Tetrahydrobiopterin has a glucose-lowering effect by suppressing hepatic gluconeogenesis in an endothelial nitric oxide synthase-dependent manner in diabetic mice

Short running title: BH4 suppresses hepatic gluconeogenesis

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ABSTRACT

Endothelial nitric oxide synthase (eNOS) dysfunction induces insulin resistance and glucose intolerance. Tetrahydrobiopterin (BH4) is an essential co-factor of eNOS that regulates eNOS activity. In the diabetic state, BH4 is oxidized to 7, 8-dihydrobiopterin, which leads to eNOS dysfunction due to eNOS uncoupling. The present study investigates the effects of BH4 on glucose metabolism and insulin sensitivity in diabetic mice. Single administration of BH4 lowered fasting blood glucose levels in streptozotocin (STZ)-induced diabetic wild-type mice, and alleviated eNOS dysfunction by increasing eNOS dimerization in the liver of these mice. Liver has a critical role in glucose-lowering effects of BH4 through suppression of hepatic gluconeogenesis. BH4 activated AMP kinase (AMPK) and the suppressing effect of BH4 on gluconeogenesis was AMPK-dependent. In addition, the glucose-lowering effect and activation of AMPK by BH4 did not appear in STZ-induced diabetic mice lacking eNOS. Consecutive administration of BH4 in ob/ob mice ameliorated glucose intolerance and insulin resistance. Taken together, BH4 suppresses hepatic gluconeogenesis in an eNOS-dependent manner, and BH4 has a glucose-lowering effect as well as an insulin sensitizing effect in diabetic mice. BH4 has a potential in the treatment of type 2 diabetes.
INTRODUCTION

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 (BH4) (2, 6-7). BH4 is an essential cofactor for eNOS catalysis, and functions as an allosteric modulator of arginine binding (7, 8). Binding of BH4 to eNOS elicits a conformational change that increases the
affinity for binding of arginine-based ligands. BH4 binding also plays a role in dimer formation of the active and stabilized form of eNOS (8). BH4 is converted to 7, 8-dihydrobiopterin (BH2) by exposure to oxidative stress such as diabetes (8, 9). Increase in BH2 induces dysfunction of eNOS as BH2 is inactive for NOS cofactor function and competes with BH4 for BH4-binding (8, 9). Furthermore, in states of diabetes and high glucose, de novo synthesis of BH4, which is rate limited by GTP cyclohydrolase I (GTPCH I), is impaired (10-13). Thus, the availability of BH4 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 BH4 can improve endothelial dysfunction by elevating the BH4/BH2 ratio leading to recoupling of eNOS, and has been used in clinical trials with patients with atherosclerotic diseases for the expected vasodilatation effects of BH4 through NO production (15). However, it is unclear whether BH4 improves glucose metabolism and insulin sensitivity in diabetic conditions.

In the present study, we investigated the effects of BH4 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 BH4 lowers fasting blood
glucose levels and suppresses gluconeogenesis in liver in an eNOS-dependent manner. In addition, BH4 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 BH4 suppresses hepatic gluconeogenesis. These data suggest that BH4 has potential as a novel therapeutic approach to diabetes.
RESEARCH DESIGN AND METHOD

Animals

Male C57/BL6 (wild-type) mice and male heterozygous Ins2\textsuperscript{Akita} (diabetic Akita) mice, which exhibit hyperglycemia with reduced β-cell mass caused by a point 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\textsuperscript{−/−} mice in the C57/BL6 mice background were obtained from Jackson Laboratories (Bar Harbor, ME). Male B6.V-Lept/J (ob/ob) mice were obtained from Charles River Japan (Yokohama, Japan). Streptozotocin (STZ)-induced diabetic mice were made by intraperitoneal injection of STZ (120 mg/kg) to 7 weeks old wild-type or eNOS\textsuperscript{−/−} mice. At 3 weeks after injection of STZ, the animals were confirmed to be diabetic by both high blood glucose levels (≥ 15 mM) and other diabetic features, including polyuria, polydispia, 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 to 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 (EC)

Mouse hepatocytes were isolated by collagenase digestion as described previously (19). Primary hepatocytes were prepared by seeding in 6-well type 1 collagen-coated plates at a density of $1.5 \times 10^6$ cells in Dulbecco's Modified Eagle Medium (DMEM) (low glucose, 5.6 mM) containing 10% (vol./vol.) FBS, 100 nM regular insulin, 50 U/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 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 6-well 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 (KRB)
medium of 119.4 mM NaCl, 3.7 mM KCl, 2.7 mM CaCl₂, 1.3 mM KH₂PO₄, 1.3 mM MgSO₄, and 24.8 mM NaHCO₃ without glucose, 2% (wt/vol.) BSA, 0.24 mM 3-isobutyl-1-methylxanthine, and gluconeogenetic substrates (1 mM pyruvate plus 10 mM lactate). Hepatocytes were treated with BH4 (Schircks Laboratories, Jona, Switzerland), sodium nitroprusside (SNP), NG-Nitro-l-Arginine Methyl Ester (l-NAME) (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 described previously (19).

Immunoblotting analysis of hepatocytes

Western blotting was performed as described previously (19). Primary hepatocytes cultured overnight were incubated in buffer A treated with BH4, SNP, sepiapterin, and EHNA. Hepatocytes were homogenized in lysis buffer. Cell lysates (50-150 µg protein per lane) were heated at 95°C for 5 min and subjected to electrophoresis on 6-10% (vol./vol.) SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. For analysis of eNOS dimerization, the samples were not heated and the temperature was maintained below 15°C during electrophoresis. Primary antibodies used were anti-phospho-AMPKα
(Thr172), anti-AMPKα, anti-Phospho-Acetyl-CoA Carboxylase (Ser79), anti-Acetyl-CoA Carboxylase, anti-Phospho-eNOS (Ser1177), anti-phospho-Akt (Ser473), 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 (1:2000 dilution; Dianova, Hamburg, Germany), anti-GTPCH I (1:3,000; kindly gifted from Prof. Ichinose, Tokyo Institute of Technology), anti-dihydrofolate reductase (DHFR) and 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, UK). The fluorescent bands were visualized using a detection system (Amersham ECL Plus; GE Heathcare) 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 synthesized by invitrogen (Carlsbad, CA). The sequence of siRNA for AMPKα1 was: 5’-UCUCUUUCCUGAGGACCCACUUUAU-3’ and
5’-UAUAAGAUGGGUCCUCAGGAAAGAGAU3’. The sequences of control siRNAs were:

5’-ACCAACACAGUUUGGGAUUAGGGA-3’ and 5’-UCCCUAUUCCCAAACUGUUGUUGGU-3’. Isolated hepatocytes in DMEM (low glucose, 5.6 mM) containing 10% (vol./vol.) FBS, 100 nM 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 CO₂ incubator. The final amounts of hepatocytes, DMEM, Opti-MEM, siRNA and Lipofectamine RNAi MAX were 5.0× 10⁵ cells /ml, 75% (v/v), 25% (v/v), 50 nM and 0.2% respectively. Medium was replaced with DMEM 6 h after transfection. 48 h after transfection, the medium was replaced with buffer A, and the cells were incubated for 60 min with or without BH4, 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, USA). 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 described previously (19).

**Measurement of adenine nucleotide content**

After primary isolated hepatocytes were incubated in buffer A with or without BH4 and SNP for 30 min, treatment was stopped by rapid addition of 0.1 ml of 2 M HClO₄, followed by mixing by vortex and sonication in ice-cold water for 3 min. Adenine nucleotide contents were measured by a luminometric method as described previously (19, 20).

**Isolation of total RNA and quantitative RT-PCR**

Total RNA was isolated from livers of 10 week-old wild-type mice, STZ-induced diabetic wild-type mice, and ob/ob mice using Trizol (Invitrogen), as described previously (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 (GAPDH) as an inner control are shown in
Supplementary Table 1. SYBR Green PCR Master Mix (Applied Biosystems, Foster, CA) was prepared for the quantative 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 STZ-induced diabetic wild-type mice were collected. To measure uptake of BH4 in liver, BH4 (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 N2, and then stored at -80°C. Total biopterin, BH4, and BH2 were measured as described previously (22).

**Effect of BH4 on blood glucose levels of STZ-induced diabetic wild-type mice, STZ-induced diabetic eNOS−/− mice, and diabetic Akita mice**
Blood glucose levels were measured in STZ-induced diabetic wild-type mice, STZ-induced diabetic eNOS\(^{-/-}\) mice, and diabetic Akita mice fasted for 16 h, and BH4 (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 BH4 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 BH4 (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 (IPGTT)**

Wild-type mice were fasted overnight for 16 h, and glucose (2 g/kg) was injected intraperitoneally with BH4 (20 mg/kg) in 0.9% (wt/vol.) sterile saline or 0.9% sterile saline alone. Ob/ob mice after 10 days treatment of saline with or without BH4 (20 mg/kg) 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 by using the formula [fasting insulin (mU/l) × fasting plasma glucose (mmol/l)] / 22.5.

**Pyruvate tolerance test (PTT)**

Pyruvate, BH4, 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 BH4 (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 (ITT)**

Ob/ob mice after 10 days treatment of saline with or without BH4 (20 mg/kg) were fasted for 6 h, and regular insulin (1 U/kg) was injected intraperitoneally 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’s $t$ test (not noted) and paired student’s $t$ 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 BH4 on blood glucose levels in diabetic mice

In STZ-induced diabetic wild-type mice, the content of BH2 was increased and the BH4/BH2 ratio was decreased in blood and respective tissues (Fig. 1A-D). To investigate whether BH4 lowers blood glucose levels, BH4 (20 mg/kg) in saline was injected intraperitoneally to STZ-induced diabetic wild-type mice. Blood glucose levels were not changed 2 h after administration of BH4 in fed STZ-induced diabetic wild-type mice, while blood glucose levels were lowered by about 2.4 mM in overnight-fasted STZ-induced diabetic wild-type mice, a change similar to that with metformin (Fig. 1E and F, Supplementary Fig. 1A). The same effects also were found in diabetic Akita mice (Supplementary Fig. 1B).

Liver tissue has an important role in glucose-lowering effects of BH4

Although the intraperitoneal glucose tolerance test (IPGTT) data in wild-type mice revealed no effects of BH4 on blood glucose levels and plasma insulin levels, the pyruvate tolerance test (PTT) data showed that BH4 decreased hepatic glucose production (Fig. 2A-C), suggesting that the suppressing effect on hepatic gluconeogenesis has a critical role in the
glucose-lowering effect of BH4. The mRNA and protein expression levels of GTPCH I, a rate limiting enzyme of the BH4 de novo synthesis pathway, were decreased in liver tissues of STZ-induced diabetic wild-type mice (Fig. 2D and E). On the other hand, uptake of BH4 into liver by its supplementation is regulated by DHFR, a rate limiting enzyme of the BH4 salvage synthesis pathway (23), and the expression of DHFR in liver tissues of STZ-induced diabetic wild-type mice was not changed (Fig. 2F and G). The uptake of BH4 in liver of wild-type mice was confirmed with a peak at 30 min by administration of BH4 (20 mg/kg) as described previously (22, 23) (Supplementary Fig. 2A). After 2-h administration of BH4, 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-induced diabetic wild-type mice (Fig. 2H-K). The mRNA expression levels of PEPCK and G6Pase in the liver of wild-type mice were not changed (Supplementary Fig. 2B and C).

**BH4 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 BH4 in suppression of hepatic gluconeogenesis using hepatocytes isolated from wild-type mice fasted for 16 h. In time course study of exposure to BH4, the suppressing effect on gluconeogenesis appeared after 60 min ($P<0.01$ vs. corresponding control; Fig. 3A). We then investigated the increment of AMPKα phosphorylation by time course exposure of BH4 to hepatocytes. AMPK was activated after 30 min by BH4 (Fig. 3B). After 60-min exposure to BH4, gluconeogenesis was dose-dependently suppressed at doses of 50 µM and 100 µM BH4 (control, 101.7±3.7 nmol/mg protein; 50 µM BH4, 72.4±7.1 nmol/mg protein, $P<0.01$ vs. control; 100 µM BH4, 60.6±4.1 nmol/mg protein, $P<0.001$ vs. control; Fig. 3C). AMPK was activated at doses of 50 and 100 µM BH4 by 30-min exposure (Fig. 3D). In accordance with the activation of AMPK, an increase in phosphorylation of Acetyl-CoA carboxylase (ACC) by BH4 was confirmed (Fig. 3B and D). To determine whether BH4 suppresses gluconeogenesis in an AMPK-dependent manner, the effect of silencing AMPK was examined (Fig. 3E). By transfection of AMPKα1 siRNA, the suppressing effect of BH4 on gluconeogenesis disappeared (Fig. 3F). The suppressing effect of BH4 on gluconeogenesis also disappeared in the presence of compound C, an AMPK inhibitor (Fig. 3G).
BH4 suppresses gluconeogenesis and increases AMPKα phosphorylation eNOS-dependently in hepatocytes.

Exposure to BH4 in hepatocytes increased NO production and eNOS phosphorylation (Fig. 4A and B). To examine whether BH4 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, BH4 did not suppress gluconeogenesis (control, 103.9±10.8 nmol/mg protein; 50 µM BH4, 98.5±11.3 nmol/mg protein; 100 µM BH4, 89.1±10.9 nmol/mg protein, \(P=\text{NS vs. control; Fig. 4C}\)). BH4 did not alter AMPKα and ACC phosphorylation in hepatocytes lacking eNOS (Fig. 4D). The suppressing effect of BH4 on gluconeogenesis and activation of AMPK also disappeared in the presence of \(l\)-NAME, a NOS inhibitor (Supplementary Fig. 4A-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. 5A-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 BH4 or SNP (Supplementary Fig. 5E).
Effect of BH4 on adenine nucleotide content in hepatocytes.

To investigate the mechanism of AMPK activation by BH4 in hepatocytes, the adenine nucleotide content with exposure of BH4 to hepatocytes was measured. BH4 and SNP significantly increased AMP content in wild-type mouse hepatocytes (Table 1). Unexpectedly, BH4 also significantly increased ATP content. To clarify the mechanism by which BH4 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 BH4. Although erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA), a known AMPD inhibitor, activated AMPK and suppressed hepatic gluconeogenesis, BH4 did not have an additive effect on EHNA (Supplementary Fig. 6A and B). These results indicate that inhibition of AMPD, at least in part, contributes to AMP accumulation by BH4 in hepatocytes.

Sepiapterin, a BH4 precursor, suppresses gluconeogenesis and increases AMPK activation.

Similarly to BH4, sepiapterin is absorbed in hepatocytes and immediately converted to BH4 via a salvage pathway of BH4 biosynthesis (23). Sepiapterin was found to suppress
gluconeogenesis and activate AMPK (Fig. 5A and B). However, these effects were abolished in hepatocytes lacking eNOS (Fig. 5A and B).

**Role of eNOS in in vivo action of BH4 on glucose metabolism**

The lowering effect of BH4 on fasting blood glucose levels disappeared in STZ-induced diabetic eNOS$^{-/-}$ mice (Fig. 6A). The PTT data showed that BH4 did not decrease hepatic glucose production in eNOS$^{-/-}$ mice (Fig. 6B). Similar results were also obtained in sepiapterin administration (Supplementary Fig. 7A and B). We then compared the effects of BH4 on phosphorylation of AMPKα in liver tissues of these diabetic mice. BH4 activated AMPK in both STZ-induced diabetic wild-type mice liver and diabetic Akita mice liver but not in STZ-induced diabetic eNOS$^{-/-}$ mice liver (Fig. 6C and D, Supplementary Fig. 8A). AMPKα phosphorylation was not changed by fasting for 16 h in liver tissues of wild-type mice (Supplementary Fig. 8B).

**Effects of BH4 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 BH4 in ob/ob mice (Fig. 7A), 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 BH4 (20 mg/kg) in saline for 10 days to ob/ob mice, fasting blood glucose levels were significantly lowered by 3.9 mM and fed blood glucose levels tended to be decreased compared to those in ob/ob mice treated with saline alone (Fig. 7B and C). Our IPGTT, homeostasis model assessment of insulin resistance (HOMA-IR), and insulin tolerance test (ITT) data suggest that consecutive administration of BH4 ameliorates glucose intolerance as well as insulin resistance (Fig. 7D-G). Phosphorylation of AMPKα, ACC, and Akt was increased in liver tissues of BH4-treated ob/ob mice compared to those in saline-treated mice (Fig. 7H and I).
DISCUSSION

The present study shows that BH4, known as a co-factor of eNOS, has a glucose-lowering effect in diabetic mice. The BH4/BH2 ratio was found to be decreased in various tissues of mice in the diabetic state, indicating deterioration of 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 BH4 ameliorates glucose dysmetabolism and insulin resistance. In addition, we found that supplementation of BH4 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, BH4 elicited a glucose-lowering effect in these mice. No such glucose-lowering effect by BH4 appeared in diabetic mice lacking eNOS. These findings clearly implicate recoupling of eNOS in the glucose-lowering effect of BH4.

We have shown that the liver plays a critical role in the glucose-lowering effect of BH4 through suppression of hepatic gluconeogenesis. It is well known that BH4 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 BH4 is known to accumulate at
higher levels in liver than other tissues including skeletal muscle (24), which also lends support to the view that BH4 readily elevates BH4/BH2 ratio and regulates glucose metabolism in the liver.

We then investigated the molecular mechanism of suppression of hepatic gluconeogenesis by BH4 using isolated mouse hepatocytes. BH4 acts directly on hepatocytes and suppresses hepatic gluconeogenesis eNOS-dependently. Several papers 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 intra-hepatocellular eNOS is essential for the effect of BH4 in suppression of hepatic gluconeogenesis. In addition, BH4 activated AMPK and the suppressing effect of BH4 on gluconeogenesis disappeared by siRNA silencing of AMPKα1 subunits in hepatocytes, indicating that AMPK is involved in the suppressing effect of BH4 on hepatic gluconeogenesis. AMPK activation by BH4 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 BH4. AMPK is a serine/threonine kinase that acts as an energy sensor and is activated by an increase in the
AMP/ATP ratio and/or AMP in response to a variety of metabolic stresses, such as hypoxia, ischemia, and exercise (31, 32). In our data, BH4 significantly increased AMP content, and tended to increase the AMP/ATP ratio. It is known that inhibition of AMP deaminase (AMPD) increases AMP in isolated hepatocytes (33). Recently, Ouyang J et. al reported that inhibition of AMPD might be involved in increased production of AMP and activation of AMPK by metformin (34). In the present study, the AMPD inhibitor EHNA was found to activate AMPK, but BH4 did not elicit an additional effect on AMPK activation in the presence of EHNA, suggesting that AMPD might be inhibited by BH4 in hepatocytes. Interestingly, BH4 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 BH4 increases ATP content is unclear, but BH4 is known to work as an anti-oxidant (36). It has been reported that BH4 preserves ATP content and has a cytoprotective effect from hypoxia on neuronal cells (37). BH4 might thus prevent cytotoxic damage from reactive oxygen species (ROS) / reactive nitrogen species (RNS) as a scavenger, keeping ATP content higher than in the absence of BH4. We therefore cannot exclude the possibility that BH4 acts as a ROS/RNS scavenger in ameliorating glucose dysmetabolism, but such effect would be limited in terms of suppressing hepatic
gluconeogenesis because the effect of BH4 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 BH4 does. Regarding the mechanism of AMPK activation by BH4 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 BH4 (40, 19). The involvement of RNS on AMPK activation by BH4 was not suggested by our present data.

Our data using ob/ob mice, a mouse model of insulin resistance, suggests that the primary physiological action of BH4 is a suppressing effect of hepatic gluconeogenesis. In addition to this effect, consecutive administration of BH4 ameliorated glucose intolerance as well as insulin resistance. A possible mechanism of these additive effects of BH4 is induction by the subsequent downstream targets of AMPK activated by BH4 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 BH4, while it raises the
possibility that Akt-dependent signaling is involved in the suppressing effect of BH4 on hepatic gluconeogenesis in ob/ob mice. Another possible mechanism of BH4 ameliorating insulin resistance would be via a direct effect of BH4 on endothelial cells. Similarly to several NO donors and NO moderating compounds (42), BH4 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 present study must be considered. Further investigations, e.g., by using liver-specific eNOS\(^{-/-}\) mice, are required to elucidate the pleiotropic effects of BH4 in lowering blood glucose levels.

The glucose-lowering effect of BH4 by single administration intraperitoneally on fasting blood glucose levels in STZ-induced diabetic mice was similar to that of metformin (250mg/kg). The dose of metformin that we used was adjusted to previous studies in mice (43), and is over 5-fold higher than that in clinical use for type 2 diabetes patients (44). We demonstrate here the lowering effects of BH4 on blood glucose levels using a dosage similar to that of BH4 used in patients with phenylketonuria (PKU) as a co-factor of phenylalanine hydroxylase (45).
Numerous clinical trials have been performed on the effect of BH4 as a co-factor of eNOS on endothelial dysfunction in a variety of vascular diseases including coronary artery disease (15). While many of the results are disappointing (46), BH4 remains as a viable candidate for clinical use if the design of the various trials is reconsidered. Several of the studies reported that BH4 levels are plainly decreased and that uncoupled eNOS is found in the diabetic state and not in non-diabetic states (47). Moreover, non-diabetic patients were included in the most of clinical trials (46); those trials should be performed to patients with diabetes. The present study furthermore clarifies a novel concept of the relationship between BH4 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, BH4 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 BH4 has a 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.Obara and A.Ohashi 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.

Dr. Nobuya Inagaki is the guarantor of this work, had full access to all the data, and takes full responsibility for the integrity of data and the accuracy of data analysis.
Parts of this study were presented in abstract form at the 71st Scientific Sessions of the American Diabetes Association, San Diego, California, 24-28 June 2011.
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267: 971-978


45. Blau N. Defining tetrahydrobiopterin (BH4)-responsiveness in PKU. J Inherit Metab Dis 2008; 31: 2–3


Table 1

Effects of BH4 on ATP, AMP, and AMP/ATP ratio in wild-type mouse hepatocytes

<table>
<thead>
<tr>
<th></th>
<th>ATP (nmol/mg protein)</th>
<th>AMP (nmol/mg protein)</th>
<th>AMP/ATP ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.66± 0.08</td>
<td>0.28 ± 0.04</td>
<td>0.44 ± 0.03</td>
</tr>
<tr>
<td>BH4</td>
<td>0.88 ± 0.04*</td>
<td>0.49 ± 0.05**</td>
<td>0.55 ± 0.04</td>
</tr>
<tr>
<td>SNP</td>
<td>0.73 ± 0.07</td>
<td>0.47 ± 0.01**</td>
<td>0.67 ± 0.07</td>
</tr>
</tbody>
</table>

Hepatocytes were incubated in KRB buffer with or without BH4 (50 µM) for 30 min, the treatment was stopped by rapid addition of 0.1 ml of 2 M HClO₄, and adenine nucleotide contents were measured. Values are means ± SE (n=5). *P <0.05, **P <0.01 vs. control.
FIGURE LEGENDS

Figure 1

Biopterin dynamics and effects of BH4 on blood glucose levels in diabetic mice. (A-D) BH2 levels and BH4/BH2 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, F) Fed blood glucose levels were not changed 2-h after intraperitoneal injection of BH4 (20mg/kg) to STZ-induced diabetic wild-type mice; fasting blood glucose levels were significantly decreased. Values are means ± SE (n=8). *P <0.05, vs. the value of pre-injection of saline with BH4 intraperitoneally, paired t test. No significant difference of fed and fasting blood glucose levels 2-h after intraperitoneal injection of saline to STZ-induced diabetic wild-type mice. Values are means ± SE (n=7).

Figure 2

Role of liver tissue in glucose-lowering effects of BH4. (A and B) IPGTT to wild-type mice. Blood glucose levels and plasma insulin levels after intraperitoneal administration of glucose (2 g/kg) with or without BH4 (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 BH4 (20 mg/kg) to wild-type mice was suppressed compared with those without BH4. Values are means ± SE (n=6), *P <0.05, vs. saline. (D) In STZ-induced diabetic wild-type mice, mRNA levels of GTPCH I expression were significantly decreased compared to that in non-diabetic wild-type mice liver. Values are means ± SE (n=5), **P <0.01 vs. non diabetic wild-type mice liver. (E) In STZ-induced diabetic wild-type mice, protein expression levels of GTPCH I were significantly decreased compared to that in non-diabetic wild-type mice liver. Values are means ± SE (n=5), **P <0.01 vs. non diabetic wild-type mice liver. (F) No significant difference of mRNA expression levels of DHFR in liver was detected between non-diabetic and STZ-induced diabetic wild-type mice. Values are means ± SE (n=10). (G) No significant difference of protein expression levels of DHFR in liver was detected between non-diabetic and STZ-induced diabetic wild-type mice. Values are means ± SE (n=5). (H and I) In liver tissues of STZ-induced diabetic wild-type mice treated with BH4, mRNA levels of PEPCK were significantly decreased compared to those treated without BH4. 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 BH4 (20 mg/kg) to STZ-induced diabetic wild type mice. 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 STZ-induced diabetic wild-type mice treated with BH4, NO content was significantly increased compared to those treated without BH4. Values are means ± SE (n=5), *P < 0.05 vs. saline.

Figure 3

BH4 suppressed gluconeogenesis and increased AMPKα phosphorylation in hepatocytes isolated from wild-type mice. (A) Time course of gluconeogenesis with exposure to BH4. Suppressing effect on gluconeogenesis by 50 µM BH4 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 BH4 (50 µM). Both AMPKα and ACC phosphorylation was stimulated after 30-min exposure to BH4 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 BH4 was detected ranging over 50 µM in hepatocytes isolated from wild-type mice. Values are means ± SE (n=6), **P < 0.01, ***P <0.001 vs. control. (D) Effect of BH4 on phosphorylation of
AMPK and ACC. After 30-min exposure to BH4, both AMPKα and ACC phosphorylation was increased by BH4 dose-dependently ranging over 50 µM 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) Transfected with AMPKα1 siRNA, protein expression of AMPKα was decreased compared to those transfected with control siRNA. Values are means ± SE (n=3), ***P <0.001 vs. control siRNA. (F) Transfected with AMPKα1 siRNA, suppressing effect of BH4 (50 µM) on hepatic glucose production was inhibited. Values are means ± SE (n=6), ***P <0.001 vs. values transfected with control siRNA without BH4. (G) Compound C (20 µM), an AMPK inhibitor, abolished the suppressing effect of BH4 (50 µM) on gluconeogenesis. Values are means ± SE (n=6), *P <0.05 vs. values without BH4 and without compound C.

Figure 4

Lack of the effect of BH4 on suppression of gluconeogenesis in eNOS−/− mouse hepatocytes. (A) BH4 (50 µM) significantly increased NO production in hepatocytes from wild-type mice. SNP (20 µM) was used as positive control. Values are means ± SE (n=5), *P <0.05 vs. control. (B) BH4 (ranging from 10 to 50 µM) increased eNOS
phosphorylation at Ser\textsuperscript{1177} in hepatocytes from wild-type mice. Values are means ± SE (n=5), \*P <0.05 vs. control. (C) BH4 (ranging from 10 to 100 µM) did not suppress gluconeogenesis after 1-h exposure in hepatocytes from eNOS\textsuperscript{−/−} mice. Values are means ± SE (n=6). (D) After 30-min exposure to BH4 ranging from 10 to 100 µM, AMPKα and ACC phosphorylation were not increased by BH4 in hepatocytes in hepatocytes from eNOS\textsuperscript{−/−} mice. Data are expressed as -fold stimulation over control. Values are means ± SE (n=3).

**Figure 5**

Effect of sepiapterin, a BH4 precursor, on gluconeogenesis and AMPK activation. (A) After 1-h exposure, sepiapterin (50 µM) significantly suppressed gluconeogenesis in hepatocytes isolated from wild-type mice. This effect was not observed in hepatocytes isolated from eNOS\textsuperscript{−/−} mice. Values are means ± SE (n=6), \*P <0.05, vs. control. (B) After 30-min exposure to sepiapterin (50 µM), AMPKα phosphorylation was increased in hepatocytes isolated from wild-type mice. AMPKα phosphorylation was not increased by sepiapterin in hepatocytes isolated from eNOS\textsuperscript{−/−} mice. Data are expressed as -fold stimulation over control. Values are means ± SE (n=3), \**P <0.01 vs. control.
Figure 6

Effects of BH4 in STZ-induced diabetic eNOS<sup>−/−</sup> mice. (A) No significant difference of fasting blood glucose levels 2h after intraperitoneal injection of saline with or without BH4 (20 mg/kg) to STZ-induced diabetic eNOS<sup>−/−</sup> mice. Values are means ± SE (n=7). (B) PTT to eNOS<sup>−/−</sup> mice. No effects of BH4 (20 mg/kg) on suppressing hepatic gluconeogenesis were detected in pyruvate tolerance test in eNOS<sup>−/−</sup> mice. Values are means ± SE (n=6). (C) AMPKα phosphorylation in liver of STZ-induced diabetic eNOS<sup>−/−</sup> mice was not changed by BH4 administration. Data are expressed as -fold stimulation over saline. Values are means ± SE (n=3). (D) AMPKα phosphorylation in liver of STZ-induced diabetic wild-type mice was significantly increased by BH4 (20 mg/kg) administration. Data are expressed as -fold stimulation over saline. Values are means ± SE (n=3), **P <0.01 vs. saline.

Figure 7

Effects of BH4 in ob/ob mice. (A) PTT to ob/ob mice with or without single administration of BH4 (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 BH4 (20 mg/kg/day) for 10 days were significantly decreased compared to those treated without BH4. 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 BH4 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 intraperitoneal administration of glucose (1 g/kg) with or without BH4 for 10 days. Values are means ± SE (n=6). *P < 0.05, **P < 0.01 vs. without BH4. (F) HOMA-IR calculated from fasting blood glucose and insulin levels from IPGTT data in ob/ob mice treated with or without BH4 for 10 days. Values are means ± SE (n=6). **P < 0.01, vs. the value of saline. (G) ITT to ob/ob mice treated with or without BH4 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 were increased by 10-day administration of BH4. Data are expressed as -fold stimulation over saline. Values are means ± SE (n=3), *P < 0.05 vs. saline.
Figure 1

A. Liver

B. Blood

C. Kidney

D. Spleen

**BH2 Levels (nmol/g)**

**BH4/BH2 Ratio**

*STZ(-) STZ(+)*
Figure 1

**E**

Fed Blood Glucose (mM)

- Saline
- BH4

**F**

Fasting Blood Glucose (mM)

- Saline
- BH4
Figure 2

A. IPGTT wild-type mice

- Blood Glucose (mM)
- Time (min)
- Saline
- BH4

B. IPGTT wild-type mice

- Insulin (ng/ml)
- Time (min)
- Saline
- BH4

C. PTT wild-type mice

- Blood Glucose (mM)
- Time (min)
- Saline
- BH4

D. GTPCH I

- Relative mRNA Expression
- STZ(-)
- STZ(+)

E. GTPCH I

- (Fold over without STZ)
- STZ(-)
- STZ(+)
Figure 2

F

DHFR

G

DHFR

β-actin

H

PEPCK

I

G6Pase

eNOS

J

dimer

monomer

β-actin

K

Nitric Oxide (µmol/l/mg protein)

Relative mRNA Expression

Saline  BH4

0

0.5

1

1.5

2

Saline  BH4

0

0.5

1

1.5

2

Saline  BH4

0

0.5

1

1.5

2

Relative mRNA Expression

Relative mRNA Expression

Relative mRNA Expression

Relative mRNA Expression

Relative mRNA Expression

Relative mRNA Expression

Relative mRNA Expression

Relative mRNA Expression

Relative mRNA Expression

Relative mRNA Expression

Relative mRNA Expression

Relative mRNA Expression
Figure 3

A. Wild-type hepatocytes

<table>
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<tr>
<th>Glucose Production (nmol/mg protein)</th>
<th>Control</th>
<th>BH4 (50 μM)</th>
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<tbody>
<tr>
<td>200</td>
<td></td>
<td></td>
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<tr>
<td>160</td>
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<td>120</td>
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<tr>
<td>80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
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</tbody>
</table>

BH4 exposure (min) 15 30 60 90

B. Wild-type hepatocytes

P-AMPKα, AMPKα, P-ACC, ACC, β-actin

<table>
<thead>
<tr>
<th>BH4 exposure (min)</th>
<th>Control</th>
<th>15</th>
<th>30</th>
<th>60</th>
<th>90</th>
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</thead>
</table>

C. Wild-type hepatocytes

<table>
<thead>
<tr>
<th>Glucose Production (nmol/mg protein)</th>
<th>Control</th>
<th>10</th>
<th>50</th>
<th>100</th>
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</thead>
<tbody>
<tr>
<td>120</td>
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<tr>
<td>80</td>
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<tr>
<td>0</td>
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</tbody>
</table>

BH4 (μM) 10 50 100

D. Wild-type hepatocytes

P-AMPKα, AMPKα, P-ACC, ACC, β-actin

<table>
<thead>
<tr>
<th>BH4 exposure (min)</th>
<th>Control</th>
<th>10</th>
<th>50</th>
<th>100</th>
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</thead>
</table>

Phosphorylation (Fold over Control)

AMPKα, ACC

<table>
<thead>
<tr>
<th>BH4 exposure (min)</th>
<th>Control</th>
<th>15</th>
<th>30</th>
<th>60</th>
<th>90</th>
</tr>
</thead>
</table>

** Statistical significance (*) and ** (p < 0.05)
Figure 3

E

AMPKα

β-actin

SiRNA Control AMPKα1
(-) SiRNA SiRNA

AMPKα

(Fold over Control SiRNA)

SiRNA Control AMPKα1
(-) SiRNA SiRNA

F

Glucose Production (nmol/mg protein)

BH4

Control SiRNA AMPKα1 SiRNA

G

Glucose Production (nmol/mg protein)

BH4

Control Compound C
Figure 4

A Wild-type hepatocytes

Nitric Oxide (μmol/l / 1.5×10^6 cells)

Glucose Production (nmol/mg protein)

B Wild-type hepatocytes

C eNOS^−/− hepatocytes

D eNOS^−/− hepatocytes

P-AMPKα
AMPKα
P-ACC
ACC
β-actin

Phosphorylation (Fold over Control)

Wild-type hepatocytes

eNOS^−/− hepatocytes

AMPKα
ACC

Phosphorylation (Fold over Control)
Figure 5

A

Glucose Production

(nmol/mg protein)

<table>
<thead>
<tr>
<th>Sepiapterin</th>
<th>Wild-type hepatocytes</th>
<th>eNOS-/- hepatocytes</th>
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</thead>
<tbody>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

B

Phosphorylation (Fold over Control)

P-AMPKα

AMPKα

β-actin

<table>
<thead>
<tr>
<th>Sepiapterin</th>
<th>Wild-type hepatocytes</th>
<th>eNOS-/- hepatocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 6

A  STZ-induced diabetic eNOS-/- mice

- Fasting Blood Glucose (mM)
  - Saline
  - BH4

B  PTT eNOS-/- mice

- Blood Glucose (mM)
  - Saline
  - BH4

C  STZ-induced diabetic eNOS-/- mice liver

- P-AMPKα
- AMPKα
- β-actin

D  STZ-induced diabetic wild-type mice liver

- P-AMPKα
- AMPKα
- β-actin

**Phosphorylation (Fold over Saline)**

- Saline
- BH4
Figure 7

A. PTT

B. Fasting Blood Glucose (mM)

C. Fed Blood Glucose (mM)

D. IPGTT

E. IPGTT

F. HOMA-IR

G. ITT

Saline  BH4

0 4 8 12

30 60 90 120 (min)

P=0.07

Saline  BH4

0 2 4 6

30 60 90 120 (min)

Saline  BH4

0 10 20 30 40

Saline  BH4
Figure 7

H

Liver

P-AMPKα
AMPKα
P-ACC
ACC
β-actin

Saline  BH4

Phosphorylation (Fold over Control)

Saline  BH4

I

Liver

P-Akt
Akt
β-actin

Saline  BH4

Phosphorylation (Fold over Control)

Saline  BH4

*
SUPPLEMENTARY DATA

Supplementary Table 1

Primer sequences used in quantitative RT-PCR

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<tr>
<th>Name</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>GTPCH I</td>
<td>GTCATCACCAGAACATCGA</td>
<td>ACGTGAGGTCGCAGCAAGCC</td>
</tr>
<tr>
<td>DHFR</td>
<td>ACCGCAATCTGTGTGAGG</td>
<td>ATGCTTTTTTCTCTCTGGAC</td>
</tr>
<tr>
<td>G6Pase</td>
<td>AAGCAGTTCCCTGTCACTCTG</td>
<td>CAAACACCGGAATCCATACG</td>
</tr>
<tr>
<td>PEPCK</td>
<td>CTAACTTGCCATGATGACC</td>
<td>CCGTTTTCTGGGTTGATAGC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>AGCTCACTGGGATGGCTGCGG</td>
<td>GCCTGCTTCACCACCTTCTTGTG</td>
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Supplementary Table 2

Characteristics of ob/ob mice

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<tr>
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<th>Body Weight (g)</th>
<th>Fed Blood Glucose (mM)</th>
<th>Fasting Blood Glucose (mM)</th>
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</thead>
<tbody>
<tr>
<td>Saline</td>
<td>46.03 ± 0.27</td>
<td>18.75 ± 2.00</td>
<td>8.01 ± 0.47</td>
</tr>
<tr>
<td>BH4</td>
<td>45.45 ± 0.39</td>
<td>19.39 ± 3.05</td>
<td>7.96 ± 0.48</td>
</tr>
</tbody>
</table>

Values are means ± SE (n=6). No significant difference between saline and BH4 groups.
LEGENDS of SUPPLEMENTARY FIGURES

Supplementary Figure 1

Effects of BH4 or metformin on blood glucose levels. (A) Fasting blood glucose levels were decreased 2 h after intraperitoneal injection of metformin (250 mg/kg) in STZ-induced wild-type mice. Values are means ± SE (n=7). *P < 0.05, vs. the value of pre-injection of saline with metformin intraperitoneally, paired t test. (B) Fasting blood glucose levels were significantly decreased 24 h after intraperitoneal injection of BH4 (20 mg/kg) to diabetic Akita mice. Values are means ± SE (n=7). *P < 0.05, vs. the value of pre-injection of saline with BH4 intraperitoneally, paired t test. No significant difference of fasting blood glucose levels 24 h after intraperitoneal injection of saline to diabetic Akita mice. Values are means ± SE (n=7).

Supplementary Figure 2

(A) Intraperitoneal injection of BH4 (20 mg/kg) significantly increased the BH4 levels with the peak at 30 min in liver tissues of wild-type mice. Values are means ± SE (n=4), *P<0.05, **P<0.01 vs. pre-administration. (B and C) The mRNA levels of PEPCK and G6Pase in liver tissues of wild-type mice treated with or without BH4. Values are means ±
Supplementary Figure 3

Immunobloting analysis of isolated ECs and hepatocytes. CD31, a known EC marker, was expressed in isolated ECs while there was no expression in isolated hepatocytes. α1-antitrypsin, a known hepatocyte marker, was expressed in isolated hepatocytes while there was no expression in ECs. ENOS was expressed both in ECs and isolated hepatocytes. Representative western blots from three independent experiments are shown.

Supplementary Figure 4

The effects of BH4 on suppression of hepatic gluconeogenesis and activation of AMPKα did not appear by exposure with L-NAME. (A) L-NAME (1 mM), an NOS inhibitor, abolished the suppressing effect of BH4 (50 µM) on gluconeogenesis. Values are means ± SE (n=6), **P <0.01 vs. values without BH4 and without compound L-NAME. (B) AMPKα phosphorylation was not increased by BH4 in hepatocytes treated with L-NAME. Data are expressed as -fold stimulation over control. Values are means ± SE (n=3).
Supplementary Figure 5

NO donor suppresses gluconeogenesis and increases AMPKα phosphorylation in hepatocytes isolated from both wild-type and eNOS−/− mice. (A) After 1-h exposure to SNP, a NO donor, SNP (20 µM) significantly suppressed gluconeogenesis in hepatocytes isolated from wild-type mice. Values are means ± SE (n=6), *P <0.05, vs. control. (B) After 30-min exposure to SNP (20 µM), AMPKα phosphorylation was increased in hepatocytes isolated from wild-type mice. Data are expressed as fold stimulation over control. Values are means ± SE (n=3), *P <0.05 vs. control. (C) Suppressing effects of SNP (20 µM) on gluconeogenesis were observed in hepatocytes isolated from eNOS−/− mice. Values are means ± SE (n=5), *P <0.05, vs. control. (D) AMPKα phosphorylation also was increased with SNP (20 µM) in hepatocytes isolated from eNOS−/− mice. Data are expressed as fold stimulation over control. Values are means ± SE (n=3), *P <0.05 vs. control. (E) Immunocytochemical staining with anti-nitrotyrosine antibody for detection of ONOO− generation in hepatocytes isolated from wild-type mice. ONOO− (10µM) incubated for 5min was used as positive control. Exposure of BH4 (50 µM) and SNP (20 µM) for 30 min did not increase staining. Confocal microscopy, magnificient ×100, scale bar 50 µm.
**Supplementary Figure 6**

Effect of AMPD on BH4 action in hepatocytes isolated from wild-type mice. (A) After 30-min exposure, EHNA (100µM), BH4 (50µM), and EHNA plus BH4 significantly increased AMPK phosphorylation. Values are means ± SE (n=6), **P <0.01, and ***P <0.001 vs. control. (B) After 1-h exposure, EHNA significantly suppressed gluconeogenesis. No additive effect of BH4 to EHNA was detected. Values are means ± SE (n=6), **P <0.01 vs. control.

**Supplementary Figure 7**

Effect of sepiapterin in PTT. (A) Elevation of blood glucose levels after intraperitoneal administration of pyruvate with sepiapterin (20 mg/kg) to wild-type mice was suppressed compared with those without sepiapterin. Values are means ± SE (n=6), *P<0.05, vs. control. (B) No effects of sepiapterin on suppressing gluconeogenesis were detected in PTT in eNOS−/− mice. Values are means ± SE (n=6).

**Supplementary Figure 8**

(A) AMPKα phosphorylation in liver tissues of diabetic Akita mice was significantly
increased by BH4 administration. Data are expressed as -fold stimulation over saline. Values are means ± SE (n=3), **$P<0.01$ vs. saline. (B) AMPKα phosphorylation in liver tissues of wild-type mice was not changed between 16 h fasting and fed state. Values are means ± SE (n=3).

**Supplementary Figure 9**

(A and B) The mRNA levels of PEPCK and G6Pase in liver tissues of ob/ob mice treated with or without single administration of BH4. Values are means ± SE (n=6).
**A** STZ-induced diabetic wild-type mice

**B** Diabetic Akita mice

**Supplementary Fig. 1**

**Diabetes**
Supplementary Fig. 2

A. Wild-type mice liver

BH4 (nmol/g)

0 10 20 30 40

30 60 120 180 (min)

**  

**

B. wild-type mice liver

PEPCK

Relative mRNA Expression

saline BH4

0.4 0.8 1.2 1.6

C. wild-type mice liver

G6Pase

Relative mRNA Expression

saline BH4

0.4 0.8 1.2 1.6
Supplementary Fig. 3

α1-antitrypsin
CD31
eNOS
β-actin

EC  Hepatocyte
Supplementary Fig. 6

A

B

Phosphorylation (Fold over Control)

Glucose Production (nmol/mg protein)

Control EHNA BH4 BH4 +EHNA

** ** **

Control EHNA BH4 BH4 +EHNA

*** *** ***

Supplementary Fig. 6

Control EHNA BH4 BH4 +EHNA

*** *** ***

Diabetes

Page 66 of 69
Supplementary Fig. 7

A  PTT wild-type mice

B  PTT eNOS\textsuperscript{-/-} mice

Blood Glucose (mM)

\begin{itemize}
\item Saline
\item Sepiapterin
\end{itemize}

(min)
Supplementary Fig. 8

A  Diabetic Akita mice liver

P-AMPKα  
AMPKα  
β-actin

Saline  BH4

Phosphorylation
(Fold over Saline)

Saline  BH4

B  Wild-type mice liver

P-AMPKα  
AMPKα  
β-actin

Fed  Fasting

Phosphorylation
(Fold over Fed Control)

Fed  Fasting
Supplementary Fig. 9

A. Relative mRNA Expression of PEPCK in ob/ob mice liver. Comparison between saline and BH4 treatment.

B. Relative mRNA Expression of G6Pase in ob/ob mice liver. Comparison between saline and BH4 treatment.