# Tetrahydrobiopterin Has a Glucose-Lowering Effect by Suppressing Hepatic Gluconeogenesis in an Endothelial Nitric Oxide Synthase–Dependent Manner in Diabetic Mice

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Endothelial nitric oxide synthase (eNOS) dysfunction induces insulin resistance and glucose intolerance. Tetrahydrobiopterin ($BH_4$) is an essential cofactor of eNOS that regulates eNOS activity. In the diabetic state, $BH_4$ is oxidized to 7,8-dihydrobiopterin, which leads to eNOS dysfunction owing to eNOS uncoupling. The current study investigates the effects of $BH_4$ on glucose metabolism and insulin sensitivity in diabetic mice. Single administration of $BH_4$ lowered fasting blood glucose levels in wild-type mice with streptozotocin (STZ)-induced diabetes and alleviated eNOS dysfunction by increasing eNOS dimerization in the liver of these mice. Liver has a critical role in glucose-lowering effects of $BH_4$ through suppression of hepatic gluconeogenesis. $BH_4$ activated AMP kinase (AMPK), and the suppressing effect of $BH_4$ on gluconeogenesis was AMPK-dependent. In addition, the glucose-lowering effect and activation of AMPK by $BH_4$ did not appear in mice with STZ-induced diabetes lacking eNOS. Consecutive administration of $BH_4$ in *ob/ob* mice ameliorated glucose intolerance and insulin resistance. Taken together, $BH_4$ suppresses hepatic gluconeogenesis in an eNOS-dependent manner, and $BH_4$ has a glucose-lowering effect as well as an insulin-sensitizing effect in diabetic mice. $BH_4$ has potential in the treatment of type 2 diabetes. ***Diabetes* 62:3033–3043, 2013**

Nitric oxide (NO) is a biological messenger produced by NO synthase (NOS), which includes endothelial (eNOS), inducible (iNOS), and neuronal (nNOS) isoforms. eNOS-derived NO is well-known to have a pivotal role in physiological regulation of endothelial function (1,2). eNOS dysfunction occurs in conditions of diabetes and is known to induce insulin resistance and glucose intolerance (3–5). Insulin resistance caused by eNOS dysfunction is thought to be induced by endothelial dysfunction, leading to decreased skeletal muscle blood flow and glucose uptake (4). On the other hand, glucose transport in isolated skeletal muscle is lower in eNOS-deficient ($eNOS^{-/-}$) mice, indicating that eNOS expressed in skeletal muscle also regulates its glucose uptake (4). Moreover, $eNOS^{-/-}$ mice are insulin resistant at the level of liver (5). These studies suggest that eNOS plays a central role in the regulation of glucose metabolism and insulin sensitivity and represents several therapeutic targets for type 2 diabetes.

The function of eNOS is regulated by multiple factors such as mRNA expression of eNOS, L-arginine, influx of $Ca^{2+}$, and tetrahydrobiopterin ($BH_4$) (2,6,7). $BH_4$ is an essential cofactor for eNOS catalysis and functions as an allosteric modulator of arginine binding (7,8). Binding of $BH_4$ to eNOS elicits a conformational change that increases the affinity for binding of arginine-based ligands. $BH_4$ binding also plays a role in dimer formation of the active and stabilized form of eNOS (8). $BH_4$ is converted to 7,8-dihydrobiopterin ($BH_2$) by exposure to oxidative stress such as diabetes (8,9). Increase in $BH_2$ induces dysfunction of eNOS, as $BH_2$ is inactive for NOS cofactor function and competes with $BH_4$ for $BH_4$ binding (8,9). Furthermore, in states of diabetes and high glucose, de novo synthesis of $BH_4$, which is rate limited by GTP cyclohydrolase I (GTPCH I), is impaired (10–13). Thus, the availability of $BH_4$ is reduced and the function of eNOS is altered so that the enzyme produces superoxide anion ($O_2^-$) rather than NO, a phenomenon called "eNOS uncoupling" (7,8,14). Supplementation of $BH_4$ can improve endothelial dysfunction by elevating the $BH_4$-to-$BH_2$ ratio, leading to recoupling of eNOS, and has been used in clinical trials with patients with atherosclerotic diseases for the expected vasodilatation effects of $BH_4$ through NO production (15). However, it is unclear whether $BH_4$ improves glucose metabolism and insulin sensitivity in diabetic conditions.

In the current study, we investigated the effects of $BH_4$ on blood glucose levels and insulin sensitivity in diabetic mice. Fasting blood glucose levels are regulated by the level of hepatic gluconeogenesis, elevation of which is the major cause of fasting hyperglycemia in diabetes (16,17). We demonstrate here that $BH_4$ lowers fasting blood glucose levels and suppresses gluconeogenesis in liver in an eNOS-dependent manner. In addition, $BH_4$ has an ameliorating effect on glucose intolerance as well as insulin resistance in diabetic mice. Using primary hepatocytes isolated from mouse liver, we have clarified the mechanism by which $BH_4$ suppresses hepatic gluconeogenesis. These data suggest that $BH_4$ has potential as a novel therapeutic approach to diabetes.

## RESEARCH DESIGN AND METHODS

Male C57/BL6 (wild-type) mice and male heterozygous $Ins2^{Akita}$ (diabetic Akita) mice, which exhibit hyperglycemia with reduced β-cell mass caused by a point

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mutation in the insulin 2 gene that leads to misfolded insulin and severe endoplasmic reticulum stress, were obtained from Shimizu (Kyoto, Japan) (18). Male $eNOS^{-/-}$ mice in the C57/BL6 mice background were obtained from The Jackson Laboratory (Bar Harbor, ME). Male B6.V-Lepob/J (*ob/ob*) mice were obtained from Charles River Japan (Yokohama, Japan). Mice with streptozotocin (STZ)-induced diabetes were made by injection of STZ (120 mg/kg i.p.) to 7-week-old wild-type or $eNOS^{-/-}$ mice. At 3 weeks after injection of STZ, the animals were confirmed to be diabetic by both high blood glucose levels (≥15 mmol/L) and other diabetic features, including polyuria, polydipsia, and hyperglycemia.

The mice were maintained in a temperature-controlled (25 ± 2°C) environment with a 12-h light/dark cycle with free access to standard laboratory chow and water. All experiments were carried out with mice aged 8–10 weeks. The animals were maintained and used in accordance with the Guidelines for Animal Experiments of Kyoto University. All experiments involving animals were conducted in accordance with the Guidelines for Animal Experiments of Kyoto University and were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University.

**Preparations and cultures of mouse hepatocyte and aortic endothelial cell.** Mouse hepatocytes were isolated by collagenase digestion as previously described (19). Primary hepatocytes were prepared by seeding in sixwell type 1 collagen–coated plates at a density of $1.5 \times 10^6$ cells in Dulbecco's modified Eagle's medium (DMEM) (low glucose, 5.6 mmol/L) containing 10% (vol/vol) FBS, 100 nmol/L regular insulin, 50 units/mL penicillin, and 50 μg/mL streptomycin. Hepatocytes were then cultured overnight in a humidified atmosphere (5% $CO_2$) at 37°C. As for mouse endothelial cells (ECs), the aorta was dissected and filled with collagenase type II solution. After incubation for 45 min at 37°C, ECs were removed from the aorta and collected by centrifugation at 1,200 rpm for 5 min. The EC was cultured in a sixwell collagen type I–coated dish for 1 week.

**Glucose production via gluconeogenesis in hepatocytes.** Freshly isolated hepatocytes from mice fasted for 16 h were treated in 24-well plates ($7.5 \times 10^5$ cells/well) in buffer A, which consisted of 0.5 mL Krebs-Ringer bicarbonate medium of 119.4 mmol/L NaCl, 3.7 mmol/L KCl, 2.7 mmol/L $CaCl_2$, 1.3 mmol/L $KH_2PO_4$, 1.3 mmol/L $MgSO_4$, and 24.8 mmol/L $NaHCO_3$ without glucose; 2% (wt/vol) BSA; 0.24 mmol/L 3-isobutyl-1-methylxanthine; and gluconeogenetic substrates (1 mmol/L pyruvate plus 10 mmol/L lactate). Hepatocytes were treated with $BH_4$ (Schircks Laboratories, Jona, Switzerland), sodium nitroprusside (SNP), NG-nitro-L-arginine methyl ester (Sigma, St. Louis, MO), sepiapterin (Schircks Laboratories), erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) (Wako, Osaka, Japan), and compound C (Sigma). Glucose production was measured by glucose oxidation method as previously described (19).

**Immunoblotting analysis of hepatocytes.** Western blotting was performed as previously described (19). Primary hepatocytes cultured overnight were incubated in buffer A treated with $BH_4$, SNP, sepiapterin, and EHNA. Hepatocytes were homogenized in lysis buffer. Cell lysates (50–150 μg protein/lane) were heated at 95°C for 5 min and subjected to electrophoresis on 6–10% (vol/vol) sodium dodecyl sulfate–polyacrylamide gels and transferred onto nitrocellulose membranes. For analysis of eNOS dimerization, the samples were not heated and the temperature was maintained at <15°C during electrophoresis. Primary antibodies used were anti–phosphorylated (phospho-) AMP kinase (AMPK)α ($Thr^{172}$), anti-AMPKα, anti–phospho-acetyl-CoA carboxylase (ACC) ($Ser^{79}$), anti-ACC, anti–phospho-eNOS ($Ser^{1177}$), anti–phospho-Akt ($Ser^{473}$), anti-Akt (all at 1:1,000 dilution; Cell Signaling Technology, Danvers, MA), anti-eNOS polyclonal antibody (1:500 dilution; BD Transduction Laboratories, San Jose, CA), anti-CD31 monoclonal antibody (1:2,000 dilution; Dianova, Hamburg, Germany), anti–GTPCH I (1:3,000; kind gift from Prof. H. Ichinose, Tokyo Institute of Technology), anti–dihydrofolate reductase (DHFR), anti–α1-antitrypsin (1:500; Santa Cruz, Delaware, CA), and anti–β-actin (1:5,000; Sigma). Secondary antibodies used were horseradish peroxidase–conjugated anti-rabbit, -mouse, -rat, or -goat antibody (GE Healthcare, Buckinghamshire, U.K.). The fluorescent bands were visualized using a detection system (Amersham ECL Plus; GE Healthcare) and quantified by densitometry using Image J software from National Institutes of Health (Bethesda, MD).

**Cell transfection and short interfering RNA.** Stealth short interfering RNA (siRNA) of AMPKα1 was purchased from Invitrogen (Carlsbad, CA). The sequences of siRNA for AMPKα1 were 5′-UCUCUUUCCUGAGGACCCAUCUUAU-3′ and 5′-AUAAGAUGGGUCCUCAGGAAAGAGA-3′. The sequences of control siRNAs were 5′-ACCAACAACAGUUUGGGAAUAGGGA-3′and 5′- UCCCUAUUCCCAAACUGUUGUUGGU-3′. Isolated hepatocytes in DMEM (low glucose, 5.6 mmol/L) containing 10% (vol/vol) FBS and 100 nmol/L regular insulin were mixed with Opti-MEM containing siRNA and Lipofectamine RNAi MAX (Invitrogen) and were plated on wells and then incubated at 37°C in a $CO_2$ incubator. The final amounts of hepatocytes, DMEM, Opti-MEM, siRNA, and Lipofectamine RNAi MAX were $5.0 \times 10^5$ cells/mL, 75% (vol/vol), 25% (vol/vol), 50 nmol/L, and 0.2%, respectively. Medium was replaced with DMEM 6 h after transfection. Forty-eight hours after transfection, the medium was replaced with buffer A, the cells were incubated for 60 min with or without $BH_4$, and the glucose content of the supernatant was measured.

**Nitrite/nitrate analysis.** Primary hepatocytes and liver tissues were homogenized in buffer A, and the amount of nitrite/nitrate in the supernatant was determined by a fluorescence method.

**Immunocytochemistry.** The hepatocytes were incubated with rabbit polyclonal anti-nitrotyrosine antibody (1:100 dilution; Millipore, Billerica, MA). Cells were then incubated with goat anti-rabbit IgG fluorescein-conjugated secondary antibody (1:100 dilution, Alexa Fluor 488; Invitrogen). Fluorescence in cells was monitored as previously described (19).

**Measurement of adenine nucleotide content.** After primary isolated hepatocytes were incubated in buffer A with or without $BH_4$ and SNP for 30 min, treatment was stopped by rapid addition of 0.1 mL of 2 mol/L $HClO_4$, followed by mixing by vortex and sonication in ice-cold water for 3 min. Adenine nucleotide contents were measured by a luminometric method as previously described (19,20).

**Isolation of total RNA and quantitative RT-PCR.** Total RNA was isolated from livers of 10 week-old wild-type mice, wild-type mice with STZ-induced diabetes, and *ob/ob* mice using Trizol (Invitrogen) as previously described (21). The mouse sequence of forward and reverse primers to detect GTPCH I and DHFR, glucose 6-phosphatase (G6Pase), phosphoenolpyruvate carboxykinase (PEPCK), and glyceraldehyde-3-phosphate dehydrogenase as an inner control are shown in Supplementary Table 1. SYBR Green PCR Master Mix (Applied Biosystems, Foster, CA) was prepared for the quantitative RT-PCR run. The thermal cycling conditions were denaturation at 95°C for 10 min followed by 50 cycles at 95°C for 15 s and 60°C for 1 min. mRNA levels were measured by real-time quantitative RT-PCR using ABI PRISM 7000 Sequence Detection System (Applied Biosystems).

**Biopterin analysis.** Tissues or whole blood of wild-type mice and wild-type mice with STZ-induced diabetes was collected. For measurement of uptake of $BH_4$ in liver, $BH_4$ (20 mg/kg) dissolved with 0.9% (wt/vol) sterile saline was administrated intraperitoneally to wild-type mice. After cervical dislocation, the mice were abdominally dissected and liver tissues were collected at 0, 30, 60, 120, and 180 min after injection. The organs were weighed, frozen immediately in liquid $N_2$, and then stored at −80°C. Total biopterin, $BH_4$, and $BH_2$ were measured as previously described (22).

**Effect of $BH_4$ on blood glucose levels of wild-type mice with STZ-induced diabetes, $eNOS^{-/-}$ mice with STZ-induced diabetes, and diabetic Akita mice.** Blood glucose levels were measured in wild-type mice with STZ-induced diabetes, $eNOS^{-/-}$ mice with STZ-induced diabetes, and diabetic Akita mice fasted for 16 h, and $BH_4$ (20 mg/kg) or metformin (250 mg/kg; Sigma) in 0.9% (wt/vol) sterile saline or 0.9% sterile saline alone was injected intraperitoneally. Blood glucose levels were measured again 2 h after injection.

**Effect of $BH_4$ on blood glucose levels of *ob/ob* mice.** Blood glucose levels and body weight of *ob/ob* mice were measured. The mice were divided into two groups shown in Supplementary Table 2, and 0.9% (wt/vol) sterile saline with or without $BH_4$ (10 mg/kg) was injected intraperitoneally twice a day for 10 days. Fed blood glucose levels were measured. After fasting overnight for 16 h, fasting blood glucose levels were measured.

**Intraperitoneal glucose tolerance test.** Wild-type mice were fasted overnight for 16 h, and glucose (2 g/kg) was injected intraperitoneally with $BH_4$ (20 mg/kg) in 0.9% (wt/vol) sterile saline or 0.9% sterile saline alone. After 10 days' treatment of saline with or without $BH_4$ (20 mg/kg), *ob/ob* mice were fasted overnight for 16 h, and glucose (1 g/kg) was injected intraperitoneally. Blood glucose levels and plasma insulin concentrations were measured at 0, 30, 60, 90, and 120 min after injection. Plasma insulin concentrations were determined by using an ELISA kit (Shibayagi, Gunma, Japan). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated with the following formula: [fasting insulin (mU/L) × fasting plasma glucose (mmol/L)]/22.5.

**Pyruvate tolerance test.** Pyruvate, $BH_4$, and sepiapterin were dissolved with 0.9% (wt/vol) sterile saline. Wild-type, $eNOS^{-/-}$, and *ob/ob* mice were fasted overnight for 16 h, and pyruvate (1 g/kg) was injected intraperitoneally with or without $BH_4$ (20 mg/kg) and sepiapterin (20 mg/kg). Blood glucose levels were measured at 0, 30, 60, 90, and 120 min after injection.

**Insulin tolerance test.** After 10 days' treatment of saline with or without $BH_4$ (20 mg/kg), *ob/ob* mice were fasted for 6 h, and regular insulin (1 units/kg i.p.) was injected with 0.9% sterile saline. Blood glucose levels were measured at 0, 30, 60, 90, and 120 min after injection.

**Statistics.** Comparison between two groups was performed using unpaired Student *t* test (not noted) and paired Student *t* test. For more than two groups, one-way or two-way ANOVA followed by post hoc Bonferroni testing was performed. A value of $P < 0.05$ was considered statistically significant.

## RESULTS

**Biopterin dynamics and effects of $BH_4$ on blood glucose levels in diabetic mice.** In STZ diabetic wild-type mice, the content of $BH_2$ was increased and the

$BH_4$-to-$BH_2$ ratio was decreased in blood and respective tissues (Fig. 1*A–D*). For investigation of whether $BH_4$ lowers blood glucose levels, $BH_4$ (20 mg/kg) in saline was injected intraperitoneally to STZ diabetic wild-type mice. Blood glucose levels were not changed 2 h after administration of $BH_4$ in fed STZ diabetic wild-type mice, while blood glucose levels were lowered by ~2.4 mmol/L in overnight-fasted STZ diabetic wild-type mice—a change similar to that with metformin (Fig. 1*E* and *F* and Supplementary Fig. 1*A*). The same effects also were found in diabetic Akita mice (Supplementary Fig. 1*B*).

**Liver tissue has an important role in glucose-lowering effects of $BH_4$.** Although the intraperitoneal glucose tolerance test (IPGTT) data in wild-type mice revealed no effects of $BH_4$ on blood glucose levels and plasma insulin levels, the pyruvate tolerance test (PTT) data showed that $BH_4$ decreased hepatic glucose production (Fig. 2*A–C*), suggesting that the suppressing effect on hepatic gluconeogenesis has a critical role in the glucose-lowering effect of $BH_4$. The mRNA and protein expression levels of GTPCH I, a rate-limiting enzyme of the $BH_4$ de novo synthesis pathway, were decreased in liver tissues of STZ diabetic wild-type mice (Fig. 2*D* and *E*). On the other hand, uptake of $BH_4$ into liver by its supplementation is regulated by DHFR, a rate-limiting enzyme of the $BH_4$ salvage synthesis pathway (23), and the expression of DHFR in liver tissues of STZ diabetic wild-type mice was not changed (Fig. 2*F* and *G*). The uptake of $BH_4$ in liver of wild-type mice was confirmed with a peak at 30 min by administration of $BH_4$ (20 mg/kg) as previously described (22,23) (Supplementary Fig. 2*A*). After 2-h administration of $BH_4$, the mRNA expression levels of PEPCK were significantly decreased, while those of G6Pase were not changed, and the eNOS dimerization and NO content were increased in the liver of STZ diabetic wild-type mice (Fig. 2*H–K*). The mRNA expression levels of PEPCK and G6Pase in the liver of wild-type mice were not changed (Supplementary Fig. 2*B* and *C*).

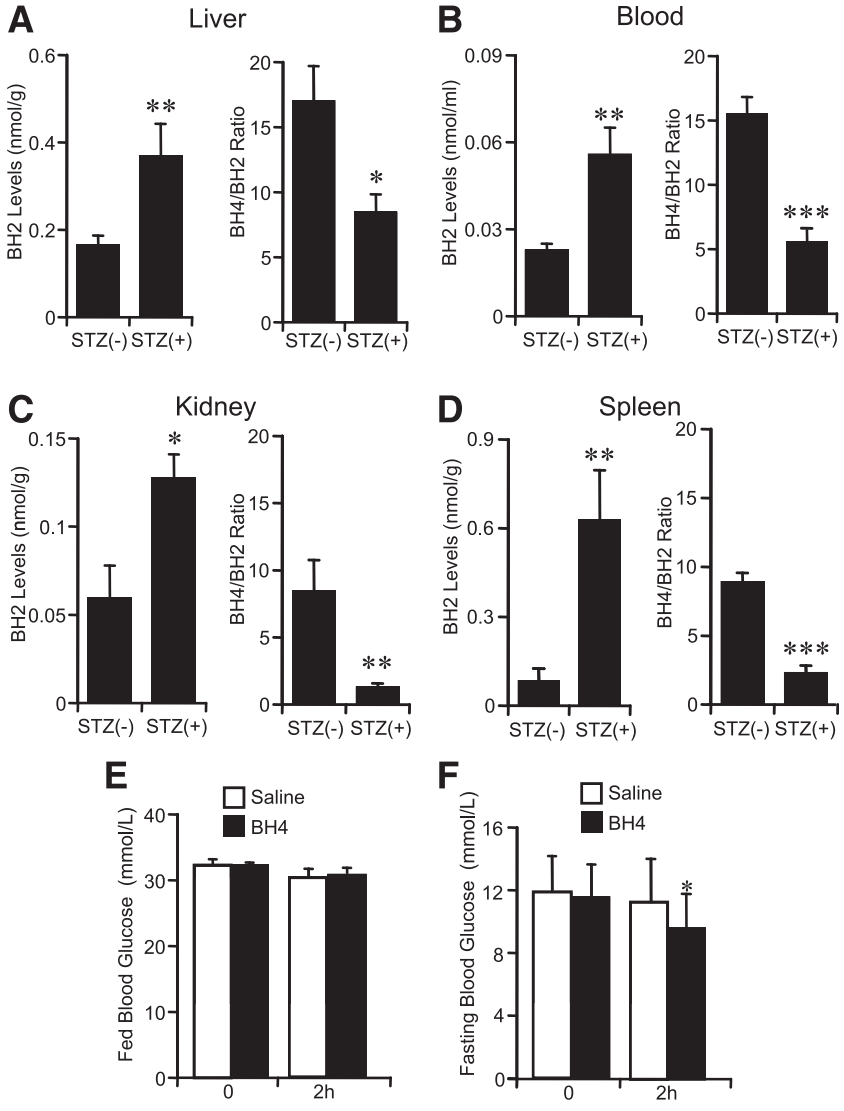


**FIG. 1. Biopterin dynamics and effects of $BH_4$ on blood glucose levels in diabetic mice. *A–D*: BH2 levels and $BH_4$-to-$BH_2$ ratio of liver, blood, kidney, and spleen. Values are means ± SE. $n$ = 7. *$P$ < 0.05, **$P$ < 0.01, ***$P$ < 0.001 vs. without STZ. *E* and *F*: Fed blood glucose levels were not changed 2 h after injection of $BH_4$ (20 mg/kg i.p.) to STZ diabetic wild-type mice; fasting blood glucose levels were significantly decreased. Values are means ± SE. $n$ = 8. *$P$ < 0.05 vs. the value of preinjection of saline with $BH_4$ intraperitoneally; paired $t$ test. No significant difference of fed and fasting blood glucose levels 2 h after intraperitoneal injection of saline to mice with STZ-induced diabetes.**

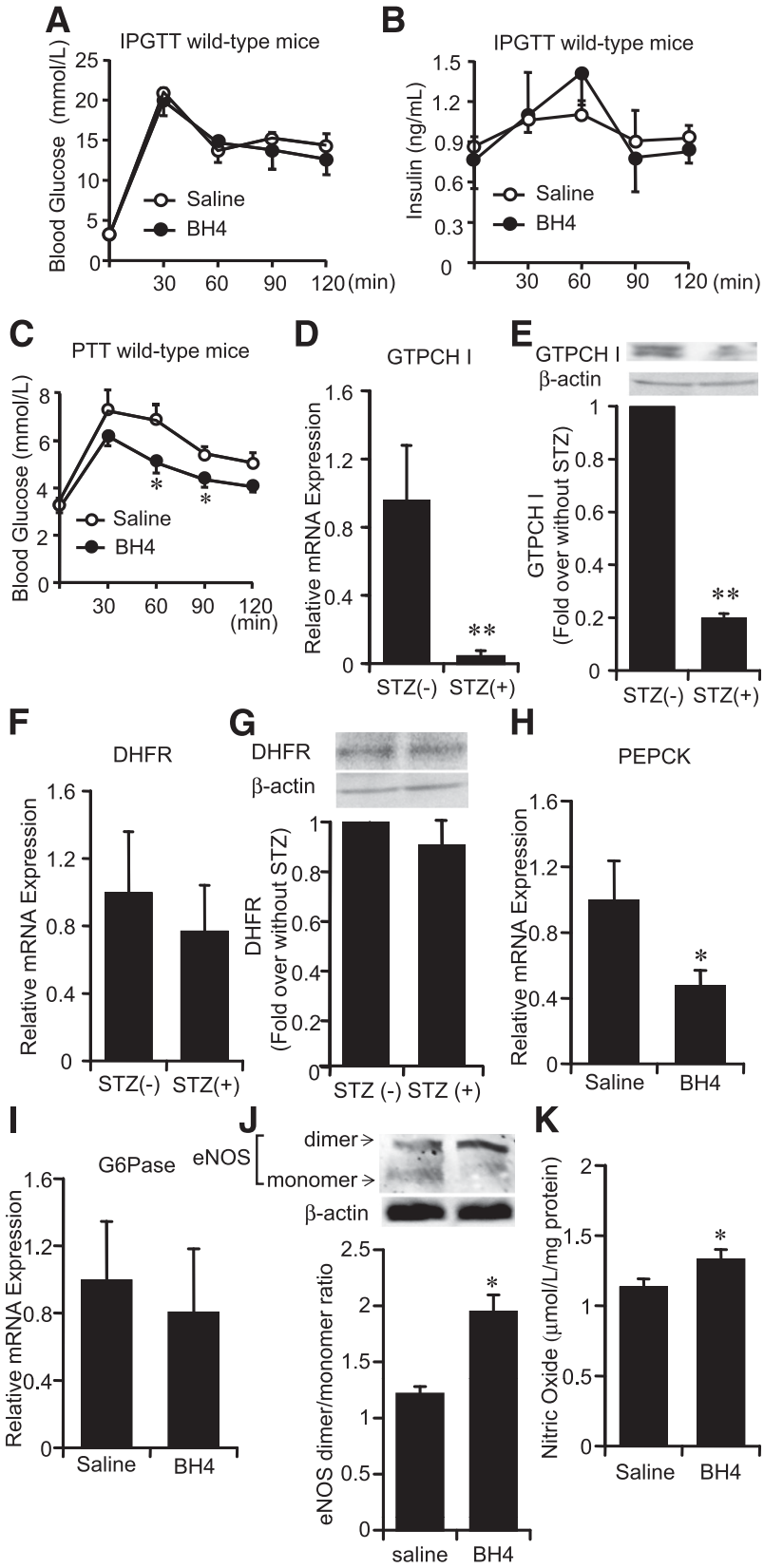


**FIG. 2. Role of liver tissue in glucose-lowering effects of $BH_4$. *A* and *B*: IPGTT to wild-type mice. Blood glucose levels and plasma insulin levels after administration of glucose (2 g/kg i.p.) with or without $BH_4$ (20 mg/kg). Values are means ± SE ($n$ = 6). *C*: PTT to wild-type mice. Elevation of blood glucose levels after intraperitoneal administration of pyruvate with $BH_4$ (20 mg/kg) to wild-type mice was suppressed compared with those without $BH_4$. Values are means ± SE ($n$ = 6). *$P < 0.05$ vs. saline. *D*: In mice with STZ-induced diabetes, mRNA levels of GTPCH I expression were significantly decreased compared with those in nondiabetic wild-type mice liver. Values are means ± SE ($n$ = 5). **$P < 0.01$ vs. nondiabetic wild-type mice liver. *E*: In wild-type mice with STZ-induced diabetes, protein expression levels of GTPCH I were significantly decreased compared with those in nondiabetic wild-type mice liver. Values are means ± SE ($n$ = 5). **$P < 0.01$ vs. nondiabetic wild-type mice liver. *F*: No significant difference**

**$BH_4$ suppresses gluconeogenesis and increases AMPKα phosphorylation in wild-type mouse hepatocytes.** As eNOS expression was confirmed in isolated hepatocytes from wild-type mice (Supplementary Fig. 3), we examined the direct effect of $BH_4$ in suppression of hepatic gluconeogenesis using hepatocytes isolated from wild-type mice fasted for 16 h. In a time course study of exposure to $BH_4$, the suppressing effect on gluconeogenesis appeared after 60 min ($P < 0.01$ vs. corresponding control) (Fig. 3*A*). We then investigated the increment of AMPKα phosphorylation by time course exposure of $BH_4$ to hepatocytes. AMPK was activated after 30 min by $BH_4$ (Fig. 3*B*). After 60 min exposure to $BH_4$, gluconeogenesis was dose-dependently suppressed at doses of 50 and 100 μmol/L $BH_4$ (control, 101.7 ± 3.7 nmol/mg protein; 50 μmol/L $BH_4$, 72.4 ± 7.1 nmol/mg protein, $P < 0.01$ vs. control; 100 μmol/L $BH_4$, 60.6 ± 4.1 nmol/mg protein, $P < 0.001$ vs. control) (Fig. 3*C*). AMPK was activated at doses of 50 and 100 μmol/L $BH_4$ by 30 min exposure (Fig. 3*D*). In accordance with the activation of AMPK, an increase in phosphorylation of ACC by $BH_4$ was confirmed (Fig. 3*B* and *D*). For determination of whether $BH_4$ suppresses gluconeogenesis in an AMPK-dependent manner, the effect of silencing AMPK was examined (Fig. 3*E*). By transfection of AMPKα1 siRNA, the suppressing effect of $BH_4$ on gluconeogenesis disappeared (Fig. 3*F*). The suppressing effect of $BH_4$ on gluconeogenesis also disappeared in the presence of compound C, an AMPK inhibitor (Fig. 3*G*).

**$BH_4$ suppresses gluconeogenesis and increases AMPKα phosphorylation eNOS dependently in hepatocytes.** Exposure to $BH_4$ in hepatocytes increased NO production and eNOS phosphorylation (Fig. 4*A* and *B*). To examine whether $BH_4$ suppresses hepatic gluconeogenesis and activates AMPK in the absence of eNOS, we performed experiments using mouse hepatocytes lacking eNOS. In hepatocytes isolated from $eNOS^{-/-}$ mice, $BH_4$ did not suppress gluconeogenesis (control, 103.9 ± 10.8 nmol/mg protein; 50 μmol/L $BH_4$, 98.5 ± 11.3 nmol/mg protein; 100 μmol/L $BH_4$, 89.1 ± 10.9 nmol/mg protein, $P$ = NS vs. control) (Fig. 4*C*). $BH_4$ did not alter AMPKα and ACC phosphorylation in hepatocytes lacking eNOS (Fig. 4*D*). The suppressing effect of $BH_4$ on gluconeogenesis and activation of AMPK also disappeared in the presence of NG-nitro-L-arginine methyl ester, an NOS inhibitor (Supplementary Fig. 4*A* and *B*). SNP, an NO donor, has suppressing effects on gluconeogenesis and increases the effects on AMPK activation both in wild-type and $eNOS^{-/-}$ hepatocytes (Supplementary Fig. 5*A*–*D*). Immunocytochemical staining of primary cultured hepatocytes from wild-type mice with anti-nitrotyrosine antibody, which detects $ONOO^-$, showed that $ONOO^-$ production was not increased by exposure with $BH_4$ or SNP (Supplementary Fig. 5*E*).

**Effect of $BH_4$ on adenine nucleotide content in hepatocytes.** For investigation of the mechanism of AMPK activation by $BH_4$ in hepatocytes, the adenine nucleotide content with exposure of $BH_4$ to hepatocytes was measured. $BH_4$ and SNP significantly increased AMP content in wild-type mouse hepatocytes (Table 1). Unexpectedly, $BH_4$ also significantly increased ATP content. To clarify the mechanism by which $BH_4$ increases AMP content and activates AMPK in hepatocytes, we examined the effect of AMP deaminase (AMPD) on activation of AMPK and suppression of gluconeogenesis by $BH_4$. Although EHNA, a known AMPD inhibitor, activated AMPK and suppressed hepatic gluconeogenesis, $BH_4$ did not have an additive effect on EHNA (Supplementary Fig. 6*A* and *B*). These results indicate that inhibition of AMPD, at least in part, contributes to AMP accumulation by $BH_4$ in hepatocytes.

**Sepiapterin, a $BH_4$ precursor, suppresses gluconeogenesis and increases AMPK activation.** Similarly to $BH_4$, sepiapterin is absorbed in hepatocytes and immediately converted to $BH_4$ via a salvage pathway of $BH_4$ biosynthesis (23). Sepiapterin was found to suppress gluconeogenesis and activate AMPK (Fig. 5*A* and *B*). However, these effects were abolished in hepatocytes lacking eNOS (Fig. 5*A* and *B*).

**Role of eNOS in in vivo action of $BH_4$ on glucose metabolism.** The lowering effect of $BH_4$ on fasting blood glucose levels disappeared in STZ-induced diabetic $eNOS^{-/-}$ mice (Fig. 6*A*). The PTT data showed that $BH_4$ did not decrease hepatic glucose production in $eNOS^{-/-}$ mice (Fig. 6*B*). Similar results were also obtained in sepiapterin administration (Supplementary Fig. 7*A* and *B*). We then compared the effects of $BH_4$ on phosphorylation of AMPKα in liver tissues of these diabetic mice. $BH_4$ activated AMPK in both STZ diabetic wild-type mice liver and diabetic Akita mice liver but not in STZ diabetic $eNOS^{-/-}$ mice liver (Fig. 6*C* and *D* and Supplementary Fig. 8*A*). AMPKα phosphorylation was not changed by fasting for 16 h in liver tissues of wild-type mice (Supplementary Fig. 8*B*).

**Effects of $BH_4$ on glucose metabolism and insulin sensitivity in *ob/ob* mice.** Our PTT data show that the suppressing effect on gluconeogenesis is also confirmed by single administration of $BH_4$ in *ob/ob* mice (Fig. 7*A*), while the mRNA expression levels of PEPCK and G6Pase in the liver (Supplementary Fig. 9*A* and *B*), fasting and fed blood glucose levels, and IPGTT data were not changed (data not shown). By consecutive administration of $BH_4$ (20 mg/kg) in saline for 10 days to *ob/ob* mice, fasting blood glucose levels were significantly lowered by 3.9 mmol/L and fed blood glucose levels tended to be decreased compared with those in *ob/ob* mice treated with saline alone (Fig. 7*B* and *C*). Our IPGTT, HOMA-IR, and insulin tolerance test data suggest that consecutive administration of $BH_4$ ameliorates glucose intolerance as well as insulin resistance (Fig. 7*D*–*G*). Phosphorylation of AMPKα, ACC, and Akt was increased in liver tissues of $BH_4$-treated *ob/ob* mice compared with those in saline-treated mice (Fig. 7*H* and *I*).

## DISCUSSION

The current study shows that $BH_4$, known as a cofactor of eNOS, has a glucose-lowering effect in diabetic mice. The $BH_4$-to-$BH_2$ ratio was found to be decreased in various tissues of mice in the diabetic state, indicating deterioration of

**of mRNA expression levels of DHFR in liver was detected between nondiabetic mice and mice with STZ-induced diabetes. Values are means ± SE ($n$ = 10). *G*: No significant difference of protein expression levels of DHFR in liver was detected between nondiabetic mice and mice with STZ-induced diabetes. Values are means ± SE ($n$ = 5). *H* and *I*: In liver tissues of wild-type mice with STZ-induced diabetes treated with $BH_4$, mRNA levels of PEPCK were significantly decreased compared with those treated without $BH_4$. The mRNA levels of G6Pase were not changed. Values are means ± SE ($n$ = 6), *$P < 0.05$ vs. saline. *J*: Liver tissues of eNOS dimer and monomer expression 2 h after intraperitoneal injection of saline with or without $BH_4$ (20 mg/kg) to wild-type mice with STZ-induced diabetes. Densitometric analysis of the ratio of eNOS dimer to monomer. Values are means ± SE ($n$ = 5). *$P < 0.05$ vs. saline. *K*: In liver tissues of wild-type mice with STZ-induced diabetes treated with $BH_4$, NO content was significantly increased compared with those treated without $BH_4$. Values are means ± SE ($n$ = 5). *$P < 0.05$ vs. saline.**

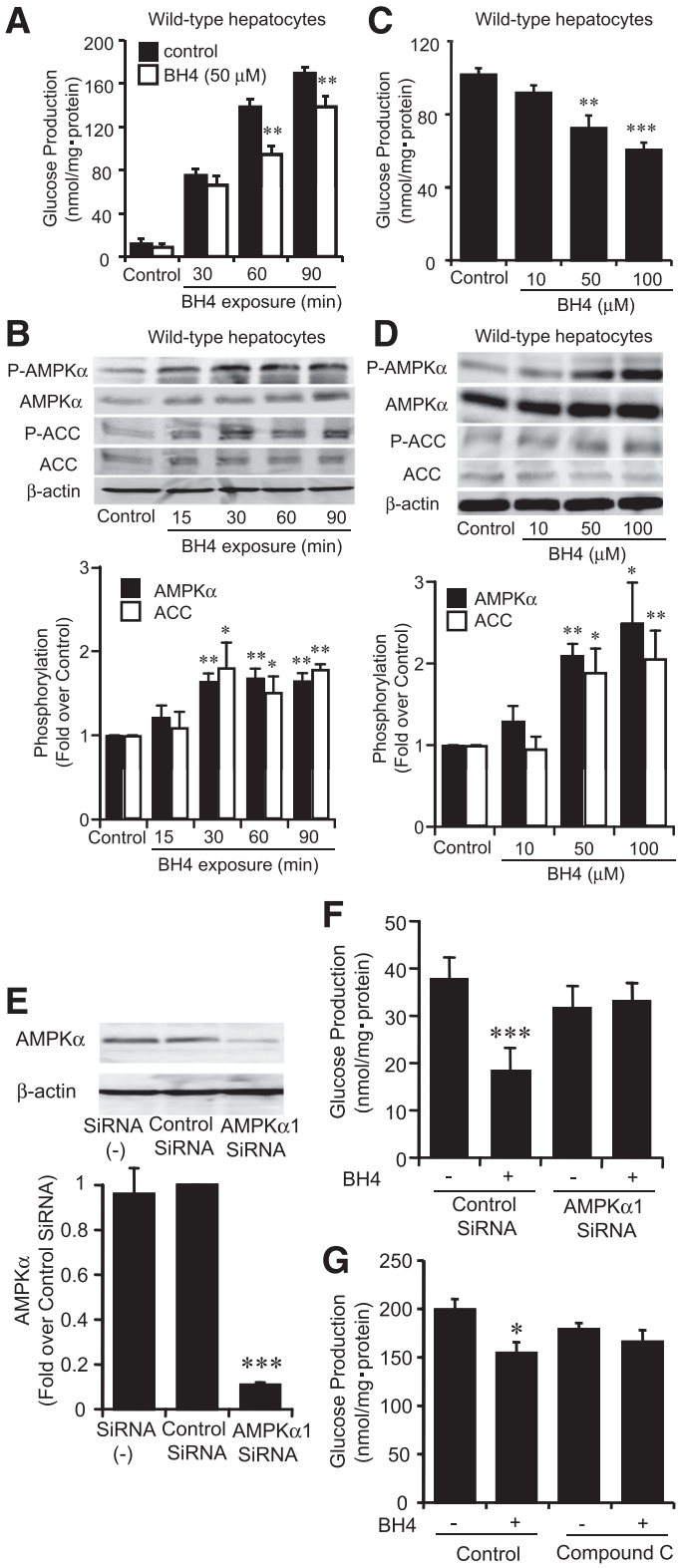


**FIG. 3. $BH_4$ suppressed gluconeogenesis and increased AMPKα phosphorylation in hepatocytes isolated from wild-type mice. *A*: Time course of gluconeogenesis with exposure to $BH_4$. Suppressing effect on gluconeogenesis by 50 μmol/L $BH_4$ compared with control was detected after 60 min in hepatocytes isolated from wild-type mice. Values are means ± SE ($n$ = 6). **$P$ < 0.01 vs. control. *B*: Time course of phosphorylation of AMPKα and ACC upon exposure to $BH_4$ (50 μmol/L). Both AMPKα and ACC phosphorylation were stimulated after 30 min exposure to $BH_4$ in hepatocytes isolated from wild-type mice. Data are expressed as fold stimulation over control. Values are means ± SE ($n$ = 3). *$P$ < 0.05, **$P$ < 0.01 vs. control. *C*: Suppressing effect on gluconeogenesis after 1 h exposure of $BH_4$ was detected ranging over 50 μmol/L in hepatocytes isolated from wild-type mice. Values are means ± SE ($n$ = 6). **$P$ < 0.01, ***$P$ < 0.001 vs. control. *D*: Effect of $BH_4$ on phosphorylation of AMPK and ACC. After 30 min exposure to $BH_4$, both AMPKα and ACC phosphorylation were increased by $BH_4$ dose dependently ranging over 50 μmol/L in hepatocytes isolated from wild-type mice. Data are expressed as fold stimulation over control. Values are means ± SE ($n$ = 3). *$P$ < 0.05, **$P$ < 0.01 vs. control. *E*: With transfection with AMPKα1 siRNA, protein expression of AMPKα was decreased compared with that of transfection with control siRNA. Values are means ± SE ($n$ = 3). ***$P$ < 0.001 vs. control siRNA. *F*: Transfected with AMPKα1 siRNA, suppressing effect of $BH_4$ (50 μmol/L) on hepatic glucose production was inhibited. Values are means ± SE ($n$ = 6). ***$P$ < 0.001 vs. values transfected with control siRNA without $BH_4$. *G*: Compound C (20 μmol/L), an AMPK inhibitor, abolished the suppressing effect of $BH_4$ (50 μmol/L) on gluconeogenesis. Values are means ± SE ($n$ = 6). *$P$ < 0.05 vs. values without $BH_4$ and without compound C.**

eNOS bioactivity by eNOS uncoupling. Previous studies have shown that impairment of eNOS function is involved in glucose dysmetabolism and insulin resistance (4,5), which lends support to the notion that alleviation of eNOS dysfunction such as by supplementation of $BH_4$ ameliorates glucose dysmetabolism and insulin resistance. In addition, we found that supplementation of $BH_4$ increased dimerization of eNOS and NO production in the liver of diabetic mice, which strongly suggests alleviation of eNOS dysfunction by recoupling of eNOS. Simultaneously with the restoration of eNOS activity, $BH_4$ elicited a glucose-lowering effect in these mice. No such glucose-lowering effect by $BH_4$ appeared in diabetic mice lacking eNOS. These findings clearly implicate recoupling of eNOS in the glucose-lowering effect of $BH_4$.

We have shown that the liver plays a critical role in the glucose-lowering effect of $BH_4$ through suppression of hepatic gluconeogenesis. It is well-known that $BH_4$ is synthesized mainly in liver (24) and that this is impaired by oxidative stress such as liver cirrhosis and diabetes (25,26). Single administration of $BH_4$ is known to accumulate at higher levels in liver than other tissues including skeletal muscle (24), which also lends support to the view that $BH_4$ readily elevates $BH_4$-to-$BH_2$ ratio and regulates glucose metabolism in the liver.

We then investigated the molecular mechanism of suppression of hepatic gluconeogenesis by $BH_4$ using isolated mouse hepatocytes. $BH_4$ acts directly on hepatocytes and suppresses hepatic gluconeogenesis eNOS dependently. Several studies reported that eNOS is found in hepatic sinusoidal and venous endothelial cells and not in hepatocytes (27,28), whereas other studies claim detection of eNOS in hepatocytes (29,30). We confirmed that eNOS is expressed in hepatocytes, which suggests that intrahepatocellular eNOS is essential for the effect of $BH_4$ in suppression of hepatic gluconeogenesis. In addition, $BH_4$ activated AMPK, and the suppressing effect of $BH_4$ on gluconeogenesis disappeared by siRNA silencing of AMPKα1 subunits in hepatocytes, indicating that AMPK is involved in the suppressing effect of $BH_4$ on hepatic gluconeogenesis. AMPK activation by $BH_4$ was not observed in $eNOS^{-/-}$ mouse hepatocytes or in the presence of NOS inhibitor, suggesting that eNOS acts upstream of AMPK activation in suppression of hepatic gluconeogenesis by $BH_4$. AMPK is a Ser/Thr kinase that acts as an energy sensor and is activated by an increase in the AMP-to-ATP ratio and/or AMP in response to a variety of metabolic stresses, such as hypoxia, ischemia, and exercise (31,32). In our data, $BH_4$ significantly increased AMP content and

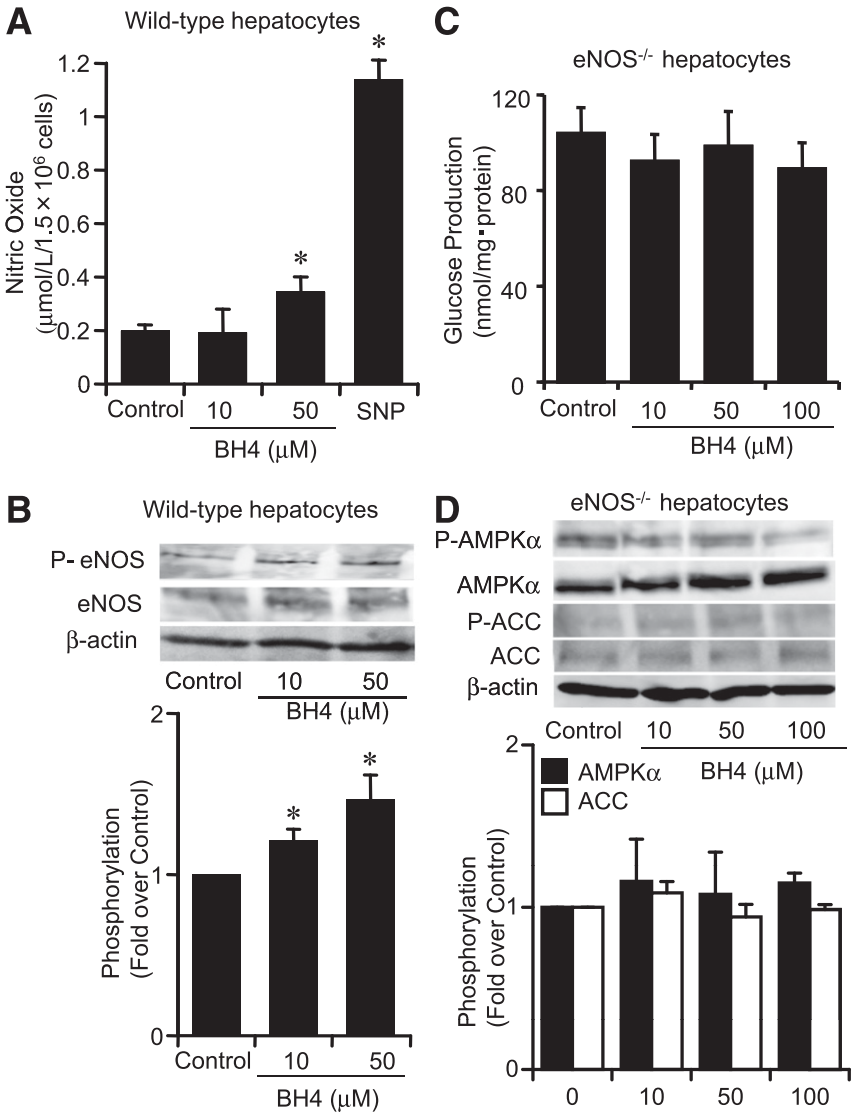


**FIG. 4. Lack of the effect of $BH_4$ on suppression of gluconeogenesis in eNOS$^{-/-}$ mouse hepatocytes. *A*: $BH_4$ (50 μmol/L) significantly increased NO production in hepatocytes from wild-type mice. SNP (20 μmol/L) was used as positive control. Values are means ± SE ($n$ = 5). *$P$ < 0.05 vs. control. *B*: $BH_4$ (ranging from 10 to 50 μmol/L) increased eNOS phosphorylation at Ser$^{1177}$ in hepatocytes from wild-type mice. Values are means ± SE ($n$ = 5). *$P$ < 0.05 vs. control. *C*: $BH_4$ (ranging from 10 to 100 μmol/L) did not suppress gluconeogenesis after 1 h exposure in hepatocytes from eNOS$^{-/-}$ mice. Values are means ± SE ($n$ = 6). *D*: After 30 min exposure to $BH_4$ ranging from 10 to 100 μmol/L, AMPKα and ACC phosphorylation were not increased by $BH_4$ in hepatocytes from eNOS$^{-/-}$ mice. Data are expressed as fold stimulation over control. Values are means ± SE ($n$ = 3).**

tended to increase the AMP-to-ATP ratio. It is known that inhibition of AMPD increases AMP in isolated hepatocytes (33). Recently, Ouyang et al. (34) reported that inhibition of AMPD might be involved in increased production of AMP and activation of AMPK by metformin. In the current study, the AMPD inhibitor EHNA was found to activate AMPK, but $BH_4$ did not elicit an additional effect on AMPK activation in the presence of EHNA, suggesting that AMPD might be inhibited by $BH_4$ in hepatocytes. Interestingly, $BH_4$ significantly increased ATP content along with the increase in AMP. This effect was not found in exposure to other potent AMPK activators, as previously reported (35). The reason why $BH_4$ increases ATP content is unclear, but $BH_4$ is known to work as an antioxidant (36). It has been reported that $BH_4$ preserves ATP content and has a cytoprotective effect from hypoxia on neuronal cells (37). $BH_4$ might thus prevent cytotoxic damage from reactive oxygen species/reactive nitrogen species (RNS) as a scavenger, keeping ATP content higher than in the absence of $BH_4$. We therefore cannot exclude the possibility that $BH_4$ acts as a reactive oxygen species/RNS scavenger in ameliorating glucose dysmetabolism, but such an effect would be limited in terms of suppressing hepatic gluconeogenesis

TABLE 1
Effects of $BH_4$ on ATP, AMP, and AMP-to-ATP ratio in wild-type mouse hepatocytes

| | ATP (nmol/mg protein) | AMP (nmol/mg protein) | AMP-to-ATP ratio |
|---|---|---|---|
| Control | 0.66 ± 0.08 | 0.28 ± 0.04 | 0.44 ± 0.03 |
| $BH_4$ | 0.88 ± 0.04* | 0.49 ± 0.05** | 0.55 ± 0.04 |
| SNP | 0.73 ± 0.07 | 0.47 ± 0.01** | 0.67 ± 0.07 |

Data are means ± SE ($n$ = 5). Hepatocytes were incubated in Krebs-Ringer bicarbonate buffer with or without $BH_4$ (50 μmol/L) for 30 min. The treatment was stopped by rapid addition of 0.1 mL of 2 mol/L $HClO_4$, and adenine nucleotide contents were measured. *$P$ < 0.05, **$P$ < 0.01 vs. control.

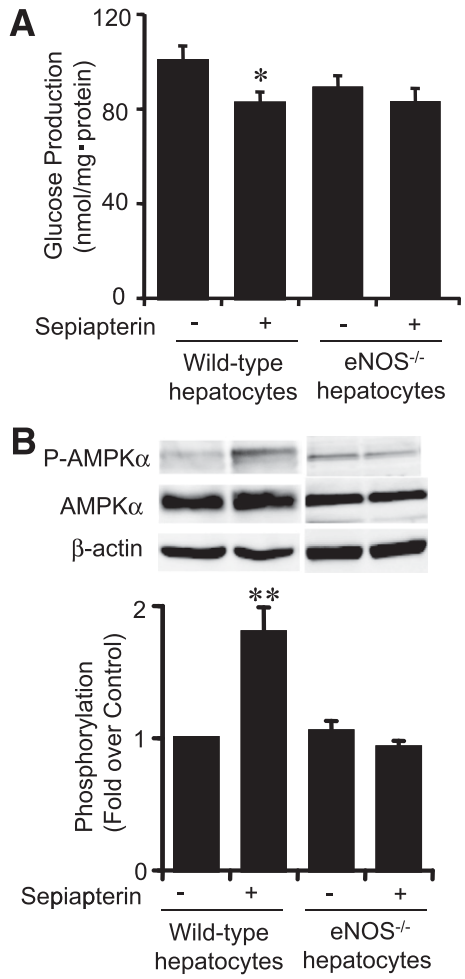


**FIG. 5. Effect of sepiapterin, a $BH_4$ precursor, on gluconeogenesis and AMPK activation. *A*: After 1 h exposure, sepiapterin (50 μmol/L) significantly suppressed gluconeogenesis in hepatocytes isolated from wild-type mice. This effect was not observed in hepatocytes isolated from eNOS$^{-/-}$ mice. Values are means ± SE ($n$ = 6). *$P$ < 0.05 vs. control. *B*: After 30 min exposure to sepiapterin (50 μmol/L), AMPKα phosphorylation was increased in hepatocytes isolated from wild-type mice. AMPKα phosphorylation was not increased by sepiapterin in hepatocytes isolated from eNOS$^{-/-}$ mice. Data are expressed as fold stimulation over control. Values are means ± SE ($n$ = 3). **$P$ < 0.01 vs. control.**

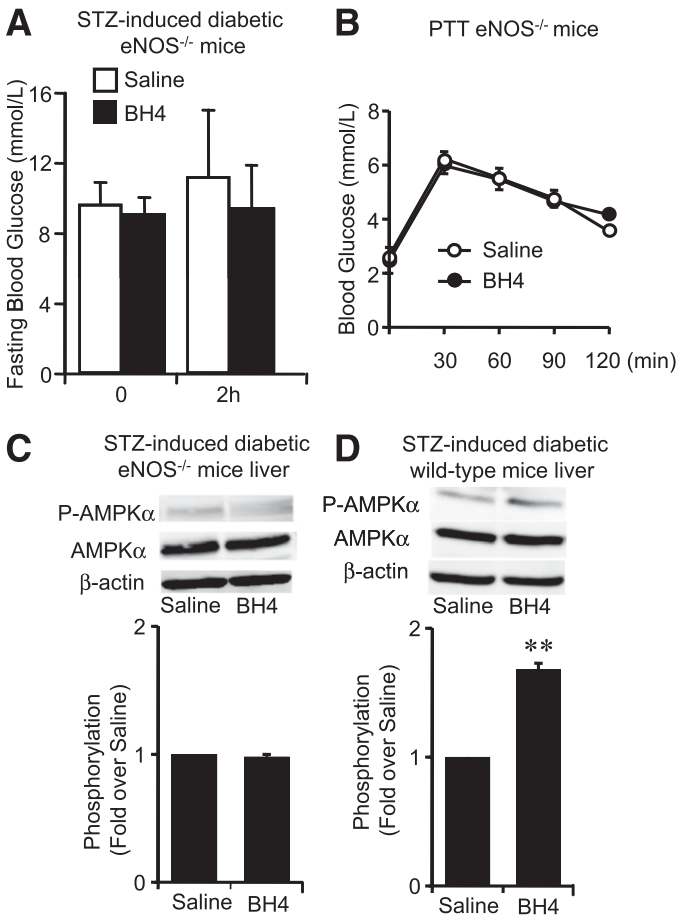


**FIG. 6. Effects of $BH_4$ in eNOS$^{-/-}$ mice with STZ-induced diabetes. *A*: No significant difference of fasting blood glucose levels 2 h after intraperitoneal injection of saline with or without $BH_4$ (20 mg/kg) to eNOS$^{-/-}$ mice with STZ-induced diabetes. Values are means ± SE ($n$ = 7). *B*: PTT to eNOS$^{-/-}$ mice. No effects of $BH_4$ (20 mg/kg) on suppressing hepatic gluconeogenesis were detected in PTT in eNOS$^{-/-}$ mice. Values are means ± SE ($n$ = 6). *C*: AMPKα phosphorylation in liver of eNOS$^{-/-}$ mice with STZ-induced diabetes was not changed by $BH_4$ administration. Data are expressed as fold stimulation over saline. Values are means ± SE ($n$ = 3). *D*: AMPKα phosphorylation in liver of wild-type mice with STZ-induced diabetes was significantly increased by $BH_4$ (20 mg/kg) administration. Data are expressed as fold stimulation over saline. Values are means ± SE ($n$ = 3). **$P$ < 0.01 vs. saline.**

because the effect of $BH_4$ was not observed in mice lacking eNOS. Previous studies found that NO has an activating effect on AMPK (38,39). Also, in our results SNP, an NO donor, activated AMPK in hepatocytes just as $BH_4$ does. Regarding the mechanism of AMPK activation by $BH_4$ via eNOS, it is possible that NO itself generated by eNOS activates AMPK; another possibility is that the RNS peroxynitrite ($ONOO^-$), an adduct of NO with superoxide, works intermediately as the activator of AMPK by $BH_4$ (19,40). The involvement of RNS on AMPK activation by $BH_4$ was not suggested by our present data.

Our data using *ob/ob* mice, a mouse model of insulin resistance, suggest that the primary physiological action of $BH_4$ is a suppressing effect of hepatic gluconeogenesis. In addition to this effect, consecutive administration of $BH_4$ ameliorated glucose intolerance as well as insulin resistance. A possible mechanism of these additive effects of $BH_4$ is induction by the subsequent downstream targets of AMPK activated by $BH_4$ such as metformin, which are known to have insulin-sensitizing effects, e.g., by modulating carbohydrate and lipid metabolism via the downstream signals of AMPK (41). It is generally known that increase in Akt phosphorylation represents an amelioration of hepatic insulin resistance. This may be applicable to the effect of $BH_4$, while it raises the possibility that Akt-dependent signaling is involved in the suppressing effect of $BH_4$ on hepatic gluconeogenesis in *ob/ob* mice. Another possible mechanism of $BH_4$ ameliorating insulin resistance would be via a direct effect of $BH_4$ on endothelial cells. Similar to several NO donors and NO-moderating compounds (42), $BH_4$ might also exert an insulin-sensitizing effect by augmenting the delivery of insulin and glucose to skeletal muscle via capillary recruitment. Since the role of eNOS in vivo was assessed using global eNOS$^{-/-}$ mice, it is difficult to exclude the possibility of indirect effects of eNOS on the liver. Therefore, limitations of the current study must be considered. Further investigations, e.g., by using liver-specific eNOS$^{-/-}$ mice, are required to elucidate the pleiotropic effects of $BH_4$ in lowering blood glucose levels.

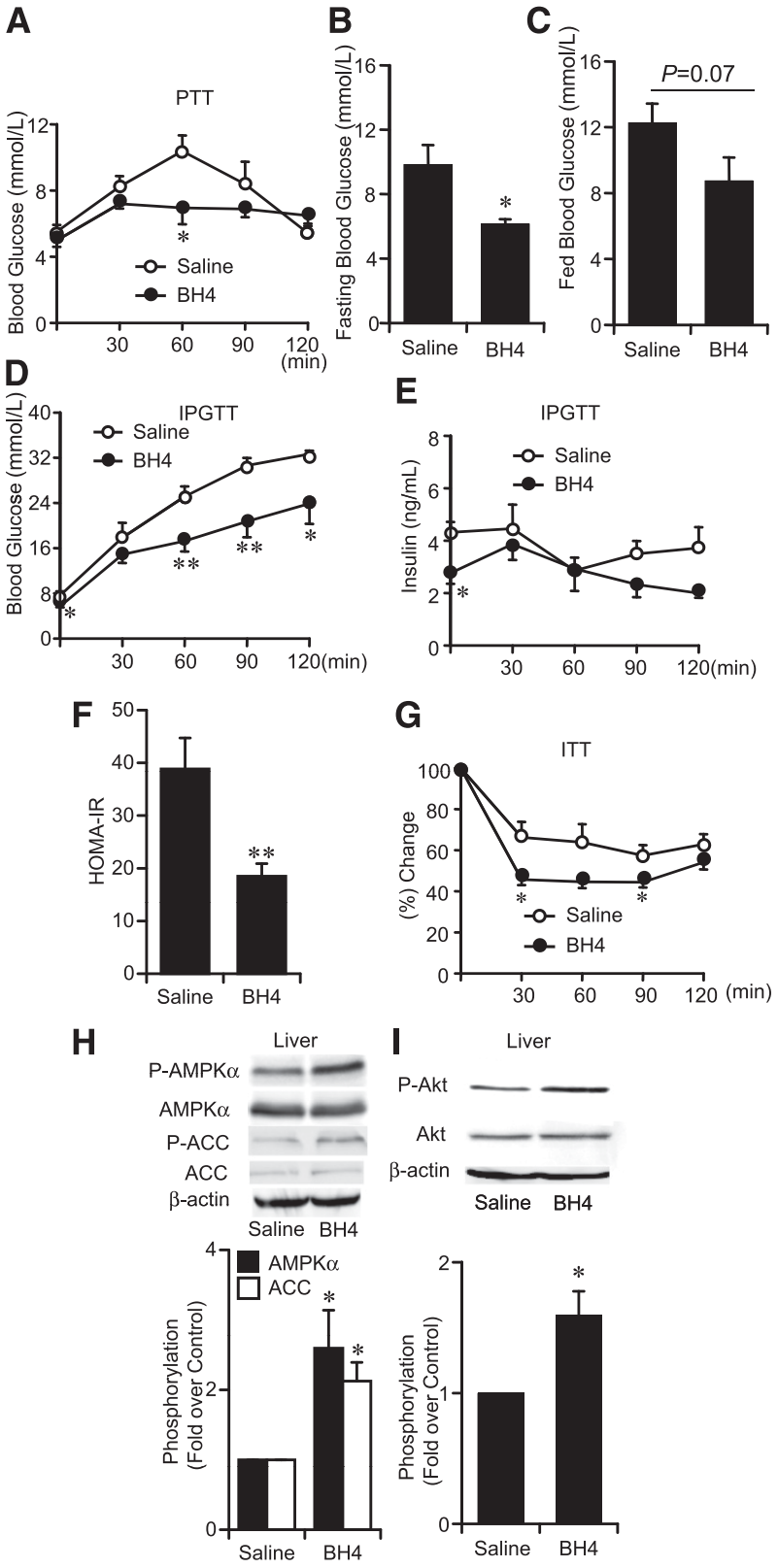


**FIG. 7. Effects of $BH_4$ in *ob/ob* mice. *A*: PTT to *ob/ob* mice with or without single administration of $BH_4$ (20 mg/kg). Values are means ± SE ($n$ = 6). *$P$ < 0.05 vs. the value of saline. *B*: Fasting blood glucose levels of *ob/ob* mice treated with $BH_4$ (20 mg/kg/day) for 10 days were significantly decreased compared with those treated without $BH_4$. Values are means ± SE ($n$ = 6). *$P$ < 0.05 vs. the value of saline. *C*: Fed blood glucose levels in *ob/ob* mice treated with or without $BH_4$ for 10 days. $P$ = 0.07 vs. the value of saline. Values are means ± SE ($n$ = 6). *D* and *E*: IPGTT to *ob/ob* mice. Blood glucose levels and plasma insulin levels after administration of glucose (1 g/kg i.p.) with or without $BH_4$ for 10 days. Values are means ± SE ($n$ = 6). *$P$ < 0.05, **$P$ < 0.01 vs. without $BH_4$. *F*: HOMA-IR calculated from fasting blood glucose and insulin levels from IPGTT data in *ob/ob* mice treated with or without $BH_4$ for 10 days. Values are means ± SE ($n$ = 6). **$P$ < 0.01 vs. the value of saline. *G*: Insulin tolerance test (ITT) to *ob/ob* mice treated with or without $BH_4$ for 10 days. Values are means ± SE ($n$ = 6). *$P$ < 0.05 vs. the value of saline. *H* and *I*: AMPKα, ACC, and Akt phosphorylation in liver tissues of *ob/ob* mice was increased by 10 days' administration of $BH_4$. Data are expressed as fold stimulation over saline. Values are means ± SE ($n$ = 3). *$P$ < 0.05 vs. saline.**

The glucose-lowering effect of $BH_4$ by single administration intraperitoneally on fasting blood glucose levels in STZ diabetic mice was similar to that of metformin (250 mg/kg). The dose of metformin that we used was adjusted to previous studies in mice (43) and is more than fivefold higher than that in clinical use for type 2 diabetic patients (44). We demonstrate here the lowering effects of $BH_4$ on blood glucose levels using a dosage similar to that of $BH_4$ used in patients with phenylketonuria as a cofactor of phenylalanine hydroxylase (45).

Numerous clinical trials have been performed on the effect of $BH_4$ as a cofactor of eNOS on endothelial dysfunction in a variety of vascular diseases including coronary artery disease (15). While many of the results are disappointing (46), $BH_4$ remains a viable candidate for clinical use if the design of the various trials is reconsidered. Several of the studies reported that $BH_4$ levels are plainly decreased and that uncoupled eNOS is found in the diabetic state and not in nondiabetic states (47). Moreover, nondiabetic patients were included in most of the clinical trials (46); those trials should be performed in patients with diabetes. The current study, furthermore, clarifies a novel concept of the relationship between $BH_4$ and glucose metabolism and insulin resistance that suggests a new approach to the prevention of macrovascular complications of diabetes induced by endothelial dysfunction as well as amelioration of the disease itself.

In conclusion, $BH_4$ has a glucose-lowering effect by suppressing hepatic gluconeogenesis in an eNOS-dependent manner and ameliorates glucose intolerance as well as insulin resistance in diabetic mice, suggesting that $BH_4$ has potential in the treatment of type 2 diabetes.

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No potential conflicts of interest relevant to this article were reported.

A.A. and Y.F. researched data, contributed to discussion, and wrote, reviewed, and edited the manuscript. A.Ob. and A.Oh. researched data and contributed to discussion. T.F., Y.S., M.O., Y.N., S.F., and M.H. contributed to discussion. H.H. researched data and contributed to discussion. N.I. contributed to discussion and wrote, reviewed, and edited the manuscript. N.I. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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