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.

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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 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 in eNOS-deicient (eNOS−/−) mice, indicating that eNOS dysfunction, as BH2 is inactive for NOS cofactor function (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 C57BL6 (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 to BH4 (1:200, Sc-13860, Santa Cruz Biotechnology, Santa Cruz, CA) and mouse monoclonal antibody to GAPDH (1:4000, BD Transduction Laboratories, San Jose, CA). The secondary antibody was horseradish peroxidase-conjugated anti-rabbit, -mouse, -rat, or -goat antibody (1:1000, Santa Cruz Biotechnology, CA). The tissue samples and primary hepatocytes were incubated with the secondary antibody and then incubated in 37°C in a CO2 incubator. At the end of incubation, the samples were fixed with 4% paraformaldehyde and then permeabilized with 0.3% Triton X-100 in PBS. The samples were incubated with Alexa Fluor 488-conjugated phalloidin (Invitrogen) to stain actin filaments.

RESULTS

Bioterin 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

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BH$_4$-to-BH$_2$ ratio was decreased in blood and respective tissues (Fig. 1A–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. 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 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. 2A–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. 2D 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. 2F 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. 2A). 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. 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 BH$_4$ on blood glucose levels in diabetic mice. A–D: BH$_2$ 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 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 AMPKα 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 independently 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 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.

Sediaperin, a BH₄ precursor, suppresses gluconeogenesis and increases AMPK activation. Similarly to BH₄, sediaperin is absorbed in hepatocytes and immediately converted to BH₄ via a salvage pathway of BH₄ biosynthesis (23). Sediaperin 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 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 sediaperin 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 show that the suppressing effect on glucose metabolism 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

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 J: In liver tissues of wild-type mice with STZ-induced diabetes treated with BH₄, mRNA levels of PEPCK were significantly decreased compared with 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 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 BH₄, NO content was significantly increased compared with those treated without BH₄. Values are means ± SE (n = 5). *P < 0.05 vs. saline.
suppressing effect on gluconeogenesis in hepatocytes isolated from wild-type mice. BH4 suppressed hepatic gluconeogenesis after 1 h exposure of BH4 was detected ranging over 50 μmol/L. BH4 acts directly on hepatocytes and without compound C. Compound C (20 μmol/L), an AMPK inhibitor, abolishes the suppressing effect of BH4 (50 μmol/L) on glucose metabolism in the liver.

eNOS bioactivity by eNOS uncoupling. Previous studies have shown that impairment of eNOS function is involved in 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-to-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 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 BH4 in suppression of hepatic gluconeogenesis. In addition, BH4 activates AMPK, and the suppressing effect of BH4 on gluconeogenesis disappeared by siRNA silencing of AMPKα 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 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, BH4 significantly increased AMP content and 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-to-BH2 ratio and regulates glucose metabolism in the liver.
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

|        | 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      |
| BH4    | 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 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 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 BH4 in lowering blood glucose levels.

**FIG. 5.** Effect of sepiapterin, a BH4 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 BH4 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 BH4 (20 mg/kg) to eNOS−/− mice with STZ-induced diabetes. Values are means ± SE (n = 7). B: PTT to eNOS−/− mice. No effects of BH4 (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 BH4 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 BH4 (20 mg/kg) administration. Data are expressed as fold stimulation over saline. Values are means ± SE (n = 3). **P < 0.01 vs. saline.
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 < 0.05 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: AMPKα, 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.
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 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 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.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 writer, 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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