-1 mice aortas \((P < 0.05;\) Fig. 8).
genetically modified mice, genetic variants of GCH gene in

**DISCUSSION**

We report several novel findings in this study. First, oxidation of BH4 was increased in the aortas but not in the mesenteric arteries of hph-1 mice. Second, superoxide anion production and 3-nitrotyrosine formation were enhanced in hph-1 mice aortas only. Third, in contrast to aorta, CuZnSOD expression was significantly elevated in the mesenteric arteries of hph-1 mice. Fourth, protein expression of catalase was increased in both the aortas and the mesenteric arteries of hph-1 mice. Fifth, GTPCH I deficiency resulted in enhanced phosphorylation of eNOS and basal cGMP levels in the hph-1 mice. These findings demonstrate that aortas and mesenteric arteries of hph-1 mice undergo a number of (mal)-adaptive changes in response to GTPCH I deficiency. Since hph-1 mice were normotensive, with normal peripheral blood cell count as well as normal circulating levels of glucose, and cholesterol, we could rule out systemic hemodynamic, metabolic, or hematologic abnormalities as contributors to observations reported in the present study.

Biochemical studies (40, 44, 45) have demonstrated that suboptimal concentration of BH4 uncouples eNOS with subsequent elevated formation of superoxide anion leading to decreased formation and release of NO. Furthermore, it is well established that diabetes, hypertension, and hypercholesterolemia reduce bioavailability of vascular BH4 thereby causing eNOS uncoupling, increased superoxide formation, and impairment of NO-mediated endothelium-dependent vasodilatation (21, 25, 27, 37). Previous studies (6, 19, 42) also demonstrated that genetic deficiency of GTPCH I in hph-1 mice is associated with significantly enhanced eNOS-derived production of superoxide anions. Consistent with observations in genetically modified mice, genetic variants of GCH gene in humans are also associated with increased levels of vascular superoxide production and endothelial dysfunction (2, 48). In the present study, we confirmed that both enzymatic activity of GTPCH I and BH4 biosynthesis were significantly reduced in both the aortas and the mesenteric arteries of hph-1 mice aortas. Because of GTPCH I deficiency, decreased biosynthesis of BH4 would be predicted to result in proportional decrease of 7,8-BH2 levels. However, 7,8-BH2 levels were increased in hph-1 mice aortas, thereby indicating that an increased oxidation of BH4 and/or reduced catabolism of 7,8-BH2 may account for this phenomenon. Consequently, the BH4-to-7,8-BH2 ratio was significantly reduced in the aorta of hph-1 mice compared with wild-type mice. Interestingly, uncoupling of eNOS and elevated concentration of eNOS-derived reactive oxygen species were observed only in the aortas of hph-1 mice. In contrast to data from conduit arteries, little is known about the eNOS-uncoupling in mesenteric arteries of hph-1 mice. Most notably, superoxide anion production was not increased, and the BH4-to-7,8-BH2 ratio was unchanged in small mesenteric arteries of hph-1 mice suggesting that elevated CuZnSOD and catalase is capable of scavenging reactive oxygen species (see below), thus preventing oxidative stress induced by eNOS uncoupling. This observation is consistent with results of several recent studies (7, 9, 38, 41) showing that a reduced BH4-to-7,8-BH2 ratio rather than absolute concentrations of BH4 is an important parameter for eNOS-uncoupling.

We observed increased 3-nitrotyrosine positive proteins in the aorta of hph-1 mice, indicating an increased formation of peroxynitrite by rapid interaction of superoxide anion with NO. However, since BH4 deficiency reduced NO availability in hph-1 mice, a portion of the eNOS-derived superoxide anion is converted to H2O2 by SOD isoforms. In addition, previous studies reported that H2O2 is a mediator of endothelium-dependent relaxations in coronary arteries depleted of BH4 (5) or in BH4-deficient aortas (6). Indeed, we detected significant increase of H2O2 release from the aorta of hph-1 mice. Expression of SOD isoforms was not affected by elevation of superoxide anion production in hph-1 mice aortas. Therefore, significant increase in expression of catalase in the hph-1 aorta is most likely an adaptive mechanism designed to minimize toxicity of higher local concentration of H2O2 (30).

**Production of H2O2.** Furthermore, despite elevation of catalase, we also detected significantly increased H2O2 release from the aorta of hph-1 mice compared with wild-type mice ($P < 0.05$; Fig. 9).
The mechanisms whereby the levels of superoxide and 3-nitrotyrosine were not significantly enhanced in the mesenteric arteries of hph-1 mice may include increased endogenous antioxidant capacity. We observed a significant increase in protein expression of CuZnSOD and catalase in the mesenteric arteries of hph-1 mice, suggesting that the vessel wall attempts to protect itself against the damaging effects of superoxide anion by increased generation of antioxidants. Surprisingly, ecSOD protein expression was very low in mesenteric arteries compared with aorta of mice. The reason for this difference between conduit and resistance arteries is not known. A previous study (13) showed that, in contrast to the aorta, production of superoxide anion was not increased and endothelium-dependent relaxations to acetylcholine were preserved in mesenteric arteries of ecSOD-deficient mice, suggesting that ecSOD is unlikely to play a role in endothelial function of small arteries. Since CuZnSOD accounts for ~80% of total SOD activity (22, 36) and has been considered the primary antioxidant defense in cells, we treated mesenteric arteries with the CuZnSOD inhibitor DDC. Treatment with DDC increased superoxide anion levels threefold in hph-1 mice mesenteric arteries demonstrating that resistance arteries possess the ability to increase production of superoxide anion when CuZnSOD is inactivated. Considering the important role of small arteries in the regulation of peripheral resistance, the preservation of endothelial response in small mesenteric arteries may help to explain preservation of normal blood pressure in hph-1 mice (13, 28).

Analysis of BH4/eNOS pathway in aorta of hph-1 mice uncovered additional quite unexpected results. Protein expression of phosphorylated eNOS at Ser^1177 was increased in hph-1 mice aortas. Phosphorylation of eNOS at Ser^1177 is a key posttranslational modification, which ensures increased production of NO (23). However, despite its potential to compensate for the reduced availability of NO in hph-1 mice, under uncoupling conditions phosphorylation of eNOS at Ser^1177 enhances superoxide anion release from eNOS (4). Indeed, in line with the previous reports (6, 19), the l-NAME-sensitive superoxide anion was significantly increased in aortas of hph-1 mice confirming that during BH4 deficiency uncoupled eNOS can function as a major enzymatic source for vascular production of superoxide anion in vivo. This conclusion is further reinforced with previously reported observation (21) that eNOS-derived production of superoxide anion caused by depletion of BH4 was abolished in eNOS-deficient mice. In addition, Khoo et al. (19) demonstrated that selective augmentation of endothelial BH4 biosynthesis protects the vascular wall against increased production of superoxide anion caused by uncoupling of eNOS in hph-1 mice.

Consistent with activation of eNOS by phosphorylation and the observed increased production of H2O2, we detected significantly higher basal levels of cyclic GMP in aorta of hph-1 mice. This unexpected observation is best explained by the well-established ability of H2O2 to phosphorylate eNOS and to directly activate guanylate cyclase (3, 20, 39). Furthermore, in our prior studies (5), we demonstrated that catalase inhibited elevation of cGMP in coronary arteries depleted of BH4. Remarkably, when stimulated with acetylcholine, the increase in cGMP levels was significantly blunted in hph-1 aorta. The reasons behind reduced ability of hph-1 aorta to generate cGMP in response to endothelium-dependent agonist are not immediately apparent. However, consistent with our findings, prior in vitro studies demonstrated that peroxynitrite increased cGMP levels under basal conditions but reduced agonist-induced cGMP formation (50). Furthermore, our findings are in line with previous report (47) demonstrating that high basal production of cyclic GMP in eNOS transgenic mice is associated with impaired endothelium-dependent relaxations to acetylcholine. The exact mechanism of this resistance to stimulation by acetylcholine is unclear and remains to be determined.

Our studies report several unexpected observations regarding differential effects of GTPCH I deficiency on large conduit and small resistance arteries. It appears that compensatory increase of CuZnSOD represents adaptive response of hph-1 mesenteric arteries intended to compensate for increased production of superoxide anion by uncoupling of eNOS. This phenomenon is absent in conduit arteries. Thus a significant increase of eNOS-derived reactive oxygen species imposes oxidative stress resulting in altered eNOS/cyclic GMP signaling in aortas of hph-1 mice.

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AUTHOR CONTRIBUTIONS
L.V.d. and Z.S.K. conception and design of research; L.V.d. and L.A.S. performed experiments; L.V.d. and Z.S.K. analyzed data; L.V.d. and Z.S.K. approved final version of manuscript.

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