GTP Cyclohydrolase I/BH4 Pathway Protects EPCs via Suppressing Oxidative Stress and Thrombospondin-1 in Salt-Sensitive Hypertension

He-Hui Xie, Shuang Zhou, Dan-Dan Chen, Keith M. Channon, Ding-Feng Su, Alex F. Chen

Abstract—Endothelial progenitor cells (EPCs) are both reduced and dysfunctional in hypertension that correlates inversely with its mortality, but the mechanisms are poorly understood. Endothelial nitric oxide synthase (eNOS) critically regulates EPC mobilization and function but is uncoupled in salt-sensitive hypertension because of the reduced cofactor tetrahydrobiopterin (BH4). We tested the hypothesis that GTP cyclohydrolase I (GTPCH I), the rate-limiting enzyme of BH4 de novo synthesis, protects EPCs and its function in deoxycorticosterone acetate (DOCA)-salt mice. EPCs were isolated from peripheral blood and bone marrow of wild-type (WT), WT DOCA-salt, endothelial-specific GTPCH transgenic (Tg-GCH), GTPCH transgenic DOCA-salt, and BH4-deficient hph-1 mice. In WT DOCA-salt and hph-1 mice, EPCs were significantly decreased with impaired angiogenesis and adhesion, which were restored in Tg-GCH DOCA-salt mice. Superoxide (O$_2^-$) and nitric oxide (NO) levels in EPCs were elevated and reduced, respectively, in WT DOCA-salt and hph-1 mice; both were rescued in Tg-GCH DOCA-salt mice. eNOS$^{-/-}$/GCH$^{+/+}$ hybrid mice demonstrated that GTPCH preserved the circulating EPC number, reduced intracellular O$_2^-$ in EPCs, and ameliorated EPC dysfunction independent of eNOS in DOCA-salt hypertension. Secreted thrombospondin-1 (TSP-1; a potent angiogenesis inhibitor) from EPCs was elevated in WT DOCA-salt and hph-1 but not DOCA-salt Tg-GCH mice. In vitro treatment with BH4, polyethylene glycol-superoxide dismutase (PEG-SOD), or Nomega-nitro-L-arginine (L-NNA) significantly augmented NO and reduced TSP-1 and O$_2^-$ levels from EPCs of WT DOCA-salt mice. These results demonstrated, for the first time, that the GTPCH/BH4 pathway critically regulates EPC number and function in DOCA-salt hypertensive mice, at least in part, via suppressing TSP-1 expression and oxidative stress. (Hypertension. 2010;56:1137-1144.) ● Online Data Supplement

Key Words: endothelial progenitor cell ● GTP cyclohydrolase ● tetrahydrobiopterin ● thrombospondin-1 ● nitric oxide synthase

Although the standard nomenclature of endothelial progenitor cells (EPCs) is still lacking, putative EPCs are a circulating, bone marrow-derived cell population that participates in vasculogenesis by differentiating into endothelial cells and have been used to successfully enhance angiogenesis under ischemia. The integrity and function of the endothelium plays a key role in the prevention of hypertension. Recent clinical studies indicate that the number of circulating EPCs may serve as a surrogate marker for cardiovascular risks, affecting the progression of cardiovascular diseases including hypertension. In addition, hypertension has been identified as a major independent predictor for impaired EPC function. Treatment with antihypertensive drugs, such as angiotensin-converting enzyme inhibitors, can increase the number of circulating EPCs in patients with cardiovascular risk factors. However, the mechanisms underlying EPC dysfunction in hypertension are poorly understood.

Endothelial nitric oxide synthase (eNOS) regulates EPC mobilization and function, and nitric oxide (NO)-mediated signaling pathways are essential for EPC mobilization. However, eNOS is uncoupled in deoxycorticosterone acetate (DOCA)-salt hypertension because of the reduced level of its essential cofactor tetrahydrobiopterin (BH4). When the BH4 level is decreased, the enzymatic reduction of molecular oxygen by eNOS is no longer coupled to L-arginine oxidation, resulting in generation of superoxide anion (O$_2^-$) rather than NO, thus exacerbating oxidative stress. Recent studies have shown that eNOS uncoupling impairs EPC number and function in diabetes. In the present study, we tested the hypothesis that BH4 overexpression, which recouples eNOS, might preserve EPCs and its function in DOCA-salt mice. Since the effects of systemic BH4 supplementation may be mediated in part by nonspecific antioxidant effects of high-

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1137
dose BH4, here we investigated the influence of endogenous BH4 on EPC dysfunction using a transgenic mouse model of endothelial-specific overexpression of GTPCH I (Tg-GCH), the rate-limiting enzyme of de novo BH4 synthesis.\textsuperscript{11,14}

Thrombospondin-1 (TSP-1) is a key inhibitor of EPC function,\textsuperscript{15} and decreased NO production has been shown to induce TSP-1 expression in cultured endothelial cells.\textsuperscript{16,17} Previous studies also demonstrated that high glucose upregulates TSP-1 expression in rat mesangial cells, and this effect is reversed by BH4.\textsuperscript{18} On the basis of these findings, we also examined the role of TSP-1 on EPCs from DOCA-salt hypertension. We demonstrate, for the first time, that the GTPCH/BH4 pathway critically regulates EPC number and function in DOCA-salt hypertensive mice, at least in part, via suppressing TSP-1 expression and oxidative stress.

Materials and Methods

Animals

Wild-type (WT) C57BL/6 male mice (10 to 12 weeks, 20 to 25 g) were obtained from Charles River Breeding Laboratories (Portage, Mich.). Tg-GCH and BH4-deficient hph-1 mice of the C57BL/6 background were bred in house.\textsuperscript{11,19} Positive expression of transgenic GTPCH was confirmed by polymerase chain reaction (PCR). Tg-GCH (GCH\textsuperscript{+/+}) and eNOS knockout (eNOS\textsuperscript{-/-}) mice were crossed to produce the offspring with the genotype of eNOS\textsuperscript{-/-}/GCH\textsuperscript{+/+}, which were further crossed with eNOS knockout mice to develop a new strain of hybrid (HY) mice with the genotype of eNOS\textsuperscript{-/-}/GCH\textsuperscript{+/+}. Potential HY mice were screened by PCR of genomic DNA from tail tips. The primer sequences were as follows: GTPCH I (5’-GGGAAAGTCGCAAAATGTTGTAGTT-3’ and 5’- GAAACCATTGTGCACCTGACG-3’); eNOS (5’-TGGCTACCCGTGATATTGCT-3’ and 5’-ATTTCTCTGTCCTCCCTGTCCT-3’, and 5’- GCCGACCTCAGGCGACATAC-3’). Both 150-bp (GCH\textsuperscript{+/+}) and 500-bp (eNOS\textsuperscript{-/-}) PCR products were identified in the HY mice (Figure S1, available at http://hyper.ahajournals.org). All animal procedures were performed according to the guidelines of the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC).

Isolation of EPCs and Characterizations

Mouse circulating and bone marrow-derived EPCs were isolated and cultured according to our described technique.\textsuperscript{20,21} All other methods are described in the online supplemental material.

Data Analysis

Values were expressed as mean±SEM. Statistical significance of difference between groups was performed using the Student’s 2-tailed unpaired t test. When more than 2 groups were compared, 1-way ANOVA was used. A value of P<0.05 was considered statistically significant.

Results

Effect of GTPCH I on Blood Pressure

Five groups of mice (WT sham, WT DOCA-salt, Tg-GCH, Tg-GCH DOCA-salt, and BH4-deficient hph-1 mice) were used in the present studies, and the mean systolic blood pressure (BP) levels of the animals were observed. There were no significant differences in baseline BP (day 0) among all the groups (data not shown). Compared to WT sham mice, BP was significantly increased in WT DOCA-salt mice after a 3-week period (107±1.6 versus 139±4.8 mm Hg on day 21, P<0.01, n=6). BP in Tg-GCH DOCA-salt mice was significantly lower than that of WT DOCA-salt mice on day 21 (123±2.8 mm Hg, P<0.01, n=6 versus WT-DOCA). BH4-deficient hph-1 mice showed a slightly higher BP level than WT sham and Tg-GCH mice, but the difference did not reach statistical significance (115±4.5 versus 107±1.6 and 106±3.4 mm Hg, P>0.05, n=6 to 7).

GTPCH I Overexpression Preserves the Number of Circulating EPCs in DOCA-Salt Mice

The number of circulating stem cell antigen-1/fetal liver kinase-1 (also known as vascular endothelial growth factor receptor 2) (Sca-1/Flk-1) double-positive cells was significantly lower in WT DOCA-salt mice compared to WT sham mice (1.52±0.13% versus 2.63±0.14%, n=6 to 12, P<0.01), and was preserved in Tg-GCH DOCA-salt mice (2.41±0.27%, n=8, P<0.05 versus WT-DOCA). EPC number was also reduced in BH4-deficient hph-1 mice (1.56±0.11%, n=8, P<0.01 versus WT sham), which was increased in Tg-GCH mice (2.30±0.21%, n=5, P<0.05 versus hph-1) (Figure 1A and 1B). No difference was found between WT sham and Tg-GCH mice. These results were also confirmed by counting Dil-acLDL and lectin double-positive adherent cells under a fluorescence microscope (Figure 1C and 1D). These results demonstrate that GTPCH I overexpression preserved the level of circulating EPCs in DOCA-salt hypertensive mice.

GTPCH I Overexpression Maintains Intracellular BH4 and NO Levels in EPCs of DOCA-Salt Mice

The intracellular BH4 level in EPCs of DOCA-salt mice was significantly decreased compared to WT sham mice (0.47 pmol/mg protein, n=6, P<0.05) and hph-1 (1.61±0.59 pmol/mg protein, n=6, P<0.01) mice compared to sham controls (7.04±0.48 pmol/mg protein, n=7). There was an ∼2-fold elevation of intracellular BH4 in EPCs of Tg-GCH mice over sham mice under control condition (14.0±1.29 pmol/mg protein, n=5, P<0.01 versus WT sham), which was maintained in Tg-GCH DOCA-salt mice (8.18±0.85 pmol/mg protein, n=6, P<0.05 versus WT sham) (Figure 2A).

The NO level in bone marrow-derived EPCs was reduced in BH4-deficient hph-1 mice (0.41±0.09, n=6, P<0.05 versus WT sham), which was increased in Tg-GCH mice (1.45±0.32, n=6, P<0.01 versus hph-1). No difference was found between WT sham and Tg-GCH mice (Figure 2B). Triple-staining flow cytometry (Sca-1/Flk-1/DAF-FM\textsuperscript{+}) showed that the NO level in circulating EPCs from WT DOCA-salt mice was significantly lower than that from WT sham mice (0.52±0.09 versus 1.0±0.06, n=5 to 8, P<0.01), which was augmented in Tg-GCH DOCA-salt mice (0.86±0.11, n=5, P<0.05 versus WT-DOCA) (Figure S2).

GTPCH I Overexpression Decreases Intracellular O₂⁻ Level in EPCs of DOCA-Salt Mice

The O₂⁻ level in bone marrow-derived EPCs was elevated in BH4-deficient hph-1 mice (2.83±0.28, n=8, P<0.01 versus WT sham), which was reduced in Tg-GCH mice (1.13±0.14, n=5, P<0.01 versus hph-1). No difference was found between WT sham and Tg-GCH mice (Figure 3A and 3B). Data from triple-staining flow cytometry (Sca-1/Flk-1/+/DHE\textsuperscript{+}) showed that the O₂⁻ level in circulating EPCs from WT DOCA-salt mice was significantly higher compared to WT.
sham mice (2.74 ± 0.27 versus 1.0 ± 0.19, n=8, P<0.01), which was reduced in Tg-GCH DOCA-salt mice (1.26 ± 0.40, n=5, P<0.01 versus WT-DOCA) (Figure S2).

GTPCH I Overexpression Preserves Circulating EPCs and Decreases Their Intracellular O$_2^-$ Level Independent of eNOS in DOCA-Salt Mice

To further elucidate the role of eNOS in the effects of GTPCH I on EPCs, a new strain of HY mice was developed that possesses the genotype of eNOS$^{-/-}$/GCH$^{+/+}$. Circulating Sca-1/Flk-1 double-positive cells in the HY mice were similar to that in WT sham mice (2.0 ± 0.66% versus 2.38 ± 0.18%, n=4 to 15, P>0.05), which was preserved in HY DOCA-salt mice (2.10 ± 0.79%, n=4 to 15, P>0.05 versus WT sham) (Figure 4A). Flow cytometry data revealed that the O$_2^-$ level of circulating Sca-1/Flk-1 double-positive cells from WT DOCA-salt mice was significantly higher than that from WT sham mice (2.74 ± 0.27 versus 1.0 ± 0.19, n=8, P<0.01), which was re-
duced in HY DOCA-salt mice (1.74±0.10, n=4 to 8, P<0.05 versus WT-DOCA). No difference was found between WT sham and HY mice (Figure 4B). These results suggest that GTPCH I overexpression preserves circulating EPCs and reduces their intracellular O$_2^-$ level independent of eNOS in DOCA-salt hypertensive mice.

**GTPCH I Overexpression Protects EPC Functions in DOCA-Salt Mice**

Both tube formation and adhesion functions in EPCs from WT DOCA-salt mice were significantly reduced compared to that from WT sham mice (24.8±4.5 versus 65.8±5.2, n=5 to 8, P<0.01; and 43.7±4.7 versus 75.0±3.9, n=7, P<0.01, respectively), which were enhanced in Tg-GCH DOCA-salt mice (61.0±3.8, n=7, P<0.01; and 69.8±4.1, n=6, P<0.01, respectively, versus WT-DOCA). Tube formation and adhesion functions in EPCs were also decreased in BH4-deficient hph-1 mice (20.7±2.5, n=5, P<0.01; and 30.3±4.3, n=8, P<0.01, respectively, versus WT sham), which was ameliorated in Tg-GCH mice (66.2±4.2, n=7, P<0.01; and 89.9±2.3, n=5, P<0.01, respectively, versus hph-1). No difference was found between WT sham and Tg-GCH mice (Figure 5A through 5D).

To further elucidate the role of eNOS in the effects of GTPCH I on EPC functions, EPCs from the HY and HY DOCA-salt mice were also assessed. Both tube formation and adhesion functions in EPCs were considerably enhanced in HY and HY DOCA-salt mice compared to WT DOCA-salt mice, and no difference was found between HY and HY DOCA-salt mice, suggesting that GTPCH I ameliorates EPC function independent of eNOS in DOCA-salt hypertensive mice (Figure 5E and 5F). However, tube formation in EPCs was significantly lower in HY and HY DOCA-salt mice than that in WT sham, Tg-GCH, and Tg-GCH DOCA-salt mice, suggesting an important role of eNOS in the angiogenic function of EPCs (Figure 5E).

**GTPCH I Overexpression Reduces Secreted TSP-1 in EPCs of DOCA-Salt Mice**

Since TSP-1 is a secreted protein and its level in EPCs was too low to be detected (data not shown), we examined the TSP-1 level in EPC media using Western blot analysis. The

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**Figure 2.** GTPCH I overexpression maintains the intracellular BH4 level (A), as determined by the high-performance liquid chromatography method, and the intracellular NO level (B), as determined by DAF-FM-staining fluorescence intensity of flow cytometry, in bone marrow-derived EPCs of DOCA-salt hypertension. n=5 to 12; *P<0.05, **P<0.01 vs WT, TG, and TG-DOCA.

**Figure 3.** GTPCH I overexpression suppresses the superoxide level, as determined by DHE-staining flow cytometry, in EPCs of DOCA-salt mice. A, Representative fluorescence-activated cell sorter analysis of DHE-staining bone marrow-derived EPCs. B, DHE fluorescence intensity in bone marrow-derived EPCs. Values were normalized to WT control. n=5 to 7; **P<0.01 vs WT, TG, and TG-DOCA.
In Vitro Treatment With BH4, Polyethylene Glycol-Superoxide Dismutase, or Omega-Nitro-L-Arginine Reduces TSP-1 and O$_2^-$ Levels in EPCs of DOCA-Salt Mice

In vitro pharmacological interventions were also performed to verify the roles of intracellular O$_2^-$ and NO in the effect of BH4 on TSP-1 expression in EPCs. The secreted TSP-1 level and intracellular O$_2^-$ level in EPCs from WT DOCA-salt mice were significantly increased compared to those from WT sham mice (1.0±0.22 versus 2.9±0.27, n=12, P<0.01; and 1.0±0.12 versus 2.17±0.13, n=5 to 7, P<0.01, respectively), which was reduced following in vitro treatment with BH4 (10 μmol/L), polyethylene glycol-superoxide dismutase (PEG-SOD) (100 U/L), or Omega-nitro-L-arginine (L-NNa) (0.8 mmol/L) (Figure 7A and 7B). In addition, the intracellular NO level in EPCs from WT DOCA-salt mice was significantly decreased compared to those from WT sham mice (1.0±0.05 versus 0.53±0.08, n=7 to 12, P<0.01), which was enhanced following in vitro treatment with BH4 (10 μmol/L) and PEG-SOD (100 U/L) but not L-NNa (0.8 mmol/L) (Figure 7C). These results suggest that restoration of intracellular O$_2^-$ and NO levels contributes to the effects of BH4 on TSP-1 expression in EPCs.

Discussion

The major new findings of the present study are as follows: (1) endothelium-specific GTPCH I overexpression ameliorates the decreased intracellular BH4 level and protects EPCs and its functions in DOCA-salt hypertensive mice; (2) BH4 attenuates the increased O$_2^-$ level and augments the decreased NO level in EPCs of DOCA-salt mice both in vitro and in vivo; (3) BH4 reduces the O$_2^-$ level and preserves EPC number and functions independent of eNOS in DOCA-salt mice; (4) TSP-1 expression in EPCs is upregulated in DOCA-salt hypertension, which is reduced by BH4 in vitro and in vivo; (5) in vitro treatment with PEG-SOD or L-NNa reduces the secreted TSP-1 level from EPCs of DOCA-salt mice; and (6) eNOS is uncoupled in EPCs from DOCA-salt hypertension.

Although clinical studies have shown that EPCs are both reduced and dysfunctional in hypertension that correlates inversely with its mortality, limited knowledge exists regarding the underlying causes. In this study, we demonstrate that the sca-1/Fk-1 double-positive cells in peripheral blood were significantly decreased in DOCA-salt mice (a salt-sensitive hypertension model with low plasma renin) compared with the normotensive controls, which was in parallel with the results of Dil-acLDL/lectin double-staining assessment. In addition, both angiogenic and adhesion functions of EPCs were impaired in DOCA-salt mice compared to sham mice. eNOS is uncoupled in DOCA-salt hypertension because of its reduced essential cofactor BH4, whereby electrons no longer flow to L-arginine to form NO but instead reduce molecular oxygen to generate O$_2^-$, resulting in exacerbated oxidative stress. It has been shown that eNOS critically regulates normal EPC mobilization and function. However, the functional state of eNOS in EPCs under hypertension was unclear. In the present study, NOS inhibitor L-NNa reduced the intracellular O$_2^-$ level, while it did not increase the NO level, suggesting eNOS uncoupling in EPCs from DOCA-salt.
mice. In addition, recent findings including ours indicate that reactive oxygen species is increased in salt-sensitive hypertension.9–11,22 The increased oxidative stress observed in both animal and clinical hypertensives may affect the survival of EPCs.23 Thus, eNOS uncoupling and the subsequent increase in intracellular O$_{2}^{-}$/H$_{2}$O$_{2}$ and reduction in intracellular NO, as observed in the present study, may represent a major mechanism underlying EPC dysfunction in DOCA-salt mice (Figure 4S). Accordingly, it is reasonable to expect that the supplement of BH4, which could recouple eNOS, may rescue EPC dysfunction in this setting. Considering the fact that BH4 is highly unstable as a potent reducing molecule, we used transgenic mice overexpressing endothelial-specific GTP cyclohydrolase I (Tg-GCH). GTPCH I overexpression markedly elevated intracellular BH4 and NO levels, reduced the intracellular O$_{2}^{-}$/H$_{2}$O$_{2}$ level, and preserved both EPC number and function in DOCA-salt Tg-GCH mice. These results demonstrate that BH4 alleviates oxidative stress-induced EPC dysfunction through reducing O$_{2}^{-}$/H$_{2}$O$_{2}$ and enhancing NO levels in DOCA-salt mice. Restoration of intracellular O$_{2}^{-}$/H$_{2}$O$_{2}$

Figure 5. GTPCH I preserves bone marrow-derived EPC functions in DOCA-salt hypertension. A, Representative images of tube formation assay in Matrigel matrix of EPCs. Bar=500 μm. B, The number of tubes that was counted at random under 4 low-power fields (magnifications ×40) for each sample. C, Representative images of adhesion assay of EPCs. Bar=200 μm. D, The number of adherent cells that was counted at random under 3 low-power fields (magnifications ×100) per well. The mean value of 4 wells was determined for each sample. n=5 to 8; *P<0.05, **P<0.01 vs WT, TG, and TG-DOCA. E and F, GTPCH I enhances EPC tube formation and adhesion independent of eNOS in DOCA-salt mice. n=4 to 12; *P<0.05, **P<0.01 vs WT, TG, TG-DOCA, HY, and HY-DOCA; #P<0.05 vs WT, TG, and TG-DOCA.
and NO levels by BH4 may be attributable to the recoupling of eNOS in EPCs (Figure 4S).

However, we suspected whether the observed effects of BH4 on EPCs were completely eNOS dependent, or whether BH4 could produce these effects partly via a non-eNOS recoupling mechanism. To address this issue, we used a new strain of HY mice with the genotype of eNOS\(^{-/-}\)GCH\(^{-/-}\)/GCH. It was demonstrated that GTPCH I could reduce the intracellular O\(_2^\bullet\) level in EPCs and thus preserve EPC number and function independent of eNOS in DOCA-salt mice, suggesting that BH4 may exert a direct effect on EPCs in addition to recoupling eNOS. The augmented BH4 level caused by GTPCH I overexpression may decrease the O\(_2^\bullet\) level by chemically reacting with O\(_2^\bullet\), which may help to explain the BH4 non-eNOS recoupling effects on EPCs. In addition, angiogenic function of EPCs was significantly lower in these HY mice compared to the WT sham and Tg-GCH mice, demonstrating the importance of eNOS for EPC-mediated angiogenesis.

It has been demonstrated that TSP-1 inhibits angiogenesis and modulates endothelial cell adhesion, motility, and growth.24 Furthermore, a study has proposed that TSP-1 acts as a key inhibitor of EPC adhesion.15 However, no direct evidence for TSP-1 upregulation has been shown in hypertensive EPCs to date. Our data demonstrate the upregulation of TSP-1 in EPCs under hypertensive conditions for the first time. In addition, we observed an inverse relationship between TSP-1 level and EPC number and function, suggesting that TSP-1 upregulation might, in part, contribute to EPC dysfunction in DOCA-salt hypertension. Consistent with this notion, our data also revealed that a downregulation of TSP-1 by BH4 in vivo was accompanied by an amelioration of EPC dysfunction in this model, indicating a negative correlation of EPC function and TSP-1 level. Collectively, our experimental observations provide the first evidence that BH4 supplement reduces TSP-1 expression in EPCs both in vivo and in vitro and enhanced EPC function in vivo in DOCA-salt hypertension. Hence, in addition to reducing the intracellular O\(_2^\bullet\) level and enhancing the NO level, BH4 could also ameliorate oxidative stress-induced EPC dysfunction by suppressing TSP-1 expression in EPCs of DOCA-salt mice (Figure 4S).

Studies have shown that decreased NO production induces TSP-1 expression in cultured endothelial cells.16,17 Augmented oxidative stress can reduce NO bioavailability by increasing NO scavenging. Since our data showed that secreted TSP-1 and intracellular superoxide levels were increased in HY mice, the decrease in secreted TSP-1 and intracellular superoxide levels by BH4 in vivo was likely related to the increased NO production in DOCA-salt hypertension.
enhanced and the intracellular NO level was reduced in EPCs of DOCA-salt mice (both ameliorated by increasing BH4 in vivo), we then tested the hypothesis that BH4 exerts its effects on TSP-1 expression in EPCs through reducing superoxide and enhancing the NO level, in a series of in vitro studies. Besides BH4, PEG-SOD and NOS inhibitor L-NNA reduced the superoxide level and TSP-1 expression in EPCs of DOCA-salt mice, suggesting that the decreased superoxide level was involved in the mechanisms underlying the effect of BH4 on TSP-1 expression in EPCs. In addition, because the intracellular NO level in EPCs of WT DOCA-salt mice was upregulated following BH4 or PEG-SOD treatment in vitro, the enhanced NO level might also contribute to the effect of BH4 on TSP-1 expression (Figure 4S).

As Tg-GCH DOCA-salt mice exhibited a significantly lower BP than WT DOCA-salt mice, possibility exists that BP reduction might also contribute to the beneficial effect of BH4 on EPCs in Tg-GCH DOCA-salt mice, considering that these in vivo studies could not exclude the effect of BP from that of BH4. However, in vitro studies possess the advantage that allowed us to determine the direct effects of BH4, PEG-SOD, and L-NNA on EPCs, which do not involve BP and other possible influences from our in vivo studies. In addition, EPCs used in the present study are heterogenous. It is possible that some of the changes detected in the present study are a consequence of alterations in cell populations induced by hypertension.

Perspectives
The present study demonstrates, for the first time, that the GTPCH/BH4 pathway critically regulates EPC number and function in DOCA-salt hypertension, at least in part, via suppressing TSP-1 expression and reducing oxidative stress. Because BH4 is highly unstable and easily oxidized (thus not suitable for chronic oral administration), our findings on how the GTPCH/BH4 pathway regulates EPC function may provide a mechanistic basis of augmenting endogenous BH4 levels by targeting GTPCH as a new rational therapeutic strategy to recouple eNOS and combat EPC and endothelial dysfunction in cardiovascular diseases, including hypertension.

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Disclosures
None.

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GTP Cyclohydrolase I/BH4 Pathway Protects EPCs via Suppressing Oxidative Stress and Thrombospondin-1 in Salt-Sensitive Hypertension

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**Fig S1.** The PCR reactions performed on DNA isolated from tail biopsies of potential HY mice. The expected 150-bp (top panel) and 500-bp (bottom panel) PCR products were identified in the HY mice. HY, hybrid mice with the genotype of eNOS\textsuperscript{+/+} / GCH\textsuperscript{+/+}. 
Fig S2. GTPCH I overexpression suppresses superoxide and increases NO levels, as determined by DHE- and DAF-FM staining flow cytometry, respectively, in circulating EPCs of DOCA-salt mice. 

A. Representative FACS analysis of freshly isolated circulating mononuclear cells that were double-positive for Sca-1 (y axis) and Flk-1 (x axis) (left panels) and DHE fluorescence intensity in circulating Sca-1/Flk-1 double-positive cells (right panels).

B. DHE fluorescence intensity in circulating Sca-1/Flk-1 double-positive cells.

C. DAF-FM fluorescence intensity in freshly isolated circulating Sca-1/Flk-1 double-positive cells.
Fig S3

A. In vitro treatment with BH4 (10 µM) increases intracellular BH4 level in EPCs from WT DOCA-salt mice. n=4-7, **P<0.01 vs. DOCA.

B. In vitro treatment with BH4 (5-20 µM) concentration-dependently reduces TSP-1 expression in EPCs from WT DOCA-salt mice. n=5-12, **P<0.01 vs. WT, DOCA+10, and DOCA+20.

Fig S3. In vitro effect of BH4 on bone marrow-derived EPCs. A, In vitro treatment with BH4 (10 µM) increases intracellular BH4 level in EPCs from WT DOCA-salt mice. n=4-7, **P<0.01 vs. DOCA. B, In vitro treatment with BH4 (5-20 µM) concentration-dependently reduces TSP-1 expression in EPCs from WT DOCA-salt mice. n=5-12, **P<0.01 vs. WT, DOCA+10, and DOCA+20.
Fig 4S. Putative mechanisms on BH4 regulation of EPC number and function in salt-sensitive hypertension. BH4 deficiency in EPCs leads to eNOS uncoupling, resulting in exacerbated oxidative stress, decreased NO, up-regulated TSP-1 expression, and EPC dysfunction. BH4 impedes these alterations via recoupling eNOS and reducing $O_2^-$ level in EPCs.
Expanded Methods

DOCA-salt hypertension

DOCA-salt hypertension was created as we previously described. Briefly, mice underwent uninephrectomy (flank incision, left side) after ligation of the renal artery and vein with 4-0 silk sutures (Ethicon Inc, Somerville, NJ). A small area between the shoulder blades was shaved and a 1-cm incision was made through which DOCA pellets were implanted s.c. to provide a giving dose of 150mg/kg DOCA. DOCA-salt mice were given water containing 1.0% NaCl and 0.2% KCl. Sham mice were also uninephrectomized but received no DOCA implant and were given tap water. All animals were individually housed in clear plastic cages with free access to both standard pelleted chow and drinking solution. Systolic blood pressure (SBP) was measured by noninvasive, tail-cuff methods, and verified by radiotelemetry.

Isolation of endothelial progenitor cells and characterizations

Mouse EPCs were isolated and cultured according to our described technique. Briefly, peripheral blood was obtained by cardiac puncture after mice were anesthetized. Peripheral blood mononuclear cells (PB-MNCs) were isolated by Histopaque-1083 (Sigma) density gradient centrifugation at 400 g for 30 min. The mononuclear fraction was collected, washed in PBS (pH 7.4), red blood cells were lysed with ammonium chloride solution (Stemcell Technologies), then washed twice with PBS and once with endothelial growth medium-2 (EGM-2, Cambrex Life Sciences Corp), $2.5 \times 10^5$ cells/cm$^2$ MNCs were seeded on vitronectin-coated culture slides and plates. Cells were cultured in EGM-2 supplemented with 5% fetal bovine serum (FBS) at 37°C, 5% CO$_2$. After 5 days
of cultivation, the supernatant including the suspended cells were removed. Adherent
cells were stained for the uptake of low-density lipoprotein from human plasma, Dil
complex (Dil-acLDL) (Molecular Probes) and lectin from Bandeiraea simplicifolia BS-1
Isolectin B4 FITC Conjugate (Sigma). Cells were first incubated with Dil-acLDL (1
µg/ml) at 37°C for 4 hours and then fixed with 2% paraformaldehyde for 10 min. After
washing, cells were reacted with lectin (1 µg/ml) for 1 hour. After staining, cells were
quantified by examining 15 random microscopic fields (magnifications ×400), and
double-positive cells were identified as EPCs and counted.²

The quantification of circulating EPCs in the peripheral blood was determined by
flow cytometry as we described.² Briefly, mononuclear cells were isolated form
peripheral blood by density centrifugation. Freshly isolated PB-MNCs were re-
suspended in 100 µl 5% albumin bovine serum (BSA)/PBS (PBS-A). The co-expressions
of Sca-1 and Flk-1 were evaluated by flow cytometry. Antibodies to the Sca-1 and Flk-1
were used, and the isotype specific conjugated anti-IgG was used as a negative control.
Cells were incubated with antibodies to Sca-1-PE (BD Pharmingen) and Flk-1-FITC (BD
Pharmingen) for 1 hour on ice. After washing and centrifugation, the cell pellets were
suspected in 200 µl 2% paraformaldehyde, and expressions of Sca-1 and Flk-1 were
determined by flow cytometry (FACScan, Becton Dickenson) gating 50,000 events,
respectively. Sca-1⁺ and Flk-1⁺ cells were gated in the mononuclear cell fraction.²

Bone marrow-derived mononuclear cells (BM-MNCs) were isolated from mouse
tibia and femur as we described,² and seeded in 6-well cell culture plates coated with rat
vitronectin (1 µg/mL, Sigma) at a density of 5×10⁶ cells /well, and cultured in EGM-2.
After 4 days of culture, nonadherent cells were removed, and the adherent cells were
further cultivated for 3 days. The culture medium was then collected for Western blot analysis, and the cells were used for in vitro studies.

**In vitro cell function assays**

*Tube formation assay*

Matrigel-Matrix (BD Biosciences) was placed in the well of a 48-well cell culture plate and a number of $5 \times 10^4$ EPCs were plated in each well with EGM-2. After 18 hours of incubation, images of tube morphology was taken and tube number was counted at random under four low power fields (magnifications $\times 40$) per sample.

*Adhesion assay*

$1 \times 10^4$ cells were plated in 96-well plates coated with 1 $\mu$g/mL mouse vitronectin. After two hours of incubation, non-adherent cells were washed away and adherent cells were fixed with 2% paraformaldehyde. Nuclei were stained with Hoechst33528 ($5 \times 10^{-6}$ mol/L, 10 min, Molecular Probes). A number of adherent cells were counted at random under three low power fields (magnifications $\times 100$) per sample, and the mean value of the four wells was determined for each sample.

**BH$_4$ measurement**

BH$_4$ levels were measured by high-performance liquid chromatography (HPLC) with fluorescence detection after iodine oxidation in acidic or alkaline conditions as we previously described. Briefly, cells were lysed in cold extract buffer (50 mM Tris-HCl of PH 7.4, 1 mM DTT, 1 mM EDTA), and centrifuged at 16 000g for 15 minutes at 4°C. Protein concentration was measured using the Bio-Rad protein assay. Proteins were
removed by adding 10 μl of a 1:1 mixture of 1.5 M HClO₄ and 2 M H₃PO₄ to 90 μl of extracts, followed by centrifugation. To determine total biopterin (BH₄, dihydropterin [BH₂], and oxidized biopterin) by acid oxidation, 10 μl of 1% iodine in 2% KI solution was added to the 90 μl protein-free supernatant. To determine BH₂ and oxidized biopterin by alkali oxidation, 10 μl of 1 M NaOH was added to 80 μl of extract, then 10 μl of 1% iodine in 2% KI solution was added. Samples were incubated at room temperature for 1 hour in the dark. Alkaline-oxidation samples were then acidified with 20 μl of 1 M H₃PO₄. Iodine was reduced by adding 5 μl of fresh ascorbic acid (20 mg/ml). Samples of 50 μl were injected into a 250-mm long, 4.6-mm-inner diameter Spherisorb ODS-1 column (5 μm particle size; Alltech Associates, Inc) isocratically eluted with a methanol-water (5:95, v/v) mobile phase running at a flow rate of 1.0 ml/min. Fluorescence detection (350 nm excitation, 450 nm emission) was performed using a fluorescence detector (RF10AXL, Shimadzu Co). BH₄ concentrations, expressed as pmol/mg protein, were calculated by subtracting BH₂ plus oxidized biopterin from total biopterins.

Intracellular superoxide measurement

Intracellular O₂⁻ level was determined using dihydroethidium (DHE) (Sigma), a membrane-permeable dye which is oxidized to ethidium bromide in the presence of O₂⁻. After 7 days of cultivation, bone marrow-derived EPCs were trypsinized and rinsed with EGM-2 twice, and then incubated with DHE (10⁻⁶ mol/L) for 30 minutes at 37°C in dark. After incubation, cells were washed with PBS-A twice and re-suspended in 200 μl 2% paraformaldehyde. The DHE fluorescence intensity in cells was determined by flow
Intracellular O$_2^-$ levels of circulating EPCs was determined using a triple-staining flow cytometry (Sca-1$^+$/Flk-1$^+$/DHE$^-$), as we described. Briefly, freshly isolated PB-MNCs were incubated with DHE (10$^{-6}$ mol/L) for 30 minutes at 37°C in dark. After incubation, cells were washed with PBS twice and re-suspended in 100 μl 5% albumin bovine serum (BSA)/PBS, then cells were incubated with antibodies to Sca-1-FITC (BD Pharmingen) and Flk-1-APC (BD Pharmingen) for 1 hour on ice. After washing and centrifugation, the cell pellets were suspended in 200 μl 2% paraformaldehyde. The DHE fluorescence intensity in Sca-1$^+$/Flk-1$^+$ cells was determined by flow cytometry.

**Intracellular nitric oxide measurement**

Intracellular NO level was determined using membrane-permeable probes 4-amino-5-methylamine-2',7'-difluorofluorescein (DAF-FM) diacetate (Molecular Probes). After 7 days of cultivation, bone marrow-derived MNCs were trypsinized and rinsed with EGM-2 twice, and then incubated with DAF-FM diacetate (10$^{-6}$ mol/L) for 30 minutes at 37°C and an additional 30 minutes at room temperature in dark. After incubation, cells were washed with PBS twice and re-suspended in 200 μl 2% paraformaldehyde. The DAF-FM fluorescence intensity in cells was determined by flow cytometry.

Intracellular NO level of circulating EPCs was determined using a triple-staining flow cytometry (Sca-1$^+$/Flk-1$^+$/DAF-FM$^+$). Briefly, freshly isolated PB-MNCs were incubated with DAF-FM diacetate (10$^{-6}$ mol/L) for 30 minutes at 37°C and an additional 30 minutes at room temperature in dark. After incubation, cells were washed with PBS twice and re-suspended in 100 μl PBS, and then cells were incubated with antibodies to
Sca-1-PE-Cy™7 (BD Pharmingen) and Flk-1-APC (BD Pharmingen) for 1 hour on ice. After washing and centrifugation, the cell pellets were suspended in 200 µl 2% paraformaldehyde. The DAF-FM fluorescence intensity in Sca-1⁺/Flk-1⁺ cells was determined by flow cytometry.²

**Western blot analysis**

Western blot analysis was performed as we previously described.¹² Briefly, collected BM-MNCs culture media were concentrated with an Amicon Ultra 4 centrifugal filter device with a 10,000 molecular weight cutoff (Millipore) according to the manufacturer’s recommendations. Protein concentrations were determined using the Bio-Rad protein assay, and samples containing equal amounts of protein were subjected to 7.5% SDS/PAGE. Gels were transferred to nitrocellulose membranes and incubated with mouse anti-TSP-1 (1:200, abcam Inc.) monoclonal antibody. Secondary antibodies included IR Dye 800 conjugated anti-mouse IgG (1:5000, Rockland). Bands were visualized using Odyssey Imager with Odyssey 1.1 software (Li-Cor) and quantified using NIH image software.

**In vitro pharmacological interventions**

*In vitro* pharmacological interventions were performed to determine the direct effects of BH4 on the TSP-1 expression in EPCs and verify the roles of intracellular O₂⁻ and NO in these effects of BH4. BH4, superoxide dismutase–polyethylene glycol (PEG-SOD), and the NOS inhibitor N(G)-nitro-L-arginine (L-NNA) were used on EPCs derived from DOCA-salt hypertensive mice. After 5-day cultivation, EPCs were incubated with BH4 (10 µM, Sigma), PEG-SOD (100 U/L, Sigma), and L-NNA (0.8 mM, Sigma) for 24 h.
After 24-hour incubation, the culture medium was collected for Western blot analysis, and the intracellular BH4, superoxide and NO levels were measured as described above.

References
