Acute Inhibition of Guanosine Triphosphate Cyclohydrolase 1 Uncouples Endothelial Nitric Oxide Synthase and Elevates Blood Pressure

Shuangxi Wang, Jian Xu, Ping Song, Yong Wu, Junhua Zhang, Hyoung Chul Choi, Ming-Hui Zou

Abstract—GTP cyclohydrolase 1 (GTPCH1) is the rate-limiting enzyme in de novo synthesis of tetrahydrobiopterin (BH4), an essential cofactor for endothelial NO synthase (eNOS) dictating, at least partly, the balance of NO and superoxide produced by this enzyme. The aim of this study was to determine the effect of acute inhibition of GTPCH1 on BH4, eNOS function, and blood pressure (BP) in vivo. Exposure of bovine or mouse aortic endothelial cells to GTPCH1 inhibitors (2,4-diamino-6-hydroxypyrimidine or N-acetyl-serotonin) or GTPCH1 small-interference RNA (siRNA) significantly reduced BH4 and NO levels but increased superoxide levels. This increase was abolished by sepiapterin (BH4 precursor) or N\(^{\text{5}}\)-nitro-L-arginine methyl ester (nonselective NOS inhibitor). Incubation of isolated murine aortas with 2,4-diamino-6-hydroxypyrimidine or N-acetyl-serotonin impaired acetylcholine-induced endothelium-dependent relaxation but not endothelium-independent relaxation. Aortas from GTPCH1-siRNA–injected mice, but not their control-siRNA injected counterparts, also exhibited impaired endothelium-dependent relaxation. BH4 reduction induced by GTPCH1 siRNA injection was associated with increased aortic levels of superoxide, 3-nitrotyrosine, and adhesion molecules (intercellular adhesion molecule 1 and vascular cell adhesion molecule 1), as well as a significantly elevated systolic, diastolic, and mean BP in C57BL6 mice. GTPCH1 siRNA was unable to elicit these effects in eNOS\(^{−/−}\) mice. Sepiapterin supplementation, which had no effect on high BP in eNOS\(^{−/−}\) mice, partially reversed GTPCH1 siRNA–induced elevation of BP in wild-type mice. In conclusion, GTPCH1 via BH4 maintains normal BP and endothelial function in vivo by preserving NO synthesis by eNOS. (Hypertension. 2008;52:484-490.)

Key Words: hypertension ■ NO ■ superoxide anions ■ endothelial NO synthase ■ tetrahydrobiopterin ■ GTP-cyclohydrolase 1 ■ oxidative stress

Nitric oxide produced by endothelial NO synthase (eNOS) is essential for cardiovascular homeostasis because of its antiinflammatory, antithrombotic, antiproliferative, and antioxidant effects.\(^ {1,2}\) A critical determinant of eNOS activity is the availability of tetrahydrobiopterin (BH4).\(^ {3}\) Recent studies\(^ {4-5}\) indicate that eNOS must be fully saturated with BH4 to completely couple reduced nicotinamide-adenine dinucleotide phosphate oxidation to NO production. Under conditions of limited BH4, eNOS functions in an “uncoupled” state in which reduced nicotinamide-adenine dinucleotide phosphate–derived electrons are added to molecular oxygen, rather than L-arginine, leading to the production of superoxide (O\(_2\)\(^{−}\)) and H\(_2\)O\(_2\).\(^ {6}\) In this uncoupled state, eNOS exacerbates oxidative stress initiated by other reactive oxygen species–generating enzymes (eg, reduced nicotinamide-adenine dinucleotide phosphate oxidase). eNOS uncoupling has been implicated in a number of vascular diseases, such as hypertension, atherosclerosis,\(^ {8}\) diabetes,\(^ {4}\) and is often accompanied by a reduction in BH4 levels. BH4 supplementation in vessel rings from animals with atherosclerosis, diabetes, or hypertension reduces endothelial dysfunction.\(^ {9-11}\) In addition, BH4 administration augments NO-mediated vasodilatation in diabetic patients\(^ {12}\) and smokers.\(^ {13}\) These findings support the hypothesis that intracellular BH4 concentrations dictate, at least in part, the balance of NO and O\(_2\)\(^{−}\) produced by eNOS in diseased blood vessels. Although the concept that BH4 deficiency uncouples eNOS is well established, how cardiovascular risk factors result in BH4 deficiency remains poorly defined.

Intracellular BH4 levels are regulated through de novo synthesis of BH4 from GTP cyclohydrolase (GTPCH) 1, a homodimeric protein consisting of 25-kDa subunits in mammalian cells.\(^ {14}\) This enzyme catalyzes the rearrangement of GTP to dihydronopterin triphosphate, a species subsequently converted to BH4 through the sequential action of 6-pyruvoyltetrahydrobiopterin synthase and sepiapterin reductase.\(^ {15}\) GTPCH1 is the rate-limiting enzyme in most tissues, making it the major determinant of intracellular BH4 content. Although several studies have investigated neuronal
regulation of the BH4-dependent enzyme tyrosine hydroxylase by GTPCH, the role of GTPCH1 in maintaining eNOS function has not yet been investigated in vivo. Several in vitro studies have shown that GTPCH1 gene transfer reverses BH4 deficiency, increases NO synthesis, and restores eNOS function in both endothelial cells and vessels isolated from diabetic rats\(^\text{16}\) and rats with low-renin hypertension.\(^\text{17}\) However, these studies did not establish a cause-effect relationship between GTPCH1 and eNOS uncoupling in vivo, because the amount of BH4 generated from the GTPCH1 overexpression system is several-fold higher than those in physiological conditions, which might increase eNOS function or NO bioactivity by scavenging \(O_2^-\) anions. In addition, the roles of GTPCH1 in the regulation of blood pressure (BP) and vascular tone were poorly characterized. The current study was undertaken to investigate the effect of acute GTPCH1 inhibition on BH4 deficiency, eNOS function, and BP in vivo.

Our results demonstrate that acute inhibition of GTPCH1 causes high BP via BH4 deficiency/eNOS uncoupling.

### Methods and Materials

A full description of materials, animals, and methods used, including cell culture, BP measurement, measurement of BH4, \(O_2^-\) anions, and NO, organ chamber, Western blot, RT-PCR, and immunohistochemistry, can be found in the online data supplement available at http://hyper.ahajournals.org.

### BP Measurement

To measure the BP in mice, a phycocerythrin-10 catheter was inserted into the right carotid artery. BP was monitored by a pressure transducer and recorded with computer software after anesthesia.

### Measurement of BH4, \(O_2^-\) Anions, and NO

The levels of BH4 and total biopterins were determined by high-performance liquid chromatography (HPLC). \(O_2^-\) production in cultured cells or mouse aortas was detected by using a dihydroethidium fluorescence/high-performance liquid chromatography assay. NO release in cultured cells was detected by using the diaminofluorescein fluorescence probe.

### Organ Chamber

Mice aorta rings were mounted in organ bath in Kreb’s solution. Contractile response was evoked by U46619. At the plateau of contraction, accumulative acetylcholine or sodium nitroprusside was added into the organ bath to induce the endothelium-dependent or -independent relaxation.

### Statistical Analysis

Data are reported as means±SEM. All of the data were analyzed using a 1- or 2-way ANOVA, followed by Bonferroni’s posttest analysis. \(P<0.05\) was considered significant.

### Results

#### GTPCH1 Inhibition Reduces Total Biopterin and BH4 Levels in Endothelial Cells

We first determined whether pharmacological inhibition of GTPCH1 alters the levels of both total biopterins and BH4 in bovine aortic endothelial cells (BAECs). Treatment of BAECs with the chemical inhibitors 2,4-diamino-6-hydroxy-pyrimidine (DAHP; 10 mmol/L) or \(N\)-acetyl-serotonin (NAS; 1 mmol/L) for 24 hours did not alter cell viability (data not shown). However, both treatments, applied alone or in combination, reduced both total biopterins (BH4+ dihydrobipterin [BH2]+ other biopterins) and BH4 levels compared with the control treatment (Figure 1A). In addition, neither DAHP nor NAS altered the ratios of BH2:BH4 (data not shown), a common index for oxidative BH4 reductin.\(^\text{18}\)

Overall, our results suggest that GTPCH1 inhibition by DAHP or NAS caused BH4 deficiency mainly by inhibiting de novo synthesis of biopterins (BH4 plus BH2).

#### Pharmacological Inhibition of GTPCH1 Uncouples eNOS in Endothelial Cells

Recent studies\(^\text{19–22}\) in transgenic mice and endothelial cells have shown that an imbalance between intracellular eNOS levels and BH4 availability leads to uncoupling of eNOS, resulting in greater \(O_2^-\) production and diminished NO release. As shown in Figure 1B, exposure of BAECs to DAHP (10 mmol/L) for 24 hours increased \(O_2^-\) production by 52.36%, as detected by dihydroethidium fluorescence. DAHP (5 and 10 mmol/L) simultaneously induced 26.70% and 39.58% decreases in NO release. Treatment of BAECs with the nonselective eNOS inhibitor \(N^\text{G}\)-nitro-L-arginine methyl ester (l-NAME) did not alter baseline \(O_2^-\) levels but...
reduced DAHP-induced $O_2^-$ release by 61.87%, showing that DAHP-induced $O_2^-$ production was eNOS dependent (Figure 1B).

Uncoupling of eNOS by GTPCH1 Inhibition Is Reversed by Sepiapterin in Endothelial Cells

To determine whether the reduced NO release observed in DAHP-treated BAECs was because of BH4 deficiency, we treated BAECs with sepiapterin, a compound that can be converted to BH4 through a GTPCH-independent salvage pathway. Sepiapterin (10 μmol/L) almost restored NO release in DAHP-treated BAECs but did not alter NO release in the absence of DAHP (Figure 1C). Similarly, sepiapterin abolished DAHP-triggered $O_2^-$ production but had no effect on $O_2^-$ levels in non-DAHP-treated BAECs (Figure 1B).

Gene Silencing of GTPCH1 Uncouples eNOS in Endothelial Cells

To exclude nonspecific effects of DAHP and NAS, we tested the effect of GTPCH1 small-interference RNA (siRNA) on $O_2^-$ and NO production. Because no specific siRNA for bovine GTPCH1 exists, mouse aortic endothelial cells (MAECs) were used in these experiments. As shown in Figure 2A, transfection of GTPCH1 siRNA, but not control siRNA, reduced GTPCH1 protein levels by 68.73%. Levels of eNOS were not affected by GTPCH1 siRNA. Analysis of $O_2^-$ production in these cells revealed that control siRNA did not alter $O_2^-$ levels. In contrast, GTPCH1 siRNA increased $O_2^-$ levels by 25.40%, with this increase being completely blocked by either L-NAME or Tempol (1 mmol/L), a superoxide dismutase mimetic (Figure 2B). GTPCH1 siRNA-induced $O_2^-$ production was accompanied by 42.23% reduction in NO release (Figure 2C) compared with control siRNA. In addition, L-NAME inhibited the NO release from control siRNA transfection MAECs but not in GTPCH1 siRNA-transfection MAECs (Figure 2C).

GTPCH1 Inhibition or Gene Silencing Results in an Impairment of Endothelium-Dependent Relaxation in Isolated Aortas That Is Sepiapterin Reversible

The effect of NAS and DAHP on endothelium-dependent and endothelium-independent vasorelaxation was determined in isolated aortas. Aortas were incubated with DAHP (10 mmol/L) or NAS (1 mmol/L) for 24 hours in endothelial cell basal medium at 37°C before the vasomotor reactivity assays. As shown in Figure S2A, acetylcholine-induced relaxation (endothelium-dependent vasorelaxation) was lower in DAHP-treated aortas (42.12±9.25%) than in vehicle-treated aortas (86.96±8.04%; P<0.05). NAS, like DAHP, suppressed acetylcholine-induced endothelium-dependent relaxation (54.53±11.07% versus 86.96±8.04%; P<0.05; Figure S2B). In contrast, sodium nitroprusside–induced relaxation (endothelium-independent vasorelaxation) was not altered by the presence of DAHP or NAS (Figure S2C and S2D).

Inclusion of sepiapterin in the chamber fluid did not affect endothelium-dependent relaxation (data not shown). However, sepiapterin partially restored relaxation in the presence of DAHP, as evidenced by an Emax (relaxation induced by
10.05 GTPCH1 siRNA vs control siRNA or control; NS

5; * /

P /H11021

P /H11005

Aortas (0.05; Figure S2A). Sepiapterin had a similar effect in DAHP-treated aortas and 42.12 ± 54.53 % of total biopterins in NAS-treated aortas (Emax: 65.96 ± 6.70% versus 54.53 ± 11.07%; P < 0.05; Figure S2B).

Next, the effects of chemical GTPCH1 inhibition on endothelium-dependent relaxation were confirmed using in vivo GTPCH1 gene silencing. Retro-orbital injection of GTPCH1 siRNA decreased aortic levels of GTPCH1 protein by 80%, whereas control siRNA had no effect (Figure 3A). Neither GTPCH1 siRNA nor control siRNA altered aortic expression of eNOS. The gene silence of GTPCH1 in vivo by GTPCH1 siRNA injection was further confirmed in RT-PCR detection of GTPCH1 mRNA. As shown in Figure 3A, GTPCH1 siRNA specially decreased the GTPCH1 mRNA level compared with control siRNA. Analysis of total biopterin and BH4 levels revealed that GTPCH1 knockdown reduced total biopterin levels in the aorta to 32.07% of the levels in the control siRNA group (P < 0.05; Figure 3B). Aortic BH4 was also reduced by GTPCH1 knockdown, with levels in this group being 60.06% of the levels in the control-siRNA group (P < 0.05; Figure 3B).

Vasoreactivity assays of aortas from siRNA-injected mice revealed that acetylcholine-induced relaxation was lower in aortas from GTPCH1 siRNA–injected mice (Emax: 67.23 ± 6.33% in sepiapterin + DAHP-treated aortas and 42.12 ± 9.21% in DAHP-treated aortas (P < 0.05; Figure S2A). Sepiapterin had a similar effect in NAS-treated aortas (Emax: 65.96 ± 6.70% versus 54.53 ± 11.07%; P < 0.05; Figure S2B).

In vivo GTPCH1 knockdown decreases both BH4 and total biopterins. A, Western blot and RT-PCR analysis of GTPCH1 or eNOS in aorta from control siRNA- and GTPCH1 siRNA-injected mice. N = 5; *P < 0.05 GTPCH1 siRNA vs control siRNA or control; NS indicates P > 0.05. B, Total biopterins and BH4 levels in aortas from siRNA-transfected mice. N = 5; *P < 0.05 GTPCH1 siRNA vs control siRNA or control. C, Endothelium-dependent relaxation in isolated aortas from control and GTPCH1 siRNA–injected mice. In a subset of experiments, aortas were incubated with sepiapterin for 1 hour. Data are expressed as means ± SEMs. N = 5; *P < 0.05 GTPCH1 siRNA vs control siRNA or control. N = 5; #P < 0.05 GTPCH1 siRNA vs GTPCH1 siRNA treated with sepiapterin.

Sodium nitroprusside–induced relaxation was identical in aortas from both groups (data not shown).

In Vivo GTPCH1 Gene Silencing Generates Aortic Oxidative Stress via eNOS Uncoupling

GTPCH1 siRNA increased aortic O$_2^-$ levels by 30.26% as compared with aortic O$_2^-$ levels in the control siRNA–treated group (Figure 4A). Accordingly, GTPCH1 siRNA, but not control siRNA, increased immunohistochemical staining for 3-nitrotyrosine (by 200%), a footprint for reactive nitrogen species such as ONOO$^-$ (a potent oxidant formed by O$_2^-$ and NO).$^{23}$ Staining for intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 was also increased (by 267% and 176%) by GTPCH1 siRNA, with control siRNA having no effect on the expressions of these proteins (Figure 4B). These results were further confirmed in Western blot analysis (Figure S3).

Next, the contribution of eNOS uncoupling in vascular oxidant stress and inflammation was investigated using eNOS-null mice. In contrast to aortas from wild-type mice, eNOS-null aortas did not exhibit increased levels of O$_2^-$ or 3-nitrotyrosine in response to GTPCH1 siRNA (Figure 4A and 4B). The expression of both ICAM-1 and VCAM-1 was slightly higher in eNOS-null mice than in wild-type mice. However, GTPCH1 siRNA did not alter levels of either protein in eNOS-null animals (Figure 4B).

In Vivo GTPCH1 Gene Silencing Elevates Systemic BP in an eNOS-Dependent and Sepiapterin-Reversible Manner

NO is known to regulate vascular tone, and eNOS depletion results in spontaneous hypertension.$^{24–27}$ Thus, we analyzed
the effect of in vivo GTPCH1 gene silencing on systemic BP. As shown in Figure 5, mean BP was greater in GTPCH1 siRNA–treated wild-type mice (136.81 ± 2.45 mm Hg) than those in the control siRNA–treated counterparts (114.28 ± 4.48 mm Hg; P < 0.05). Systolic pressure and diastolic pressure also showed significant differences between control siRNA–treated and GTPCH1 siRNA–treated wild-type mice. The mean, systolic, and diastolic BPs were significantly elevated in eNOS-null mice compared with those in C57BL/6 wild-type animals (Figure 5), similar to the results of Huang et al. 24 As expected, administration of neither GTPCH1 siRNA nor control siRNA altered the mean, systolic, and diastolic BPs in eNOS-null mice.

Administration of sepiapterin had no effects on the mean, systolic, and diastolic BPs in control siRNA- or GTPCH1 siRNA–treated eNOS-null mice (Figure 5). Similar results were obtained in untreated or control siRNA–treated wild-type mice (data not shown). However, in wild-type animals receiving GTPCH1 siRNA, sepiapterin reduced the mean, systolic, and diastolic BPs by 15.46% (P < 0.05; Figure 5).

**Discussion**

The major finding of the present study is that GTPCH1 likely plays an essential role in maintaining normal BP. In wild-type mice, eNOS uncoupling induced by GTPCH1 inhibition significantly elevated systemic BP. Sepiapterin partially reversed this effect in these animals but did not alter BP in eNOS-null mice. These findings indicate that the BP-lowering effect of sepiapterin is eNOS-dependent and that sepiapterin has no direct effect on BP. Because conduit arteries like aorta do not regulate total peripheral resistance, the hypertensive phenotype of mice administered GTPCH1 siRNA and the BP-lowering effect of sepiapterin in these animals may be attributable to changes in resistance artery structure and function. Indeed, initial studies of the vascular phenotype in the hyperphenylalaninemic mouse mutant (hph-1), which displays a 90% GTPCH1 deficiency, of the hph-1 mouse have demonstrated that BH4 deficiency causes pul-
monary hypertension, even under normoxic conditions, and greatly increases susceptibility to hypoxia-induced pulmonary hypertension. In contrast, augmentation of endothelial BH4 synthesis through targeted transgenic overexpression of GTPCH1 prevents hypoxia-induced pulmonary hypertension.28 Similarly, restoration of endothelial BH4 in hph-1 mice by crossing these animals with GTPCH1 transgenic mice rescues pulmonary hypertension induced by systemic BH4 deficiency.28 In this study, lung BH4 availability dose-dependently controlled pulmonary vascular tone, right ventricular hypertrophy, and vascular structural remodeling under both normoxic and hypoxic conditions. We have found that GTPCH inhibition has dual effects (ie, increased oxidative stress and decreased NO release) that lead to high BP in vivo. This finding is in line with a recent study in humans showing that the common GTPCH1 variant, C+243T, predicted NO excretion, with the most extreme diastolic and systolic BP values occurring in females independently of catecholamine secretion.29 In contrast, genetic variation in eNOS (Glu298Asp) does not influence the renal NO excretion (P>0.1).30 Our results are consistent with these reports, because they suggest that GTPCH1 is the rate-limiting enzyme determining in vivo NO biosynthesis and, consequently, vascular tone. Thus, GTPCH1 might play an essential role in maintaining endothelial function through the regulation of eNOS function.

Perspectives
It has been observed in animal models of cardiovascular diseases, including hypertension and diabetes, that the eNOS, crucial in maintaining endothelium homeostasis, has been transformed from a protective enzyme to a contributor of oxidative stress, known as “eNOS uncoupling.” It is generally agreed that the lack of BH4, the essential cofactor of eNOS, plays a causal role in the development of eNOS uncoupling. However, how BH4 shortage is developed is poorly understood. In this publication, we have discovered that selective inhibition of GTP cyclohydrolase I, a rate-limiting enzyme in BH4 de novo synthesis, causes hypertension and vascular inflammation. The clinical implication of the present study is supported by a recent study29 showing a common GTPCH1 variant, C+243T, in the 3′-untranslated region predicts NO excretion with the most extreme BP values of diastolic and systolic BP in females, although it does not predict catecholamine secretion. Our results support human data that GTPCH1 is a key player in maintaining eNOS function and systolic BP. The finding that GTPCH1 inhibition increases systemic BP through eNOS uncoupling may have broad applications for cardiovascular diseases, because endothelial GTPCH1 protein levels, GTPCH1 activity, and BH4 levels are reduced in a number of vascular diseases, including atherosclerosis.51,32 and diabetes.16,33–35 GTPCH1 inhibition might be a common mechanism for vascular endothelial dysfunction in vascular diseases, including hypertension, diabetes, and atherosclerosis.

Sources of Funding
This work was supported by National Institutes of Health grants (HL079584, HL074399, HL080499, and HL089920), a research award from the American Diabetes Association, a research award from the Juvenile Diabetes Research Foundation, a research award from the Oklahoma Center for Advancement of Science and Technology, a research award from Research Management Group, and the Travis Endowed Chair in Endocrinology, University of Oklahoma Health Sciences Center. J.X. is supported by postdoctoral fellowship from the American Heart Association.

Disclosures
None.

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Hypertension. 2008;52:484-490; originally published online July 21, 2008;
doi: 10.1161/HYPERTENSIONAHA.108.112094

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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Supplemental materials and data:

Materials and Methods

Reagents
Polyclonal or monoclonal antibodies against GTPCH1, eNOS, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM1), and β-actin were obtained from Santa Cruz Biotechnology, and 3-nitrotyrosine (3-NT) antibody was from Upstate Biotechnology. Secondary antibodies were purchased from Cell Signaling Technology. Mouse GTPCH1 siRNA and control siRNA were purchased from Ambion RNA Company. Dihydroethidium (DHE) and diaminofluorescein (DAF) were purchased from Calbiochem (USA). Sepiapterin and 9,11-dideoxy-11,9-epoxymethano-prostaglandin F2 (U46619) were obtained from Cayman Chemical. N-acetylserotonin (NAS), 2,4-Diamo-no-6-hydroxypyrimidine (DAHP), acetylcholine (Ach), sodium nitroprusside (SNP), and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Bovine aortic endothelial cells (BAECs) were obtained from Clonetics Inc. (Walkersville, MD).

Animals
Male wild-type (C57BL6) and eNOS\(^{-/-}\) mice, 8-12 weeks of age and weighing 20-25 g, were obtained from Jackson Laboratory (Bar Harbor, ME). Mice were housed in temperature-controlled cages under a 12 h light-dark cycle and given free access to water and normal chow. The siRNA sequences targeting murine GTPCH1 (GenBank\(^{TM}\) accession number NM_008102) were as follows: 5’-GGGACAUUUUCUUCUUUAUtt-3’ (sense) and 5’-AUAAAGAAGAAAGCCCtg-3’ (antisense). Scrambled siRNA served as a negative control. Mice were transfected with siRNA as previously described.\(^1\) Briefly, 25 µg of GTPCH1 siRNA was combined with in vivo-jetPEI\(^{TM}\)
(Polyplus Transfection, France) at N/P ratio of 5 (total volume, 100 µl). The mixture was incubated at room temperature for 15 min and retro-orbitally injected into mice. Seven days later, aortas were isolated to measure endothelium-dependent and -independent relaxation via organ chamber studies. Alternatively, aortas were used for biochemical assays described below. The animal protocol was reviewed and approved by the University of Oklahoma Institute Animal Care and Use Committee.

**Cell culture**

BAECs were grown in EBM (Clonetics Inc. Walkersville, MD) supplemented with 2% fetal bovine serum, penicillin (100 u/ml), and streptomycin (100 µg/ml). All cells were grown at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Experiments were performed on early-passage cells (passage 3 – 8) grown to at 70-80% confluence.

For isolation of primary mouse aortic endothelial cells (MAECs), collected aortas were washed twice with PBS at 4°C, carefully freed from all fat and connective tissue, and cut into 3 mm-long sections. These sections were then incubated in a 0.2% collagenase solution at 37°C with frequently shaking to detach endothelial cells from the aorta. MAECs were pelleted from solution through centrifugation at 1000 rpm for 15 min at 4°C, washed with PBS, and seeded onto culture plates containing EBM. Purity of MAEC cultures was confirmed through positive staining for eNOS, ICAM-1, and VCAM-1. MAECs at passages 3-5 were used for siRNA transfection.

**Transfection of siRNA into endothelial cells**

MAECs were transfected in 6-well plates according to a previously described protocol.² Briefly, a 10 µM stock solution of siRNA was prepared in 20 mM KCl, 6.0 mM HEPES (pH 7.5), and 0.2 mM MgCl₂. For each transfection, 100 µl transfection media (Gibcol) containing 4 µl siRNA stock solution was incubated with 100 µl transfection media containing 4 µl transfection reagent (Lipofectamine 2000, Invitrogen) for 30 min at room temperature. The siRNA-lipid complex was then added to each well, which contained 1 ml transfection media. After incubation for 6 h at 37°C, the transfection media was replaced with normal growth media, and cells were cultured for an additional 48 h.

**Blood pressure measurement**

Blood pressure was determined by a carotid catheter method.³ Mice were anesthetized with a ketamine and xylazine mixture (70:6 mg/kg, intramuscular injection) and placed
under warm light (37°C). A catheter was inserted into the left common carotid artery, with the aid of a dissecting microscope, to measure arterial blood pressure. For catheter insertion, the left common carotid artery was carefully exposed via a 0.5- to 1.0-cm midline incision in the ventral neck region. The tip of the artery toward the head was ligated with a suture (5–0 silk), and the tip toward heart was occluded with a microclip (no. 18055–03; Fine Science Tool, Foster City, CA). A small cut was then made in the vessel wall using microscissors (no. 15000–08, Fine Science Tool). A 60-cm catheter (PE10 tubing, A-M Systems) containing a sterile 10% heparin-90% saline solution was inserted into the artery a distance of 0.65 cm toward the thorax. The arterial clip was removed, and the catheter was tied in place. Blood was directed to a pressure transducer through the catheter to obtain computerized blood pressure measurements (AD instruments). The mice were allowed to recover and the mean, systolic, and diastolic blood pressures were monitored for at least 30 min in conscious states.

**Measurement of Biopterins**

The levels of BH4 and total biopterins were determined as previously described with some modification. Briefly, homogenates of aorta or cell lysates were suspended in distilled water containing 5 mM dithioerythrol, centrifuged at 12000g at 4°C for 10 min, and then subjected to oxidation in acid or base. To 100 µl aliquot of supernatant, 20 µl of 0.5 M HCl and 0.05 M iodine were added for acidic oxidation, and 20 µl of 0.5 M NaOH plus 0.05 M iodine were added for basic oxidation. After incubation for 1h in the dark at room temperature, 20 µl HCl was added to the basic oxidation only. All mixtures received 20 µl 0.1 M ascorbic acid for the reduction of excess iodine. Samples were then centrifuged for 10 min at 12000g at 4°C. Biopterin concentrations were determined by HPLC with a PR-C18 column. Elution was at a rate of 1.0 ml/min of 50 mM potassium phosphate buffer, pH 3.0. Fluorescence was detected with an excitation at 350 nm and emission at 440 nm. BH4 concentrations were calculated as the difference in results from oxidation in acid and base.

**Detection of ROS**

ROS production in culture cells or mice aortas was detected using the fluorescent probe DHE as described previously. Briefly, before the end of treatment, 10 µM DHE was added to the medium and incubated for 30 min at 37°C, then washing with PBS twice.
The DHE fluorescent intensity in cells was recorded by fluorescent reader at the wave of excitation (485 nm) and emission (645 nm). The DHE fluorescence intensity in homogenates of aorta was assayed by HPLC according to the method we used before. Control was setup as 100%.

**Detection of NO**

NO production in culture cells was detected using the fluorescent probe DAF as described previously. Briefly, before the end of treatment, 10 µM DAF was added to the medium and incubated for 30 min at 37°C, then washing with PBS twice. The DAF fluorescent intensity was recorded by fluorescent reader at the wave of excitation (485 nm) and emission (545 nm). Control was setup as 100%.

**Organ chamber**

Organ chamber study was performed as described previously. Rings (3 mm in length) from mice aorta, free of fat and connective tissue, were mounted in organ bath by 2 stainless hook in 5 ml Kreb’s solution (mM): NaCl 118.3, KCl 4.7, MgSO41.2, KH2PO41.2, CaCl2 2.5, NaHCO3 25.0, EDTA 0.026 and glucose 11.0 at 37 °C, gassed with 95%O2+5%CO2, under a tension of 0.8 g, for 1 h equilibration period. During this period, the Kreb’s solution was changed every 15 min. After the equilibration, tissues were contracted with 60 mM high-potassium salt solution. After washing and another 30 minutes equilibration period, contractile response was evoked by U46619 (30 nM) to elicit reproducible responses. At the plateau of contraction, accumulative Ach (0.01, 0.1, 1, 10, 100 µM) or SNP (0.0001, 0.001, 0.01, 0.1, 1 µM) was added into the organ bath to induce the endothelium dependent or independent relaxation.

**Western blot**

Aortic tissues or endothelial cells were homogenized on ice in cell-lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na3VO4, 1 µg/ml leupeptin, and 1 mM PMSF. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed using specific antibodies. Band intensity (area × density) was measured by densitometry (model GS-700, Imaging Densitometer; Bio-Rad). Background intensity was subtracted from all calculated areas.

**Reverse Transcription–Polymerase Chain Reaction**
Total cellular RNA was isolated from mice aorta using RNA extract kit from invitrogen. The procedures for semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) were performed by using forward (5’-GCCATGCAGTACTTCACCAA-3’) and reverse (5’-AGGCTTTCTGTGATGGCC ACCG-3’) primers corresponding to murine GTPCH1 mRNA. Reactions were run for 30 cycles at conditions as follows: denaturation for 30 seconds at 94°C, annealing for 30 seconds at 57°C, and extension for 30 seconds at 72°C. Constitutively expressed GADPH mRNA was amplified as control.

**Immunohistochemistry**

Isolated aortas were immediately fixed in 4% paraformaldehyde. Immunohistochemical staining for 3-NT, ICAM1, and VCAM1 was performed as described previously.8

**References**


Figure S1. Inhibition of GTPCH1 by DAHP decreases NO release and increases $O_2^-$ productions in BAECs. BAECs were incubated with DAHP for 24 hours as indicated concentration. The NO release and $O_2^-$ productions were detected by DAF and DHE fluorescence respectively. Data are expressed as mean±SEM., N=5, $^*P<0.05$ DAHP vs. control.
Figure S2. Pharmacological inhibition of GTPCH1 impairs the sepiapterin-reversible, endothelium-dependent relaxation in murine aortas. (A) Ach-induced endothelium–dependent relaxation of aortic rings incubated with EBM with 2% FBS for 24 h in the presence of DAHP (10 mM) ± sepiapterin (10 µM). Data are expressed as mean±SEM. N=6, *P<0.05 DHAP vs. control; #P<0.05 DHAP vs. DHAP plus sepiapterin. (B) Endothelium–dependent relaxation of aortic rings incubated with EBM with 2% FBS for 24 h in the presence of NAS (1 mM) ± sepiapterin (10 µM). Data are expressed as mean±SEM. N=6, *P<0.05 NAS vs. control; #P<0.05 NAS vs. NAS plus sepiapterin. (C) and (D) SNP-induced endothelium–independent relaxation of aortic rings incubated with EBM with 2% FBS for 24 h in the presence DAHP or NAS ± sepiapterin. Data are expressed as mean±SEM.
Figure S3. *In vivo* GTPCH1 knockdown induces eNOS-dependent increases of ICAM-1 and VCAM-1. Aortas from control or GTPCH1 siRNA-injected wild type (WT) or eNOS<sup>−/−</sup> mice were homogenated. The homogenates were subjected to perform western blot analysis of VCAM-1 and ICAM-1. The blot is a representative of at least three blots from three independent experiments.