Endothelium-Specific GTP Cyclohydrolase I Overexpression Attenuates Blood Pressure Progression in Salt-Sensitive Low-Renin Hypertension

Yan-Hua Du, MD; Yong-Yuan Guan, MD; Nicholas J. Alp, MD; Keith M. Channon, MD; Alex F. Chen, MD, PhD

Background—Tetrahydrobiopterin (BH4) is an essential cofactor of endothelial nitric oxide synthase (eNOS). When BH4 levels are decreased, eNOS becomes uncoupled to produce superoxide anion (O2−) instead of NO, which contributes to endothelial dysfunction. Deoxycorticosterone acetate (DOCA)–salt hypertension is characterized by a suppressed plasma renin level due to sodium retention but manifests in eNOS uncoupling; however, how endogenous BH4 regulates blood pressure is unknown. GTP cyclohydrolase I (GTPCH I) is the rate-limiting enzyme for de novo BH4 synthesis. This study tested the hypothesis that endothelium-specific GTPCH I overexpression retards the progression of hypertension through preservation of the structure and function of resistance mesenteric arteries.

Methods and Results—During 3 weeks of DOCA-salt treatment, arterial blood pressure was increased significantly in wild-type mice, as determined by radiotelemetry, but this increase was attenuated in transgenic mice with endothelium-specific GTPCH I overexpression (Tg-GCH). Arterial GTPCH I activity and BH4 levels were decreased significantly in wild-type DOCA-salt mice, but both were preserved in Tg-GCH mice despite DOCA-salt treatment. Significant remodeling of resistance mesenteric arteries (≈100-μm outside diameter) in wild-type DOCA-salt mice exists, evidenced by increased medial cross-sectional area, media thickness, and media-lumen ratio and overexpression of tenascin C, an extracellular matrix glycoprotein that contributes to hypertrophic remodeling; all of these effects were prevented in DOCA-salt–treated Tg-GCH mice. Furthermore, NO-mediated relaxation in mesenteric arteries was significantly improved in DOCA-salt–treated Tg-GCH mice, in parallel with reduced O2− levels. Finally, phosphorylation of eNOS at serine residue 1177 (eNOS-S1177), but not its dimer-monomer ratio, was decreased significantly in wild-type DOCA-salt mice compared with sham controls but was preserved in DOCA-salt–treated Tg-GCH mice.

Conclusions—These results demonstrate that endothelium-specific GTPCH I overexpression abrogates O2− production and preserves eNOS phosphorylation, which results in preserved structural and functional integrity of resistance mesenteric arteries and lowered blood pressure in low-renin hypertension. (Circulation. 2008;117:1045-1054.)

Key Words: GTP cyclohydrolase ▪ 5,6,7,8-tetrahydrobiopterin ▪ oxidative stress ▪ mesenteric arteries ▪ hypertension

Endothelial dysfunction contributes to the pathogenesis and progression of hypertension and is an independent predictor of cardiovascular risk. A cardinal feature of endothelial dysfunction is the loss of the protective actions of nitric oxide (NO) due to reduced synthesis from endothelial NO synthase (eNOS) and/or increased scavenging by reactive oxygen species (ROS). More recent evidence indicates that endothelial dysfunction also results from eNOS dysfunction due to a reduced level of its essential cofactor, tetrahydrobiopterin (BH4). When BH4 levels are decreased, the enzymatic reduction of molecular oxygen by eNOS is no longer coupled to L-arginine oxidation, which results in generation of superoxide anion (O2−) rather than NO, thus contributing to vascular oxidative stress and endothelial dysfunction. Therefore, it is important to understand the dynamic regulation of BH4 synthesis in vascular disease. GTP cyclohydrolase I (GTPCH I) is the rate-limiting enzyme for de novo synthesis of BH4. Inhibition of GTPCH I activity leads to BH4 deficiency and increases blood pressure (BP).

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Recent studies have shown that there are markedly increased levels of arterial O2− and decreased BH4 in deoxycorticosterone acetate (DOCA)–salt hypertension, a model that features typical eNOS uncoupling. Although oral BH4...
supplementation lowers BP in this model, the mechanistic relationship between endogenous BH4 levels and BP regulation in vivo is still unknown. In addition, the effects of systemic pharmacological BH4 supplementation may be mediated in part by nonspecific antioxidant effects of high-dose BH4. Here, we delineated the influence of endogenous BH4 on hypertension development using a transgenic mouse model with endothelium-targeted overexpression of GTPCH I (Tg-GCH). Arterial BH4 levels in this mouse strain are increased 3-fold, without elevation of plasma BH4 levels. Because total peripheral resistance plays a crucial role in BP regulation, we targeted resistance mesenteric arteries in vivo to determine the influence of constitutive GTPCH I on their structure and function in vivo. Because the catalytic activity of eNOS is associated with its homodimerization and/or phosphorylation of serine 1177,9 we further determined whether eNOS dimerization or phosphorylation is involved in the molecular mechanisms of endothelial dysfunction in DOCA-salt hypertension.

**Methods**

**DOCA-Salt Hypertensive Mice**

Wild-type (WT) C57BL/6 male mice (10 to 12 weeks old, weight 20 to 25 g) were obtained from Charles River Breeding Laboratories (Portage, Mich). Tg-GCH mice of C57BL/6 background were bred in-house. DOCA-salt hypertension was created as described previously (Data Supplement). BP was measured by both noninvasive tail-cuff and radiotelemetry methods. All arteries were collected 3 weeks after DOCA implantation, a late stage of hypertension in this model in which BP has been well-established. For radiotelemetry, arteries were surgically implanted with a TA11PA-C10 radiotelemetry transmitter (Data Sciences, Laurel, Md) for 24-hour recording of arterial pressure and heart rate with a radiotelemetry data-acquisition program (Dataquest ART 3.1, Data Sciences). Hemodynamic measurements were sampled for 10 seconds every 10 minutes for the 3-week duration. Data were reported as 24-hour average. Seven to 10 days after implantation surgery, mice underwent DOCA-salt or sham surgery. All animal procedures were in accordance with the institutional guidelines of the Michigan State University.

**In Vitro Study of Resistance Mesenteric Arteries**

A standard in vitro arterial preparation was used to study mesenteric vessels as described previously. Briefly, a section of the third-order mesenteric arteries (100 μm outside diameter) was isolated for study by computer-assisted video microscopy. After vessel preparation, all drugs were added in stated concentrations to the superfusing Krebs’ solution. Endothelium-dependent relaxation was assessed with cumulative doses of acetylcholine (Ach; 10^-9 to 10^-5 mol/L) after precontraction with 50 mmol/L KCl in the presence of the cyclooxygenase inhibitor indomethacin (10 μmol/L). NO-mediated relaxation was verified by the NO synthase (NOS) inhibitor Nω-nitro-L-arginine (L-NNA; 0.3 mmol/L, preincubated for 15 minutes). In complementary studies, endothelium-independent responses to sodium nitroprusside (SNP; 10 μmol/L) were studied. Relaxation responses were expressed as a percent relaxation of 10-μmol/L SNP-induced maximal relaxation. To determine the contribution of endothelium-derived hydrogen peroxide (H2O2) in ACh-induced relaxation, the effect of polyethylene-glycolated catalase (250 U/mL, preincubated for 15 minutes), a membrane-permeable specific scavenger of H2O2, was examined.

**Morphological Study**

Arteries were subjected to in vitro measurement of vascular remodeling as described previously. Mice were anesthetized and perfusion-fixed at a constant pressure (100 mm Hg) via the left ventricle with PBS followed by 4% paraformaldehyde for 10 minutes. Arteries were isolated, further fixed with 4% paraformaldehyde for 24 hours in situ, processed for paraffin embedding, and cut into 7-μm transverse sections for hematoxylin-eosin staining (Sigma, St Louis, Mo). Morphometric analysis was performed with NIH Image J software by measuring the circumference (C) of the external elastic lamina and internal elastic lamina. External (De) and internal (Di) diameters were calculated as C/π, assuming a circular structure under in vivo conditions, and medial cross-sectional area (CSA) was obtained by subtraction of the internal CSA from the external CSA: CSA=(π/4)*(De^2-Di^2). The media thickness, media-lumen ratio, and medial CSA were calculated to assess the degree of remodeling. Data from 5 sections per mice were averaged. In complementary studies, mouse heart was collected and weighed 3 weeks after the DOCA-salt regimen. Left ventricle weight was measured after the right ventricle and atria were cleared away. Left ventricle-to-body weight and left ventricle-to-right ventricle ratios were calculated to assess the degree of cardiomyopathy (Data Supplement).

**Immunohistochemistry**

Immunohistochemistry was performed to detect the expression of tenasin-C (Tn-C), an extracellular matrix glycoprotein that contributes to vascular remodeling. After being blocked with 5% goat serum for 1 hour, frozen sections were incubated with the primary rabbit polyclonal Tn-C antibody (1:50, Santa Cruz Biotechnology, Santa Cruz, Calif) at 4°C overnight, followed by incubation with secondary goat anti-rabbit antibody (1:200, Vector Laboratories, Burlingame, Calif). In negative controls, normal goat serum in PBS was substituted for the primary antibody. Positive immunostaining was determined with the Vectastain Elite ABC system and visualized with diaminobenzidine substrate (Vector Laboratories), followed by counterstaining with hematoxylin (Sigma).

**Superoxide Measurements**

Detection of arterial O2- was performed by both dihydroethidium fluorescence confocal microscopy and lucigenin (5 μmol/L)-enhanced chemiluminescence as described previously. Superoxide levels were expressed as nmol·min^-1·mg tissue^-1. To further verify the role of eNOS in O2- production, some vessels were preincubated with 10^-4 mol/L L-NNA for 30 minutes before being treated with dihydroethidium or lucigenin.

**Western Blot Analysis**

Western blot analysis was performed by conventional or low-temperature SDS/PAGE as described previously. Briefly, to detect eNOS dimerization, arteries were homogenized, and a nonboiled sample that contained 30 μg of protein was subjected to 6% SDS/PAGE at 4°C. To investigate total eNOS, phosphorylated eNOS, and actin, 30 μg of protein was resolved on 7.5% Tris gels at room temperature. Gels were transferred to nitrocellulose membranes and incubated with mouse anti-eNOS monoclonal antibody at 1:1500 dilution (BD Transduction Laboratories, Lexington, Ky), rabbit polyclonal anti-phosphorylated eNOS (Ser-1177) antibody at 1:1000 dilution (Cell Signaling Technology, Danvers, Mass), or mouse anti-actin (C-20) at 1:1000 dilution (Santa Cruz Biotechnology). Secondary antibodies included IRDye 800-conjugated anti-mouse antibody (1:5000, Rockland Immunochemicals, Inc, Gilbertsville, Pa) and Alexa Fluor 680 goat anti-rabbit IgG antibody (1:2000, Invitrogen, Carlsbad, Calif). Bands were visualized with an Odyssey Imager and quantified with NIH Image J software.

**Data Analysis**

All values are expressed as mean±SEM. Statistical significance of differences between groups was determined with Student’s 2-tailed unpaired t test. When >2 treatment groups were compared, including BH4 and O2- levels, GTPCH activity, BP, and vascular remodeling, 1-way ANOVA was used. Repeated-measures ANOVA was used to analyze concentration-response curves. In all tests, P<0.05 was taken as statistically significant.
The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

**Results**

**Effect of GTPCH I on BP and Body Weight**

The effects of GTPCH I on BP and heart rate responses to DOCA-salt in mice implanted with radiotelemetry transmitters are shown in Figure 1. There were no significant differences in baseline BP (day 0) and heart rate among the groups. Compared with sham mice, mean BP was increased significantly in WT DOCA-salt mice over a 3-week period (107±0.7 versus 146±3.9 mm Hg at day 21, P<0.01). Mean BP in DOCA-salt Tg-GCH mice was significantly lower than that of WT DOCA-salt mice beginning on day 11 (125±3.2 versus 135±4.3 mm Hg, P<0.05) and continuing through day 21 (124±4.3 mm Hg). Systolic and diastolic BP also showed significant differences between DOCA-salt–treated WT and TG mice starting on day 7 and day 18, respectively, through day 21. Similar BP results were observed by noninvasive tail-cuff methods (Data Supplement). Body weight before DOCA-salt treatment was similar for WT and Tg-GCH mice (22.0±0.5 versus 21.5±0.6 g); however, after 3 weeks of DOCA-salt treatment, WT mice had significantly lower body weight than sham controls (19.1±0.42 versus 24.9±0.51 g, P<0.01), which was significantly improved in DOCA-salt Tg-GCH mice (21.7±0.51 g, P<0.05).

**Effect of GTPCH I Overexpression on Arterial BH4 Levels and GTPCH Activity in DOCA-Salt Hypertension**

There was significantly decreased arterial GTPCH I activity, as well as decreased BH4 levels, in WT DOCA-salt mice compared with sham controls. Endothelium-targeted GTPCH I overexpression exhibited an 3-fold elevation of GTPCH I activity and BH4 levels over sham mice under control conditions, both of which were preserved in DOCA-salt Tg-GCH mice (Figure 2).

**GTPCH I Decreases O2− Levels in DOCA-Salt Hypertension**

In situ detection of O2− indicated that ethidium fluorescence in mesenteric artery of WT DOCA-salt mice was increased 3-fold compared with sham mice; this was decreased significantly by pretreatment with the NOS inhibitor L-NNA, which suggests that eNOS is a significant source of O2−. Furthermore, O2− levels in DOCA-salt–treated Tg-GCH mice were also significantly reduced, which suggests that endogenous BH4 supplementation by GTPCH overexpression inhibits eNOS uncoupling (Figure 3A and 3B). Consistently, lucigenin chemiluminescence revealed that arterial O2− levels in DOCA-salt mice were significantly diminished by endothelium-targeted GTPCH overexpression in Tg-GCH mice (Figure 3C).

**GTPCH I Preserves NO-Mediated Endothelial Relaxation in Mesenteric Artery in DOCA-Salt Hypertension**

There was no difference in vascular contractions to KCl in mesenteric artery among the 4 groups of mice; however, endothelium-dependent relaxations to ACh were significantly impaired in WT DOCA-salt mice compared with sham controls. In contrast, endothelium-dependent relaxations remained normal in DOCA-salt–treated Tg-CGH mice (Figure 4A). The relaxation response to 10−6 mol/L ACh was signifi-
Supplement). Developed over 3 weeks of DOCA-salt treatment (Data Supplement). GTPCH overexpression did not appear to exert a significant protective effect on cardiac hypertrophy, vasculature, GTPCH overexpression did not appear to exert a significant protective effect on cardiac hypertrophy. In contrast to the vasculature, GTPCH overexpression did not appear to exert a significant protective effect on cardiac hypertrophy. Developed over 3 weeks of DOCA-salt treatment (Data Supplement).

GTPCH I Reduces Expression of Tn-C
Representative photomicrographs of immunohistochemistry indicated that Tn-C staining was negative or only weakly presented in mesenteric arteries from sham mice. In arteries from WT DOCA-salt mice, strong Tn-C staining was scattered throughout the media, and this was markedly reduced in DOCA-salt–treated Tg-GCH mice (Figure 6).

Effect of GTPCH I on eNOS Dimerization and Phosphorylation
Because eNOS phosphorylation and dimerization are associated with eNOS activity, we determined the expression of these proteins. Our results showed that total eNOS expression was similar among groups, as indicated by the ratio of total eNOS to actin. The ratio of phosphorylated eNOS to total eNOS at the serine 1177 residue (eNOS-S1177) was significantly decreased in aortas from WT DOCA-salt mice compared with sham control; however, the ratio of phosphorylated eNOS to total eNOS was preserved in DOCA-salt–treated Tg-GCH mice. Interestingly, we observed no difference in the eNOS dimer-monomer ratio among the groups (Figure 7). These results suggest that endothelial dysfunction in DOCA-salt mice may be related to the decreased eNOS phosphorylation but not its monomerization. Augmentation of BH4 levels by endothelium-targeted GTPCH overexpression in vivo preserved eNOS phosphorylation.

Discussion
The results of the present study in resistance mesenteric arteries (third-order arteries with an outside diameter of 100 μm) demonstrate the following for the first time: (1) In vivo GTPCH I overexpression rescued the decreased arterial GTPCH activity and BH4 levels induced by DOCA-salt treatment. (2) Endothelium-specific GTPCH I overexpression was associated with increased NO bioavailability, as demonstrated by improved endothelium-dependent relaxation in DOCA-salt hypertensive mice. (3) There was significant vascular remodeling and O$_2^-$ production in WT hypertensive mice, which was markedly inhibited in Tg-GCH mice. (4) eNOS phosphorylation of serine 1177, but not the dimer-monomer ratio, was significantly decreased in WT hypertensive mice and was preserved by GTPCH I overexpression. (5) Progression of hypertension in DOCA-salt–treated Tg-GCH mice was significantly retarded.

Endogenous GTPCH I Inhibits Remodeling of Resistance Mesenteric Arteries but Not the Heart
Morphological studies showed that mesenteric arteries of WT DOCA-salt mice presented significant changes relative to controls in 3 weeks. DOCA-salt treatment resulted in a dramatic increase in media thickness and media-to-lumen ratio. Medial CSA was also significantly enhanced in WT DOCA-salt mice, a typical feature of hypertrophic remodeling. Vascular structure alterations were significantly reduced by endothelium-specific GTPCH I overexpression in DOCA-salt–treated Tg-GCH mice (Figure 5). In contrast to the vasculature, GTPCH overexpression did not appear to exert a significant protective effect on cardiac hypertrophy developed over 3 weeks of DOCA-salt treatment (Data Supplement).
oxygen to generate $O_2^-$, which results in exacerbated oxidative stress.\textsuperscript{1,4} Thus, eNOS uncoupling contributes to endothelial dysfunction not only by directly reducing NO production but also by increasing NO scavenging due to $O_2^-$ formation. The present results show that both arterial GTPCH activity and BH4 levels were decreased significantly in WT DOCA-salt mice. Arterial $O_2^-$ production was increased significantly in WT DOCA-salt mice compared with sham control, and this was significantly inhibited by the NOS inhibitor L-NNA. This finding is consistent with a previous report in aortas\textsuperscript{4} and further demonstrates that uncoupled eNOS is an important source of increased vascular ROS production in resistance mesenteric arteries of this model. Importantly, the increased $O_2^-$ levels caused by DOCA-salt were attenuated in Tg-GCH mice, in which both arterial GTPCH activity and BH4 levels were preserved, which suggests that eNOS uncoupling could be prevented by endothelium-specific GTPCH overexpression in vivo. Hence, the ability to maintain sufficient BH4 levels by GTPCH overexpression may provide a basis for a new strategy to combat oxidative stress–induced endothelial dysfunction in vivo.

An important question that remains to be answered is how the DOCA-salt hypertensive state affects BH4 levels. BH4 oxidation by ROS may be one of the mechanisms that contributes to BH4 deficiency. The initial source of ROS in hypertension that leads to BH4 oxidation may be NADPH oxidase, because in mice that lack the critical component of this oxidase complex (p47\textsuperscript{phox}−/−), eNOS uncoupling is prevented despite DOCA-salt treatment.\textsuperscript{4} Our recent studies also showed that endothelin-1–induced $O_2^-$ via the ET\textsubscript{A}/NADPH oxidase pathway led to BH4 deficiency in this model.\textsuperscript{3,12} It is known that $O_2^-$ reacts with NO to form peroxynitrite (ONOO\textsuperscript{-}), a radical species that oxidizes BH4. It is thus possible that $O_2^-$ may yield to ONOO\textsuperscript{-} to oxidize BH4, which sustains eNOS uncoupling, thereby creating a vicious circle of oxidative stress. Conversely, augmented BH4 levels induced by GTPCH I overexpression may decrease ONOO\textsuperscript{-} levels via chemical reactions with ONOO\textsuperscript{-} or inhibition of $O_2^-$ formation from uncoupled eNOS, contributing to lowered oxidative stress and nitrination of vascular proteins. Finally, the decreased arterial GTPCH activity in WT DOCA-salt mice may help to explain the BH4 deficiency in DOCA-salt hypertension in addition to the oxidative-reduction mechanisms.

Because resistance arteries play a major role in regulating peripheral vascular resistance and thus the development of
hypothesis, we targeted resistance mesenteric arteries (≈100-μm outside diameter) to determine their function. The present results showed that ACh-induced relaxations were markedly impaired in DOCA-salt mice compared with sham controls, and they were preserved in DOCA-salt–treated Tg-GCH mice. The restored relaxation response to ACh in Tg-GCH mice treated with DOCA-salt might best be explained by increased NO bioavailability, because it was attenuated by the NOS inhibitor L-NNA, accompanied by blunted O2− formation. Together, these observations suggest that endothelium-targeted GTPCH I overexpression maintains eNOS in its coupled state, as demonstrated by normalized NO-mediated relaxations and reduced O2− levels, which may account in part for the reduced BP level (Figure 8).

The relaxation responses in resistance arteries involve factors in addition to NO, such as endothelium-derived hyperpolarizing factors (EDHFs). The current major candidates for EDHF include epoxyeicosatrienoic acids, metabolites of the arachidonic P450 epoxygenase pathway, K+ ions, and electrical communication through myoendothelial gap junctions.13 Recently, H2O2 has been suggested as an EDHF that mediates endothelium-dependent relaxation in certain vascular beds.4,13 However, there are conflicting reports about the relaxation effect of H2O2, because some studies have reported negligible inhibitory effects by catalase on endothelium-dependent relaxations.14 Even under pathological conditions in which increased ROS production is expected to occur, such as diabetes mellitus, it has been shown that H2O2 does not mediate endothelium-dependent relaxations in response to ACh in either mouse aortas or small mesenteric arteries.15 Other studies have reported that endothelium-dependent relaxation to ACh is improved after treatment with catalase, which suggests that H2O2 is a source of ROS.16 In the present study, we did not observe any significant inhibitory effect of polyethylene-glycolated catalase on ACh-induced relaxations. In fact, such relaxation was actually slightly greater after treatment with polyethylene-glycolated catalase in WT DOCA-salt mice, which suggests that H2O2 is not a major EDHF in the mesenteric arteries of this model. It is not likely that EDHF plays a major role in relaxing responses for the following reasons. First, it has been shown that EDHF-mediated relaxations are only temporarily enhanced to compensate for the reduced NO-mediated relaxation under pathological conditions, whereas the initial responses are subsequently reduced during the same processes,17 which may help to explain the inconsistent results observed in previous studies. In the present study, mice were euthanized 3 weeks after the DOCA-salt regimen, a late stage of hypertension in this model, which makes it unlikely that EDHF plays a major role in sustaining endothelium-dependent relaxations. More importantly, the present results clearly demonstrated that ACh-induced relaxation was blunted by L-NNA (Figure 4), which provides strong evidence that NO plays a key role in response to ACh in this model.

The combination of reduced NO and increased ROS production not only may contribute to endothelial dysfunction but also could have vascular proliferative effects. NO inhibits platelet and leukocyte adhesion, as well as smooth muscle proliferation.
and migration, and promotes endothelial survival and proliferation, whereas ROS appear to counter these processes. It has been shown that ROS (such as O$_2^{-}$ and H$_2$O$_2$) play a crucial role in smooth muscle growth and hypertrophy. Treatment with superoxide dismutase mimetics or the antioxidants vitamin C and vitamin E has been shown to prevent vascular remodeling in resistance arteries in salt-loaded stroke-prone spontaneously hypertensive rats. Endothelin-1– and angiotensin II–induced remodeling are also related to increased O$_2^{-}$ production. Conversely, the protective effects of NO on vascular structure have been revealed in both N$^\text{G}$-nitro-L-arginine methyl ester–treated and eNOS-deficient mice, in which hypertrophy of cerebral vasculature is observed. The results of the present study revealed that resistance mesenteric arteries of DOCA-salt mice developed important adaptive changes in their structure after just 3 weeks of hypertension, including an increase in media thickness, media-lumen ratio, and medial CSA of the vessel wall (volume per unit length), which indicates hypertrophic remodeling consistent with that observed in DOCA-salt rats. The altered vascular structure not only may provide an explanation for hypertension development via increasing total peripheral resistance but also may contribute to vascular complications. One of the most important new findings of the present study is that the observed hypertrophic remodeling in WT hypertensive mice was significantly attenuated in Tg-GCH mice (Figure 5). It is likely that in addition to reduced oxidative stress from uncoupled eNOS, endothelium-targeted GTPCH I overexpression also preserved NO bioavailability to inhibit vascular remodeling in DOCA-salt–treated Tg-GCH mice (Figure 8).

The proliferation and migration of smooth muscle cells play a fundamental role in growth of the media of a blood vessel. Smooth muscle cell growth is facilitated by several extracellular matrix proteins. Tn-C is one of the extracellular matrix proteins that have been implicated in vascular smooth muscle cell proliferation and migration. Increased expression of Tn-C is associated with altered vascular structure in experimental and clinical pulmonary hypertension and angiotensin II–induced hypertension. It is well-established that ROS can stimulate extracellular matrix synthesis and accumulation; however, the role of ROS in Tn-C expression is much less understood. In pulmonary arterial endothelial cells, the monocrotaline-induced alteration in Tn-C expres-
sion is mediated in part by oxidative stress. Because of the small size of the mesenteric artery, we examined Tn-C expression by immunohistochemistry. The present study provides a new finding that Tn-C contributes to vascular hypertrophic remodeling in mesenteric arteries of DOCA-salt mice characterized by excessive oxidative stress, which is blunted by GTPCH I overexpression.

Another interesting finding of the present study is that although total expression of eNOS was similar, eNOS phosphorylation was reduced in WT DOCA-salt mice compared with sham controls but was normal in Tg-GCH mice. In contrast, there was no difference in eNOS dimerization among the groups. Previous studies reported that GTPCH I gene transfer increases eNOS activity by redistribution of the enzyme into an active homodimer form in aortas of diabetic mice and hyperglycemic human endothelial cells. However, this mechanism is controversial, because in vitro homodimer formation has been shown to be essentially insensitive to BH4 availability. Furthermore, the dimer-monomer ratio is not directly related to the functional uncoupling of eNOS, a condition that leads to eNOS-mediated O$_{2}^{-}$ production instead of NO, because only the dimeric form is biochemically active and able to generate either NO or O$_{2}^{-}$.

eNOS is not a monomer even in the uncoupled state, because the oxidase activity of the monomer is limited. Thus, it might be possible that with the similar dimer-monomer ratio, eNOS of sham mice produces NO with sufficient levels of BH4, whereas eNOS of DOCA-salt mice produces O$_{2}^{-}$ due to BH4 deficiency. Consistent with this stipulation, a recent study demonstrates that the homodimer-monomer ratio is not dependent on changes in bipterin levels in either BH4-free purified eNOS or bovine aorta endothelial cells. In contrast, inhibition of de novo and salvage pathways of BH4 synthesis (with inhibitors of GTPCH and sepiapterin reductase, respectively) led to decreased eNOS phosphorylation. Phosphorylation of eNOS at serine 1177 (eNOS-S$^{1177}$) has been shown to increase eNOS catalytic activity by reducing Ca$^{2+}$ dependence, but this is reduced in arteries from DOCA-salt rats and in hypoxia-induced pulmonary hypertension. In the present study, we demonstrated for the first time that eNOS phosphorylation was decreased in arteries of DOCA-salt mice and was rescued by GTPCH I overexpression. These findings may represent an important mechanism of GTPCH I modulation of eNOS activity and NO bioavailability in the vasculature by a mineralocorticoid. Recent studies showed that phosphatidylinositol 3-kinase and its downstream effector,
Akt, are implicated in eNOS function, because phosphorylation of eNOS and Akt was significantly reduced in the left ventricle of DOCA-salt rats. Because increased blood flow causes coordinated upregulation of arterial eNOS-S1177, phosphorylated Akt, and GTPCH I expression in vivo, it is possible that Akt activation might be involved in eNOS phosphorylation by BH4. Future studies are warranted to determine the detailed mechanisms underlying GTPCH/BH4 regulation of eNOS-S1177 phosphorylation in this model. In addition, studies are also needed to examine the influences of GTPCH I on renal function in DOCA-salt mice, because this is also important in BP regulation.

In summary, the present study demonstrates for the first time that endothelium-specific GTPCH I overexpression in vivo abrogates O$_2$ production and maintains BH4 levels and eNOS phosphorylation, which results in preserved structural and functional integrity of resistance mesenteric arteries and lowered BP in low-renin hypertension. These findings may provide a mechanistic basis of increasing endothelial BH4 or protecting it against oxidation as a rational therapeutic strategy to rescue eNOS function and combat endothelial dysfunction in hypertension.

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Disclosures
None.

References
Hypertension affects ~25% of the adult population worldwide, of whom ~1 in 3 are salt sensitive. It is the major risk factor for stroke and atherosclerosis. Endothelial dysfunction contributes to hypertension pathogenesis, and a cardinal feature is the loss of the protective actions of nitric oxide (NO). Recent evidence indicates that endothelial NO synthase (eNOS) is a bifunctional enzyme: When its cofactor, tetrahydrobiopterin (BH4), is reduced by oxidative degradation, eNOS becomes “uncoupled” to produce superoxide anion (O2·−) instead of NO. In this way, eNOS uncoupling has a major impact on the redox state and function of blood vessels. Low-renin deoxycorticosterone acetate (DOCA)–salt hypertension exhibits typical eNOS uncoupling with exaggerated vascular oxidative stress; however, the way in which endogenous BH4 regulates blood pressure in vivo is incompletely understood. In the present study, we found that transgenic mice with endothelium-specific overexpression of GTP cyclohydrolase I (GTPCH), the rate-limiting enzyme for de novo BH4 synthesis, had preserved arterial BH4 levels that retarded eNOS uncoupling and hypertension despite DOCA-salt regimens. In addition, endothelium-dependent NO-mediated relaxation and vascular remodeling in resistance mesenteric arteries was largely protected.

**CLINICAL PERSPECTIVE**

Hypertension affects ~25% of the adult population worldwide, of whom ~1 in 3 are salt sensitive. It is the major risk factor for stroke and atherosclerosis. Endothelial dysfunction contributes to hypertension pathogenesis, and a cardinal feature is the loss of the protective actions of nitric oxide (NO). Recent evidence indicates that endothelial NO synthase (eNOS) is a bifunctional enzyme: When its cofactor, tetrahydrobiopterin (BH4), is reduced by oxidative degradation, eNOS becomes “uncoupled” to produce superoxide anion (O2·−) instead of NO. In this way, eNOS uncoupling has a major impact on the redox state and function of blood vessels. Low-renin deoxycorticosterone acetate (DOCA)–salt hypertension exhibits typical eNOS uncoupling with exaggerated vascular oxidative stress; however, the way in which endogenous BH4 regulates blood pressure in vivo is incompletely understood. In the present study, we found that transgenic mice with endothelium-specific overexpression of GTP cyclohydrolase I (GTPCH), the rate-limiting enzyme for de novo BH4 synthesis, had preserved arterial BH4 levels that retarded eNOS uncoupling and hypertension despite DOCA-salt regimens. In addition, endothelium-dependent NO-mediated relaxation and vascular remodeling in resistance mesenteric arteries (tertiary branch outside diameter of ~100 μm), which critically regulate total peripheral resistance, were largely protected through adequate eNOS phosphorylation. Because BH4 is highly unstable and easily oxidized (and thus not suitable for chronic oral administration), our findings may provide a mechanistic basis for augmentation of endogenous BH4 levels by targeting GTPCH as a rational therapeutic strategy (eg, by statins and peroxisome proliferator–activated receptor agonists) to recouple eNOS and combat endothelial dysfunction in hypertension and other cardiovascular diseases.
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