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 (BH4) is an essential cofactor of eNOS that regulates eNOS activity. In the diabetic state, BH4 is oxidized to 7,8-dihydrobiopterin, which leads to eNOS dysfunction owing to eNOS uncoupling. The current 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 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 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 mice with STZ-induced diabetes 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 potential in the treatment of type 2 diabetes. 


Nitreric oxide (NO) is a biological messenger produced by NO synthase (NOS), which includes endothelial (eNOS), inducible (iNOS), and neuronal (nNOS) isozymes. 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 Ca2+, 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 (O2−) rather than NO, a phenomenon called “eNOS uncoupling” (7,8,14). Supplementation of BH4 can improve endothelial dysfunction by elevating the BH4-to-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 current 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 METHODS

Male C57/BL6 (wild-type) mice and male heterozygous Ins2Akita (diabetic Akita) mice, which exhibit hyperglycemia with reduced β-cell mass caused by a point
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 antibody against AMPK (Cell Signaling Technology, Danvers, MA), anti-ACC (Ser79), anti-AMPK (Thr172), anti-phospho-acetyl-CoA carboxylase (ACC) (Ser79), anti-AMPK, and anti-AMPK (Thr172), and 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 BH4 and SNP for 30 min, treatment was stopped by rapid addition of 0.1 mL of 2 mol/L HClO4, followed by mixing by vortex and sonication in ice-cold water for 3 min. Adenine nucleotide contents were measured by a luminoometric 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 DHRF, glucose 6-phosphatase (G6Pase), phosphoenolpyruvate carboxykinase (PEPK), 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).

**Bioprotein analysis.** Tissues or whole blood of wild-type mice and wild-type mice with STZ-induced diabetes was collected. For measurement of 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 liver was dissected and crushed in liquid nitrogen. Blood glucose levels were measured again 2 h after injection.

**Effect of BH4 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 and ob/ob mice, diabetic Akita mice (ob/ob), and ob/ob mice with STZ-induced diabetes. 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. Blood glucose levels in diabetic mice 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 (mIU/L) × fasting plasma glucose (mM/L)]/22.5.

**Pyruvate tolerance test.** 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.** After 10 days’ treatment of saline with or without BH4 (20 mg/kg), ob/ob mice were fasted for 6 h, and insulin (1 units/kg ip) 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**

Bioprotein dynamics and effects of BH4 on blood glucose levels in diabetic mice. In STZ diabetic wild-type mice, the content of BH2 was increased and the
BH₄-to-BH₂ ratio was decreased in blood and respective tissues (Fig. 1A–D). For investigation of whether BH₄ lowers blood glucose levels, BH₄ (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 BH₄ 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. 1E and F and 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 glucoseogenesis 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 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 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 previously described (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 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).

**FIG. 1.** Biopterin dynamics and effects of BH₄ on blood glucose levels in diabetic mice. A–D: BH2 levels and BH₄-to-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 and F: Fed blood glucose levels were not changed 2 h after injection of BH₄ (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 BH₄ 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 BH4. 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 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 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
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 a 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 and 100 μmol/L BH₄ (control, 101.7 ± 3.7 nmol/mg protein; 50 μmol/L BH₄, 72.4 ± 7.1 nmol/mg protein, P < 0.01 vs. control; 100 μmol/L BH₄, 60.6 ± 4.1 nmol/mg protein, P < 0.001 vs. control) (Fig. 3C). AMPK was activated at doses of 50 and 100 μmol/L BH₄ by 30 min exposure (Fig. 3D). In accordance with the activation of AMPK, an increase in phosphorylation of ACC by BH₄ was confirmed (Fig. 3B and D). For determination of 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 in eNOS-deficient mice. 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 μmol/L BH₄, 98.5 ± 11.3 nmol/mg protein; 100 μmol/L BH₄, 89.1 ± 10.9 nmol/mg protein, P = 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 NG-nitro-l-arginine methyl ester, an NOS inhibitor (Supplementary Fig. 4A 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. 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. For investigation of 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 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 diabetic wild-type mice liver and diabetic Akita mice liver but not in STZ diabetic eNOS−/− mice liver (Fig. 6C and D and 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 showed 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. 9A 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 mmol/L and fed blood glucose levels tended to be decreased compared with those in ob/ob mice treated with saline alone (Fig. 7B and C). Our IPGTT, HOMA-IR, and insulin tolerance test 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 with those in saline-treated mice (Fig. 7H and I).

Discussion
The current study shows that BH₄, known as a cofactor of eNOS, has a glucose-lowering effect in diabetic mice. The BH₄-to-BH₃ ratio was found to be decreased in various tissues of mice in the diabetic state, indicating deterioration of
BH4suppresses hepatic gluconeogenesis

FIG. 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 μmol/L 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 μmol/L). Both AMPKα and ACC phosphorylation were 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 μmol/L in hepatocytes isolated from wild-type mice. Values are means ± SE (n = 6). ***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 were increased by BH4 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 < 0.01, ***P < 0.001 vs. control. E: With transfection with AMPKα 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 AMPKα siRNA, suppressing effect of BH4 (50 μmol/L) on hepatic glucose production was inhibited. Values are means ± SE (n = 6), ***P < 0.001 vs. values transfected with control siRNA. G: Compound C (20 μmol/L), an AMPK inhibitor, abolished the suppressing effect of BH4 (50 μmol/L) on gluconeogenesis. Values are means ± SE (n = 6), *P < 0.05 vs. values without BH4 and without compound C.
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 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 antioxidant (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/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 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 BH4 ON ATP, AMP, AND AMP-TO-ATP RATIO IN WILD-TYPE MOUSE HEPATOCYTES

<table>
<thead>
<tr>
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<th>ATP (nmol/mg protein)</th>
<th>AMP (nmol/mg protein)</th>
<th>AMP-to-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>
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Data are means ± SE (n = 5). Hepatocytes were incubated in Krebs-Ringer bicarbonate buffer with or without BH4 (50 μmol/L) for 30 min. The treatment was stopped by rapid addition of 0.1 mL of 2 mol/L HClO4, and adenine nucleotide contents were measured. *P < 0.05, **P < 0.01 vs. control.
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 (19,40). 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, suggest 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. Similar 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 eNOS²⁻/² 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 BH₄ in lowering blood glucose levels.
FIG. 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 with 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 administration of glucose (1 g/kg i.p.) 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: Insulin tolerance test (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: AMPK, ACC, and Akt phosphorylation in liver tissues of ob/ob mice was increased by 10 days' administration of BH4. Data are expressed as fold stimulation over saline. Values are means ± SE (n = 3). *P < 0.05 vs. saline.
BH₄ suppresses hepatic gluconeogenesis

The glucose-lowering effect of BH₄ 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 BH₄ on blood glucose levels using a dosage similar to that of BH₄ used in patients with phenylketonuria as a cofactor of phenylalanine hydroxylase (45).

Numerous clinical trials have been performed on the effect of BH₄ 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), BH₄ remains 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, 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 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 potential in the treatment of type 2 diabetes.

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A.A. and Y.F. researched data, contributed to discussion, and wrote, reviewed, and edited the manuscript. A.Oh. and A.Ob. 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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