GTP cyclohydrolase I prevents diabetic-impaired endothelial progenitor cells and wound healing by suppressing oxidative stress/thrombospondin-1

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Tie L, Chen LY, Chen DD, Xie HH, Channon KM, Chen AF. GTP cyclohydrolase I prevents diabetic-impaired endothelial progenitor cells and wound healing by suppressing oxidative stress/thrombospondin-1. Am J Physiol Endocrinol Metab 306: E1120–E1131, 2014. First published March 18, 2014; doi:10.1152/ajpendo.00696.2013.—Endothelial progenitor cell (EPC) dysfunction is a key contributor to diabetic refractory wounds. Endothelial nitric oxide synthase (eNOS), which critically regulates the mobilization and function of EPCs, is uncoupled in diabetes due to decreased cofactor tetrahydrobiopterin (BH4). We tested whether GTP cyclohydrolase I (GTPCH I), the rate-limiting enzyme of BH4 synthesis, preserves EPC function in type 1 diabetic mice. Type 1 diabetes was induced in wild-type (WT) and GTPCH I transgenic (Tg-GCH) mice by intraperitoneal injection of streptozotocin (STZ). EPCs were isolated from the peripheral blood and bone marrow of WT, Tg-GCH, and GTPCH I-deficient hph-1 mice. The number of EPCs was significantly lower in STZ-WT mice and hph-1 mice and was rescued in STZ Tg-GCH mice. Furthermore, GTPCH I overexpression improved impaired diabetic EPC migration and tube formation. EPCs from WT, Tg-GCH, and STZ-Tg-GCH mice were administered to diabetic excisional wounds and accelerated wound healing significantly, with a concomitant augmentation of angiogenesis. Flow cytometry measurements showed that intracellular nitric oxide (NO) levels were reduced significantly in STZ-WT and hph-1 mice, paralleled by increased superoxide anion levels; both were rescued in STZ-Tg-GCH mice. Western blot analysis revealed that thrombospondin-1 (TSP-1) was significantly upregulated in the EPCs of STZ-WT mice and hph-1 mice and suppressed in STZ-treated Tg-GCH mice. Our results demonstrate that the GTPCH I/BH4 pathway is critical to preserve EPC quantity, function, and regenerative capacity during wound healing in type 1 diabetic mice at least partly through the attenuation of superoxide and TSP-1 levels and augmentation of NO level.

guanosine triphosphate cyclohydrolase I; endothelial progenitor cells; oxidative stress; wound healing; diabetes

IMPAIRED CUTANEOUS WOUND HEALING in diabetic patients is a serious complication that often leads to amputation (27). It is estimated that worldwide every 30 s a lower limb is lost as a result of diabetes due to limited treatment regimens (3). Numerous studies have targeted current powerful technologies, such as sustained topical delivery and tissue engineering, to help eliminate amputations in diabetic patients (4). Recently, studies have focused intensively on bone-marrow (BM)-derived endothelial progenitor cells (EPCs), which circulate in the blood and exhibit characteristic features consistent with endothelial cells (2). EPCs’ capability of being recruited to arterial injury sites and contributing to vasculogenesis has been shown (15). Our previous studies have shown that normal EPC transplantation could effectively improve diabetic wound healing and suggest that normal EPC transplantation could be applied as a novel therapeutic intervention in diabetic wound healing (20). However, reduced EPC function is found in patients with type 1 and type 2 diabetes (18, 31), which would be a major problem in diabetic EPC transplantation.

Endothelial nitric oxide synthase (eNOS) has been shown to critically regulate EPC function (33). Tetrahydrobiopterin (BH4) is an essential cofactor of eNOS (7). BH4 reduction critically regulates EPC function (33). Tetrahydrobiopterin (BH4) is an essential cofactor of eNOS (7). BH4 reduction results in eNOS uncoupling and the generation of superoxide instead of nitric oxide (NO) (5). Intracellular BH4 levels are regulated by the de novo biosynthetic pathway from guanosine triphosphate (GTP), and GTP cyclohydrolase I (GTPCH I) is the rate-limiting enzyme (32). Ex vivo GTPCH gene transfer has been shown to reverse BH4 deficiency and rescue the ability of endothelial cells to produce NO in diabetic rats (21). In vivo studies have indicated that an increased BH4 levels inhibit vascular oxidative stress and improve eNOS function in type 1 diabetes in a transgenic mouse model with endothelium-targeted GTPCH I overexpression (Tg-GCH) (11). More recently, downregulation of BH4 and subsequent eNOS uncoupling have been shown in EPCs from diabetic rats (32). Therefore, the first aim of the current study is to determine whether GTPCH I overexpression could preserve the quantity and function of EPCs by eNOS recoupling in diabetic mice. Thrombospondin-1 (TSP-1) is a potent endogenous angiogenesis inhibitor (16, 23). TSP-1 suppresses endothelial cells’ responses to NO in a cGMP-dependent manner (16). Furthermore, TSP-1 mRNA and protein levels were found to be significantly increased in the vessel walls of type 2 diabetic rats (29). Based on these findings, the second aim of this study was to determine TSP-1’s role in BH4’s effects on EPCs from type 1 diabetic mice. Our results demonstrate for the first time that GTPCH I overexpression increased the quantity of EPCs and rescued their impaired function in type 1 diabetic mice. GT-PCH I overexpression increased the quantity of EPCs and rescued their impaired function in type 1 diabetic mice. GTPCH I overexpression could also effectively rescue the regenerative capacity of diabetic EPCs during wound healing in streptozotocin (STZ) mice. The putative mechanisms include attenuation of superoxide and TSP-1 levels.

MATERIALS AND METHODS

Animals and induction of type 1 diabetes. Wild-type (WT) C57BL/6 male mice were obtained from Charles River Breeding Laboratories (Portage, MI). Tg-GCH mice with C57BL/6 background

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were bred in house. The breeding pair of hph-1 mice was a kind gift from Dr. Keith Hyland (Institute of Metabolic Disease, Baylor University Medical Center, Dallas, TX), and the offspring were bred in house. Diabetes was induced in WT and Tg-GCH 10- to 12-wk-old mice (20–25 g) by intraperitoneal injection of 60 mg/kg STZ (Sigma) in 50 mM sodium citrate, pH 4.5, daily for 5 days (22). Control mice were treated with daily injections of citrate buffer. Blood glucose was measured from mouse tail veins using a One Touch Blood Glucose Meter (Lifescan). Once they reached 250 mg/dl, mouse blood glucose levels were measured daily for 1 wk prior to use in experiments. As shown in Table 1, STZ administration-induced diabetes in mice was shown by hyperglycemia (blood glucose levels: 414 ± 8 vs. 167 ± 3 mg/dl in WT mice, P < 0.001, and 342 ± 11 vs. 148 ± 3 mg/dl in Tg-GCH mice, P < 0.001). Blood glucose levels did not differ between hph-1, Tg-GCH, and WT mice. Male genetically diabetic (db/db) and male heterozygous littermate control (db/+; C57BLKS/J) between hph-1, Tg-GCH, and WT mice. Male genetically diabetic (db/db) and male heterozygous littermate control (db/+; C57BLKS/J) on rat vitronectin-coated (Sigma) culture slides and cultured in EBM-2 (Clonetics) medium with supplements (SingleQuot steps. Mononuclear cells isolated from BM (5 × 10^6) with ammonium chloride solution and purified with three washing steps. Mononuclear cells were isolated from BM (5 × 10^6 cells/cm^2) were cultured in EBM-2 (Clonetics) medium with supplements (SingleQuot Kit; Clonetics) on rat vitronectin-coated (Sigma) culture slides and plates. After 4 days in culture, nonadherent cells were removed and adherent cells further cultivated for 3 days. EPCs were characterized as adherent cells double positive for 1,1'-diacetylene-3,3',3'-tetramethylindocarbocyanine-labeled acetylated low density lipoprotein (DiI-AcLDL; Molecular Probes) uptake and fluorescein isothiocyanate (FITC)-conjugated lectin from Bandeiraea simplicifolia BS-1 (Griffonia simplicifolia; Sigma). Described briefly, adherent cells were incubated with 10 μg/ml Dil-ac-LDL for 4 h and later fixed with 2% paraformaldehyde for 10 min. Cells were washed and then reacted with 5 μg/ml FITC-BS-1 lectin for 1 h. For nuclear staining, cells were incubated for 5 min at room temperature with Hoechst 33258 (Sigma). The triple-stained cells, considered EPCs, were observed using a Nikon fluorescent microscope.

Excisional wound model and EPC therapy. One week after blood glucose reached 250 mg/dl (11), STZ mice and citrate-treated control mice were anesthetized with a halothane-oxygen vapor mixture (1.0–1.5%), and hair was shaved from their dorsum. A full thickness excisional wound was created on the dorsomedial back of each animal, using a 4-mm punch biopsy (Acuderm) as described previously (19, 34). Full-thickness skin was removed, exposing the underlying muscle. Immediately after surgery, BM-derived EPCs from different groups of mice were transplanted at 1 × 10^5 in 10 μl of PBS onto the wounds of diabetic mice, and then the wounds were covered with a biocclusive transparent dressing (Johnson & Johnson). Wound closure rate was measured by tracing the wound area every other day onto the biocclusive dressing. The tracings were digitized, and the areas were calculated in a blinded fashion with the use of a computerized algorithm (Sigma Scan; Jandel Scientific). By using a laser Doppler flowmeter (Transonic Systems), tissue blood flow in regions of the dorsum wound area was measured every other day. The mean tissue perfusion rate (ml·min⁻¹·100 g tissue⁻¹) was analyzed by using axoscope 9.0 software (Axon Instruments).

EPC functional assay: migration and tube formation assay. EPC migratory capacity was investigated using the modified Boyden chamber assay (33). Explained briefly, 5 × 10^6 cells were cultured in insets, which were placed in 24-well culture dishes containing EGM-2 and 50 mg/ml vascular endothelial growth factor (VEGF). After 24 h of incubation at 37°C and 5% CO₂, migrated cells were fixed and stained with Hoechst 33258 (Sigma). The number of cells on the lower side of the membrane was counted at magnification ×100, and the mean value of five different areas was determined for each sample.

For tube formation assay, Matrigel-Matrix (BD Biosciences) was placed in the well of a 48-well cell culture plate, and 5 × 10^6 cells were plated in each well with EGM-2 (13). After 24 h of incubation, images of tube morphology were taken by inverted microscope (Nikon), and tube lengths were measured at four random low-power fields (×40 magnification) per sample.

Fluorescence-activated cell sorting analysis. The quantification of circulating EPCs in the peripheral blood was also determined by flow cytometry. Peripheral blood mononuclear cells (PB-MNCs) were used for fluorescence-activated cell sorter (FACS) analysis. Cells were incubated with Sca-1 (PE-conjugated rat anti-mouse Ly-6A/E, 4 μg/ml; BD Pharmingen) and Flk-1 antibodies (FITC-conjugated rat anti-mouse Flk-1, 10 μg/ml; BD Pharmingen) for 1 h on ice. Isotype-matched mouse immunoglobulins served as negative controls. After incubation, the cells were washed with PBS and fixed in 2% paraformaldehyde before analysis with a FACS Calibur (Vantage SE turbo). The mean fluorescence intensity was detected using FlowJo software (version 6.4.3; Treestar).

Intracellular superoxide measurement. Intracellular superoxide (O₂⁻) levels of circulating EPCs were determined by a triple-staining flow cytometry assay using dihydroethidium (DHE; Sigma), a membrane-permeable dye that is oxidized to ethidium bromide in the presence of O₂⁻. Described briefly, freshly isolated PB-MNCs were incubated with DHE (10⁻⁴ mol/l) for 30 min at 37°C in the dark. After incubation, cells were washed with PBS (pH 7.4) to remove excess probe and then reacted with Sca-1 (10 μg/ml FITC-conjugated rat anti-mouse Ly-6A/E; BD Pharmingen) and Flk-1 antibodies (10 μg/ml APC-conjugated rat anti-mouse Flk-1; BD Pharmingen) for 1 h on ice. After incubation, cells were
washed with PBS and fixed in 2% paraformaldehyde before analysis with a FACS Calibur. The DHE fluorescence intensities of Sca-1 and Flk-1 double-positive cells were analyzed using FlowJo software.

*Intracellular nitric oxide measurement.* Intracellular NO levels were determined by a triple-staining flow cytometry assay using the membrane-permeable probes 4-amino-5-methylamino-2′ and 7′-difluorofluorescein (DAF-FM) diacetate (Molecular Probes). Freshly isolated PB-MNCs were incubated with the diluted DAF-FM diacetate (10⁻⁶ mol/l) for 30 min at 37°C and for an additional 30 min at room temperature in the dark to allow complete deesterification of the intracellular diacetates. After incubation, cells were washed with PBS, resuspended in EGM-2, and then reacted with Sca-1 (10 µg/ml PE-conjugated rat anti-mouse Ly-6A/E; BD Pharmingen) and Flk-1 antibodies (10 µg/ml APC-conjugated rat anti-mouse Flk-1; BD Pharmingen) for 1 h on ice.

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**Fig. 1.** A: bone marrow (BM) endothelial progenitor cells (EPCs) stained with Dil-acLDL (red), FITC-lectin (green), and Hoechst 33258 (blue) and BM EPCs under light microscopy. Scale bar, 10 µm. B: circulating EPC levels in Tg-GCH mice, hph-1 mice, and wild-type (WT) littermates with or without streptozotocin (STZ) treatment. Sca-1 and Flk-1 double-positive cells from each group were assessed by flow cytometry. Data are expressed as means ± SE; n = 4–6/group. *P < 0.05 vs. WT and Tg-GCH mice; #P < 0.05 vs. WT-STZ mice. PE FL 2-A, Flk-1 positive.
After incubation, cells were washed with PBS and fixed in 2% paraformaldehyde before their analysis with a FACS Calibur. The DAF-FM fluorescence intensity of Sca-1- and Flk-1-double-positive cells was analyzed using FlowJo software.

**BH4 measurement.** EPC BH4 levels were measured by high-performance liquid chromatography (HPLC) with fluorescence detection after iodine oxidation in acidic or alkaline conditions, as described previously (38). Cell pellets were lysed in cold extract buffer (50 mM Tris-HCl, pH 7.4, 1 mM DTT, and 1 mM EDTA). Protein concentration was measured using the BCA protein assay. Protein was 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 alkaline oxidation, 10 μl of 1 M NaOH was added to 80 μl of extract, and then 10 μl of 1% iodine in 2% KI solution was added. Samples were incubated at room temperature for 1 h 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) that was isocratically eluted with a methanol-water (5:95, vol/vol) 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). BH4 concentrations, expressed as picomoles per milligram of protein, were calculated by subtracting BH2 plus oxidized biopterin from total biopterins.

**Immunoblot analysis.** After 7 days in culture, collected media were concentrated with an Amicon Ultra 4 centrifugal filter device with a 0.45-μm pore size. Membranes were blocked in Odyssey Blocking Buffer (OBB) for 1 h at room temperature and then incubated overnight at 4°C with thrombospondin antibody (1:200, Abcam) overnight at 4°C with thrombospondin antibody (1:200, Abcam) diluted in OBB and 0.1% Tween. After incubation with the IR Dye 800 conjugated anti-mouse (1:5,000; Rockland) antibody, bands were visualized using Odyssey Imager and quantified using National Institutes of Health Image J software.

**Statistical analysis.** Data were expressed as means ± SE. The significance of differences between groups was evaluated by unpaired Student’s t-test. When more than two treatment groups were compared, one-way ANOVA with Newman-Keuls test was used. A probability level of P < 0.05 was considered statistically significant.

**RESULTS**

**GTPCH I overexpression increased intracellular BH4 levels.** To investigate intracellular BH4 levels, BM-derived EPCs were isolated as described previously (Fig. 1A), and their BH4 levels were evaluated using HPLC (Fig. 2). Intracellular BH4 levels were reduced significantly in WT STZ mice compared with the nondiabetic controls. Endothelium-targeted GTPCH I overexpression resulted in a significant increase in intracellular BH4 levels in the EPCs of Tg-GCH mice, approximately twofold higher compared with WT mice. Intracellular BH4 levels of EPCs from hph-1 mice were markedly decreased compared with WT mice. BH4 levels in EPCs from Tg-GCH STZ mice were significantly elevated compared with those from WT-STZ mice.

**EPC transplantation improved wound healing in diabetic mice.** EPCs could promote new blood vessel formation and increase angiogenesis. We next determined the effect of EPC transplantation on impaired wound healing in STZ mice by using full-thickness excisional wounds. EPCs (1 × 105 cells) from different groups were transplanted onto the wounds of WT-STZ mice. As shown in Fig. 3A, compared with control mice, the wound closure rate was significantly delayed in STZ mice (67.23 ± 3.84 vs. 34.82 ± 9.27% on day 6, P < 0.05). WT or Tg-GCH EPC transplantation significantly accelerated the rate of wound closure by as much as 2 days and continued through day 8 after injury (64.44 ± 4.13 and 68.78 ± 3.07% on day 6). The percentage of wound closure in STZ mice transplanted with EPCs, WT-STZ or hph-1, was significantly lower than that of WT-STZ mice beginning on day 2 and continuing through day 6 after injury (53.90 ± 2.40 and 51.43 ± 4.62% on day 6; Fig. 3, B and C). However, Tg-STZ EPC transplantation could accelerate the rate of wound closure as WT EPC transplantation. The mean perfusion rate around the wound was analyzed by using scanning laser Doppler flowmetry and was found to be the same as the wound healing rate (Fig. 3D).

**GTPCH I overexpression improved EPC function in diabetes.** Next, we determined the effects of GTPCH I overexpression on EPC function in vitro. As shown in Fig. 4A, EPC migratory capacity was impaired in WT STZ mice, and EPC migration drastically improved in STZ Tg-GCH mice. Additionally, EPC migration in BH4-deficient hph-1 mice also decreased. Matrigel tube formation assay was performed to...
investigate EPCs’ ability to integrate into tubule networks. Similarly, fewer diabetic EPCs or hph-1 EPCs formed tubes compared with control EPCs. Also, EPCs from STZ-induced Tg-GCH diabetic mice showed significant improvement in tube formation (Fig. 4B).

GTPCH I decreased EPC intracellular O$_2^-$ and NO level in diabetic mice. To determine the intracellular O$_2^-$ levels of circulating Sca-1+/Flk-1+ EPCs, a triple-staining flow cytometry assay was used to detect DHE fluorescence in Sca-1+/Flk-1+ EPCs. The results showed that the intracellular O$_2^-$
levels of circulating EPCs from WT STZ mice were increased compared with control mice (203.5 ± 29.25 vs. 91.60 ± 8.01, \( P < 0.05 \)). However, these were significantly attenuated in Tg-GCH diabetic mice when compared with those of WT-STZ mice (127.3 ± 16.98; Fig. 5A). Consistently, increased intracellular \( \text{O}_2^- \) levels were observed in circulating EPCs from hph-1 mice (230.8 ± 20.91).

Then we measured intracellular NO levels in circulating Sca-1\(^+\)/Flk-1\(^+\) EPCs by using DAF-2. Endothelium-targeted GTPCH I overexpression resulted in a significant increase in intracellular NO levels in EPCs from Tg-GCH mice compared with WT mice (196.5 ± 37.60 vs. 110.2 ± 20.33, \( P < 0.05 \)). In contrast, intracellular NO levels of circulating EPCs from hph-1 mice were significantly attenuated compared with those in WT mice (62.84 ± 10.66). The decreased intracellular NO levels were also observed in STZ WT mice compared with control mice; however, this was rescued in EPCs from STZ Tg-GCH mice (47.09 ± 11.01 vs. 150.2 ± 42.90, \( P < 0.05 \); Fig. 5B).

**GTPCH I overexpression suppressed TSP-1 secretion.** TSP-1 acts as an endogenous inhibitor of angiogenesis and is a key inhibitor of EPC function. TSP-1 protein was almost undetectable in cell lysates (data not shown), but a high TSP-1 level could be detected in the medium, so we examined TSP-1 levels in EPC media using Western blot. As shown in Fig. 6A, TSP-1 was significantly upregulated in
Fig. 4. A: EPC migration ability was impaired in WT-STZ and hph-1 mice compared with WT and GTPCH I transgenic mice. Data are expressed as means ± SE and are shown as %control; n = 7–9/group. Scale bar, 20 μm. B: EPC tube formation was impaired in WT-STZ mice and hph-1 mice compared with WT and GTPCH I transgenic mice. Data are expressed as means ± SE and are shown as %control; n = 4–6/group. Scale bar, 50 μm. *P < 0.05 vs. WT and Tg-GCH mice; #P < 0.05 vs. WT mice treated with STZ (WT-STZ).
EPC media from WT STZ and hph-1 mice compared with WT mice (165.5 ± 25.79 and 186.6 ± 35.93 vs. 100.0 ± 9.690, P < 0.05). Tg-GCH mice and STZ Tg-GCH mice showed a lower level of TSP-1 in EPCs (95.60 ± 14.50 and 96.86 ± 17.47).

High glucose incubation increased TSP-1 secretion significantly in EPCs (100.0 ± 18.03 vs. 175.4 ± 19.99, P < 0.05), and coincubation with membrane-permeable antioxidative enzymes [polyethylene glycol-superoxide dismutase (Peg-SOD)], eNOS inhibitor L-NNA, or BH4 prevented the increased TSP-1 secretion in high glucose treatment (Fig. 6B). Moreover, those coincubations also suppressed the number of DHE-positive EPCs under the stimulus of high glucose (Fig. 6C). Additionally, TSP-1 secretion was also markedly increased in db/db mice compared with db/+ mice (100.0 ± 18.93 vs. 203.4 ± 23.07, P < 0.05). BH4 incubation could dose-dependently attenuate the secretion of TSP-1 in db/db EPCs (Fig. 6D).

**DISCUSSION**

The results of this study demonstrate for the first time that 1) GTPCH I overexpression significantly prevented decreased EPC BH4 level and dysfunction in STZ-induced diabetic mice; 2) cell therapy, in which normal EPCs are transplanted onto the wounds of diabetic mice, ameliorated the wound-healing delay, paralleled with a marked acceleration of blood flow; 3) GTPCH I overexpression significantly decreased O2− levels and increased intracellular NO levels in EPCs from diabetic mice; and 4) augmentation of BH4 decreased EPC TSP-1 secretion in diabetic mice.

STZ is widely used for the induction of diabetes in animals by causing destruction of pancreatic β-cells. The glucosamine-nitrosourea compound STZ is taken up into the
cell via the GLUT2 glucose transporter, which is present at high levels in the insulin-producing pancreatic β-cells (25). However, in addition to its cytotoxic action on pancreatic β-cells, administration of STZ can produce toxic actions on other body organs. In this study, to avoid the difference induced by STZ’s direct effects on EPCs, we used multiple small doses of STZ to generate the diabetic animal model in both WT mice and Tg-GCH mice.

Normal circulating EPC levels are relatively low, but increases occur in response to ischemia or trauma injury, where...
EPCs mobilize from the BM into the peripheral blood. These cells then migrate to sites of injured endothelium and differentiate into mature endothelial cells in situ (12, 35). To date, it has been demonstrated that EPC numbers were reduced and EPC migration and vasculogenesis capacity were impaired in patients with type 1 and type 2 diabetes (18, 31). However, the mechanisms underlying such reduction remain poorly understood. Diabetes-induced impaired EPC mobilization from the BM leads to decreased levels of circulating EPCs; dysfunctional EPCs may also result in impaired reendothelialization (14).

Other studies show that several factors were responsible for EPC mobilization. eNOS and NO are additionally important factors. eNOS-deficient mice show reduced EPC mobilization under various stimuli such as VEGF and G-CSF (1). Although the amount of total eNOS protein did not change in diabetic EPCs, eNOS activity was impaired in EPCs from diabetic mice. Impaired phosphorylation of eNOS was found in the BM from diabetic mice, which directly impacts EPC mobilization from the BM into circulation (11). Moreover, in response to various stimuli, diabetic EPC migration decreases markedly but can be reversed by exogenous administration of physiological concentrations of NO (26). Using hyperbaric oxygen to induce tissue level hyperoxia results in increased NO levels in the BM and hence, enhanced mobilization of EPCs into the peripheral blood (11). These studies agree with our present findings and together suggest that under diabetic conditions, and in the absence of sufficient BH4 level, eNOS becomes uncoupled from arginine oxidation and produces superoxide rather than NO. Therefore, uncoupled eNOS may result in lower NO bioavailability in STZ mice. In hph-1 mice with decreased EPC BH4 levels, migration ability was inhibited. In diabetic Tg-GCH mice, the intracellular BH4 level was maintained to restore NO level, and migration was preserved. Furthermore, the diabetes-induced impairment of EPC quantity and function was not mediated by insulin levels. Evidence suggests that insulin treatment could not reverse the impairment of eNOS phosphorylation and NO production in the EPCs of WT-STZ mice (11).

Compared with mature human umbilical vein endothelial cells and human microvascular endothelial cells, EPCs exhibited a significantly lower basal reactive oxygen species (ROS) concentration and held higher expression levels of the antioxidative enzymes catalase, glutathione peroxidase, and manganese superoxide dismutase (9, 10). However, EPCs from type 1 and type 2 diabetic animal models showed high ROS levels; elevated ROS could exhaust the EPC antioxidant system (9, 10). eNOS uncoupling has been seen as a major source of local superoxide production (24). With these findings, our results show that EPCs from diabetic individuals or hph-1 mice had substantially increased superoxide production and decreased intracellular NO; increased intracellular O2·− level in EPCs from diabetic mice was diminished by GTPCH I overexpression in Tg-GCH mice. eNOS inhibitor L-NNA could partly attenuate overproduction of superoxide. However, Chen et al. (6) reported that various antioxidants failed to reverse the high-glucose-induced inhibitory effect on EPC quantity and proliferation. These results could suggest that ROS is a part of the reason to induce EPC impairment, and antioxidant use is not enough to abolish the impaired effects on EPC function under diabetes. BH4 could ameliorate EPC dysfunction in diabetes in two processes. Besides alleviating the intracellular oxidative stress, BH4 could also restore the eNOS activities by augmenting NO level (Fig. 7).

Another important finding of our study is that WT or Tg-GCH EPC transplantation improved impaired wound healing significantly in STZ mice. In support of this possibility, one study reported that human EPC transplantation accelerates the wound closure rate in nude mice (30). The study showed that EPCs moved into the wound and directly incorporated with newly formed capillaries in the granulation tissue. In light of this finding, another study showed that wound treatment with human CD34+ peripheral blood mononuclear cells could decrease wound size in diabetic mice and accelerate wound revascularization compared with the CD34+ peripheral blood mononuclear cells (28). Here, we provide evidence that EPC transplantation could significantly increase blood perfusion around wounds, as high blood perfusion levels were maintained in WT and Tg-GCH and Tg-STZ EPC-transplanted mice. These results suggest that EPC transplantation could promote skin wound vascularization and healing. However, the percentage of wound closures in STZ mice transplanted with EPCs from WT-STZ or hph-1 was significantly lower than that of WT mice. Tg-STZ EPC transplantation could accelerate the wound closure rate the same as WT EPCs. These findings indicate that isolating EPCs from the peripheral blood of diabetic patients and correcting them by ex vivo gene transfer may be an effective cell therapy for accelerated wound healing.

TSP-1 is a secreted glycoprotein that is made and secreted by cells in response to injury and stress. It has been shown that TSP-1 is a potent inhibitor of angiogenesis (16, 23). It is rapidly secreted at high levels in inflamed and damaged tissues (23). It has been demonstrated that endothelial cell adhesion, growth, motility, and survival are clearly regulated by TSP-1 (16). Our previous studies have shown that isolated early EPCs from TSP-1-knockout mice demonstrated improved migration, adhesion, and tube formation ability (36). Recombinant TSP-1 type 1 repeats, which interact with CD36, mimicked TSP-1 to

![Fig. 7. Schematic illustration of possible mechanisms contributing to GTPCH I regulation of EPC function in type 1 diabetes. GTPCH I overexpression improved the impaired function of EPCs in type 1 diabetes by increasing intracellular BH4 and NO levels and decreasing O2·− levels in diabetic mice.](http://ajpendo.physiology.org/doi/10.1152/ajpendo.00696.2013/fig/7)
block NO/cGMP signaling in endothelial cells to inhibit NO-stimulated accumulation of cGMP (16). TSP-1 mRNA and protein levels were significantly increased in the vessel walls of a type 2 diabetic animal model (29). Furthermore, TSP-1 expression increased in response to glucose stimulation in endothelial cells (8). Our study showed that GTPCH I overexpression-induced elevated BH4 level in EPCs could abolish the increased TSP-1 levels in diabetic mice. Additionally, the inhibition of superoxide could minimize TSP-1 levels. In support of this view, it has been reported that peroxynitrite-induced damage was mediated by upregulation of TSP-1 in retinal microvascular endothelial cells (17).

In summary, the findings of this study demonstrate that GTPCH I overexpression improved impaired EPC function in diabetic mice at least in part by attenuating superoxide and suppressing TSP-1 expression. These findings may provide a mechanistic basis for targeting the endogenous GTPCH/BH4 pathway as a potential therapeutic strategy to combat EPC dysfunction and refractory wound healing in diabetes.

DISCLOSURES
None of the authors have conflicts of interest, financial or otherwise, to disclose.

AUTHOR CONTRIBUTIONS
L.T., D.-D.C., and H.-H.X. performed experiments; L.T. analyzed data; L.T. prepared figures; L.T. drafted manuscript; L.T., D.-D.C., and A.F.C. conceived and designed of research; A.F.C. interpreted results of experiments.

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