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

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

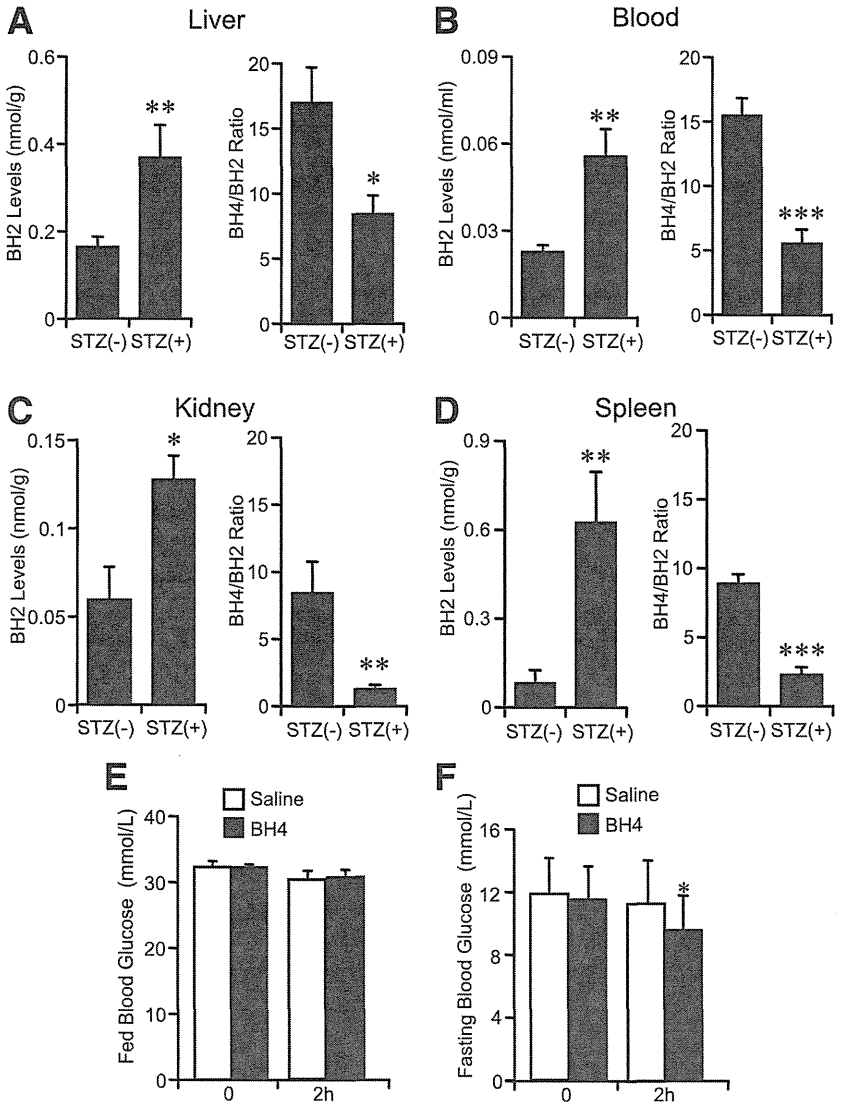

FIG. 1. Biopterin dynamics and effects of BH_4 on blood glucose levels in diabetic mice. *A–D*: BH2 levels and BH_4-to-BH_2 ratio of liver, blood, kidney, and spleen. Values are means ± SE. $n = 7$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. without STZ. *E* and *F*: Fed blood glucose levels were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

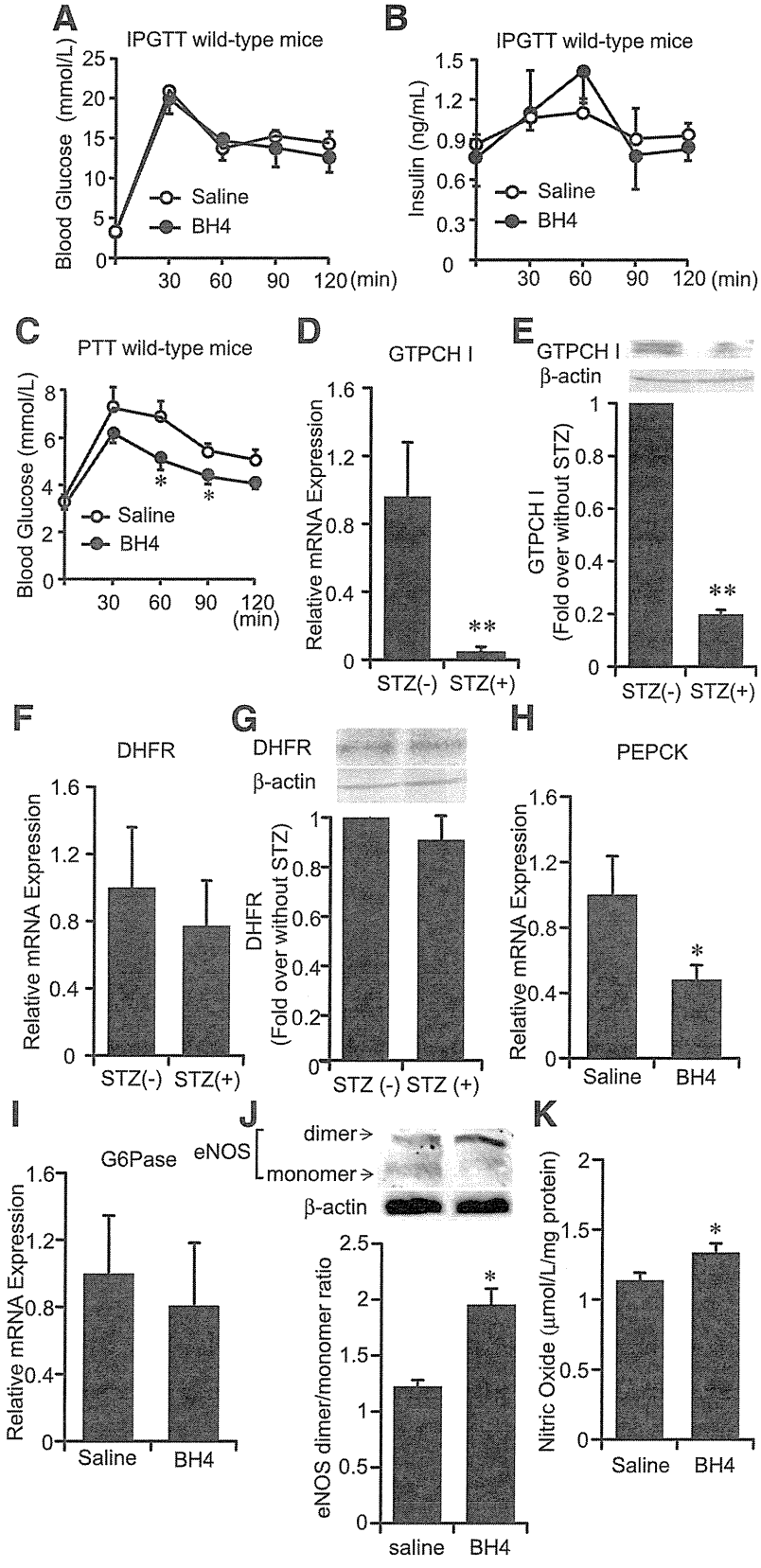

FIG. 2. Role of liver tissue in glucose-lowering effects of BH_4. *A* and *B*: IPGTT to wild-type mice. Blood glucose levels and plasma insulin levels after administration of glucose (2 g/kg i.p.) with or without BH_4 (20 mg/kg). Values are means ± SE (n = 6). *C*: PTT to wild-type mice. Elevation of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

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

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

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

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

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

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

DISCUSSION

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

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

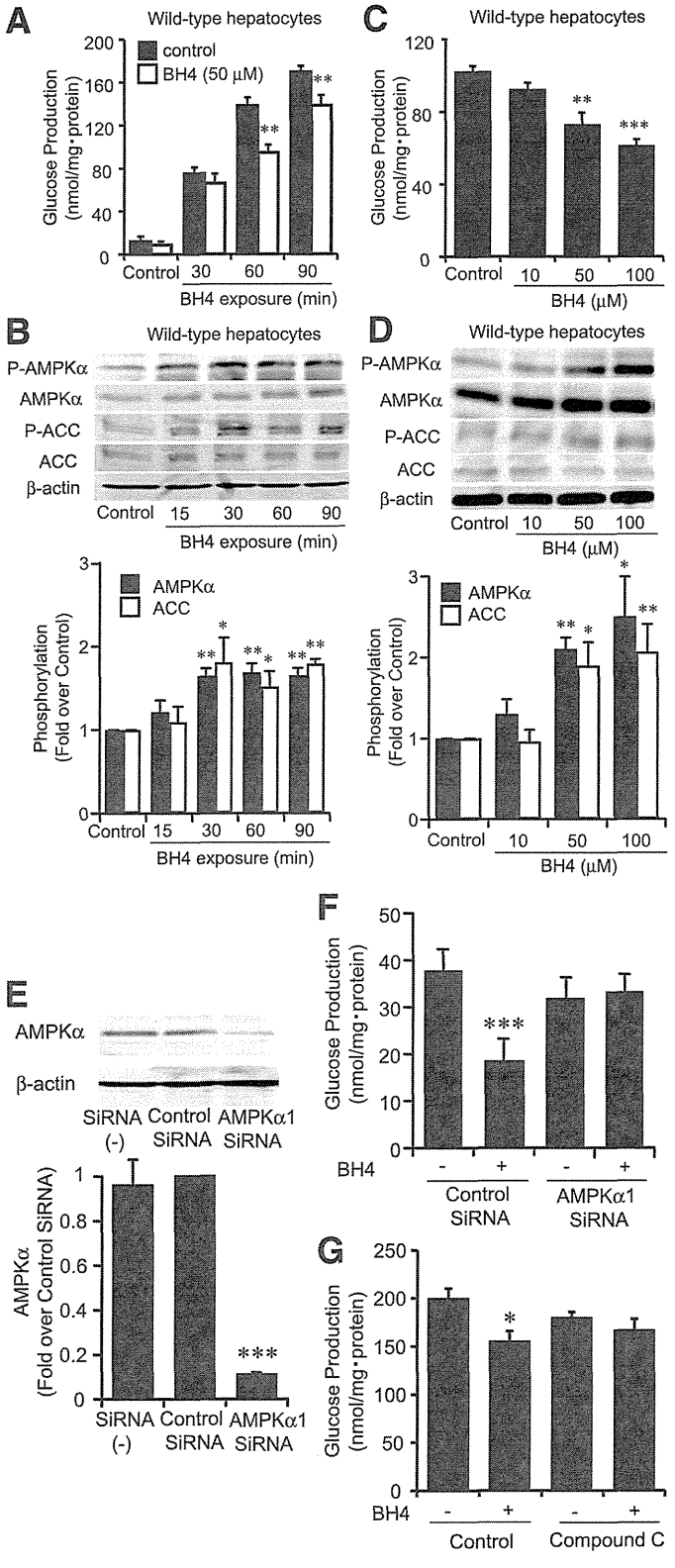

FIG. 3. BH_4 suppressed gluconeogenesis and increased AMPKα phosphorylation in hepatocytes isolated from wild-type mice. *A*: Time course of gluconeogenesis with exposure to BH_4. Suppressing effect on gluconeogenesis by 50 μmol/L BH_4 compared with control was detected after 60 min in hepatocytes isolated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

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

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

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

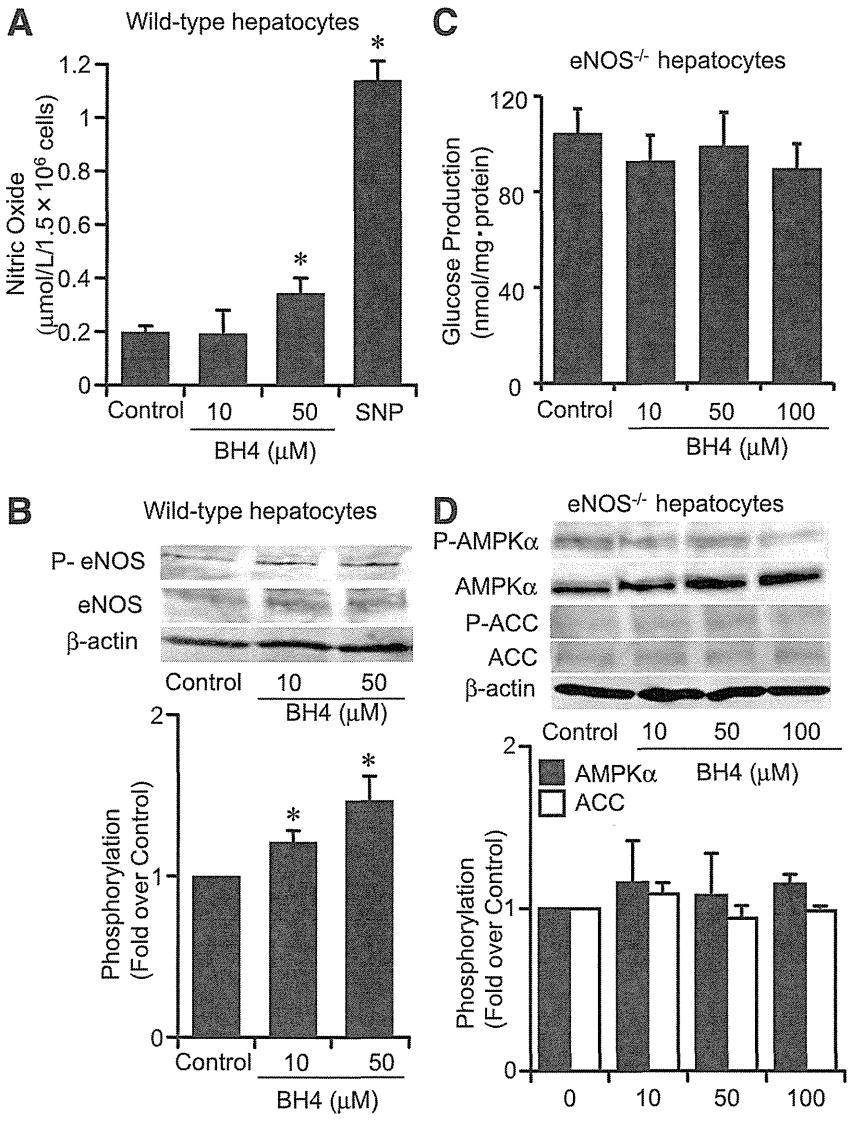

FIG. 4. Lack of the effect of BH_4 on suppression of gluconeogenesis in $eNOS^{-/-}$ mouse hepatocytes. *A*: BH_4 (50 μmol/L) significantly increased NO production in hepatocytes from wild-type mice. SNP (20 μmol/L) was used as positive control. Values are means ± SE (n = 5). *P < 0.05 vs. control. *B*: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

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

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

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

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

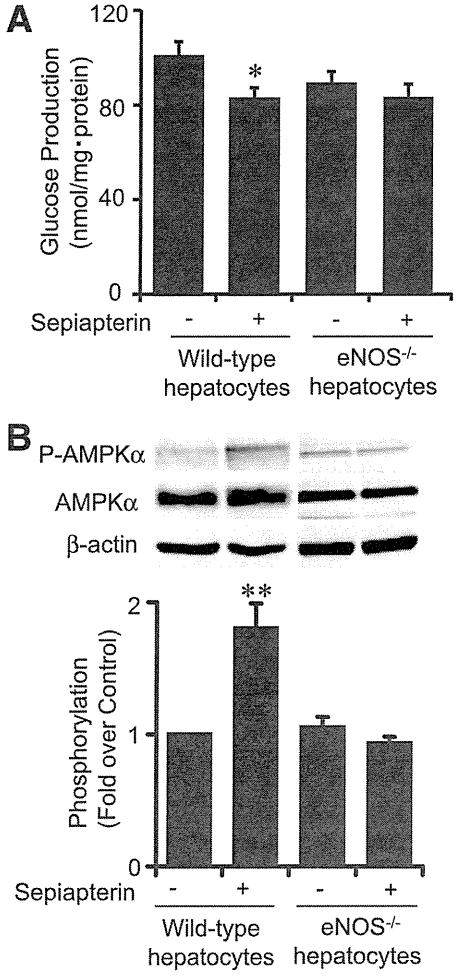

FIG. 5. Effect of sepiapterin, a BH_4 precursor, on gluconeogenesis and AMPK activation. *A*: After 1 h exposure, sepiapterin (50 μmol/L) significantly suppressed gluconeogenesis in hepatocytes isolated from wild-type mice. This effect was not observed in hepatocytes isolated from $eNOS^{-/-}$ mice.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

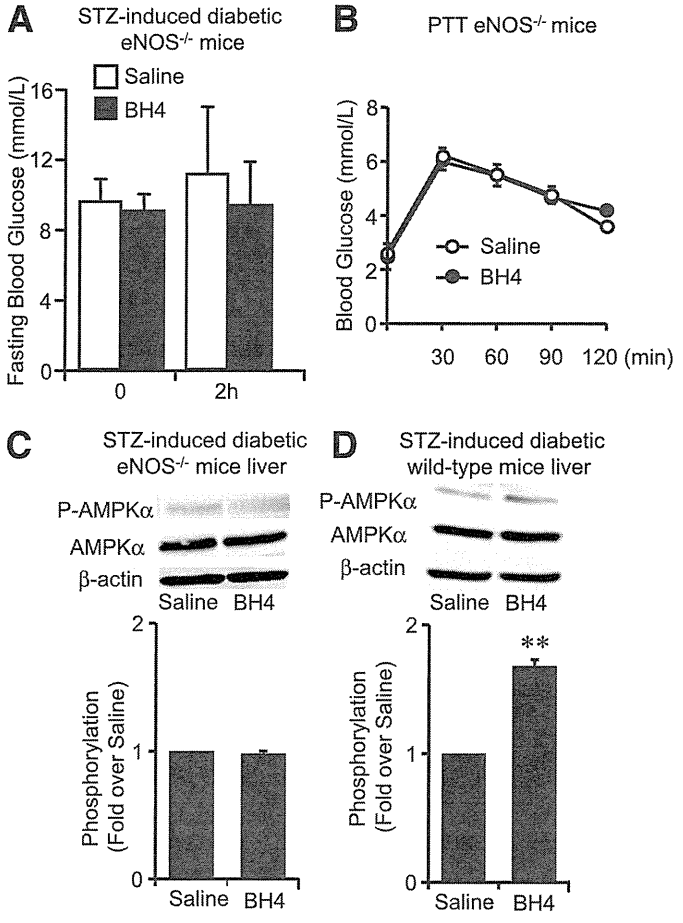

FIG. 6. Effects of BH_4 in $eNOS^{-/-}$ mice with STZ-induced diabetes. *A*: No significant difference of fasting blood glucose levels 2 h after intraperitoneal injection of saline with or without BH_4 (20 mg/kg) to $eNOS^{-/-}$ mice with STZ-induced diabetes. Values are means ± SE (n = 7). *B*: PTT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

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

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

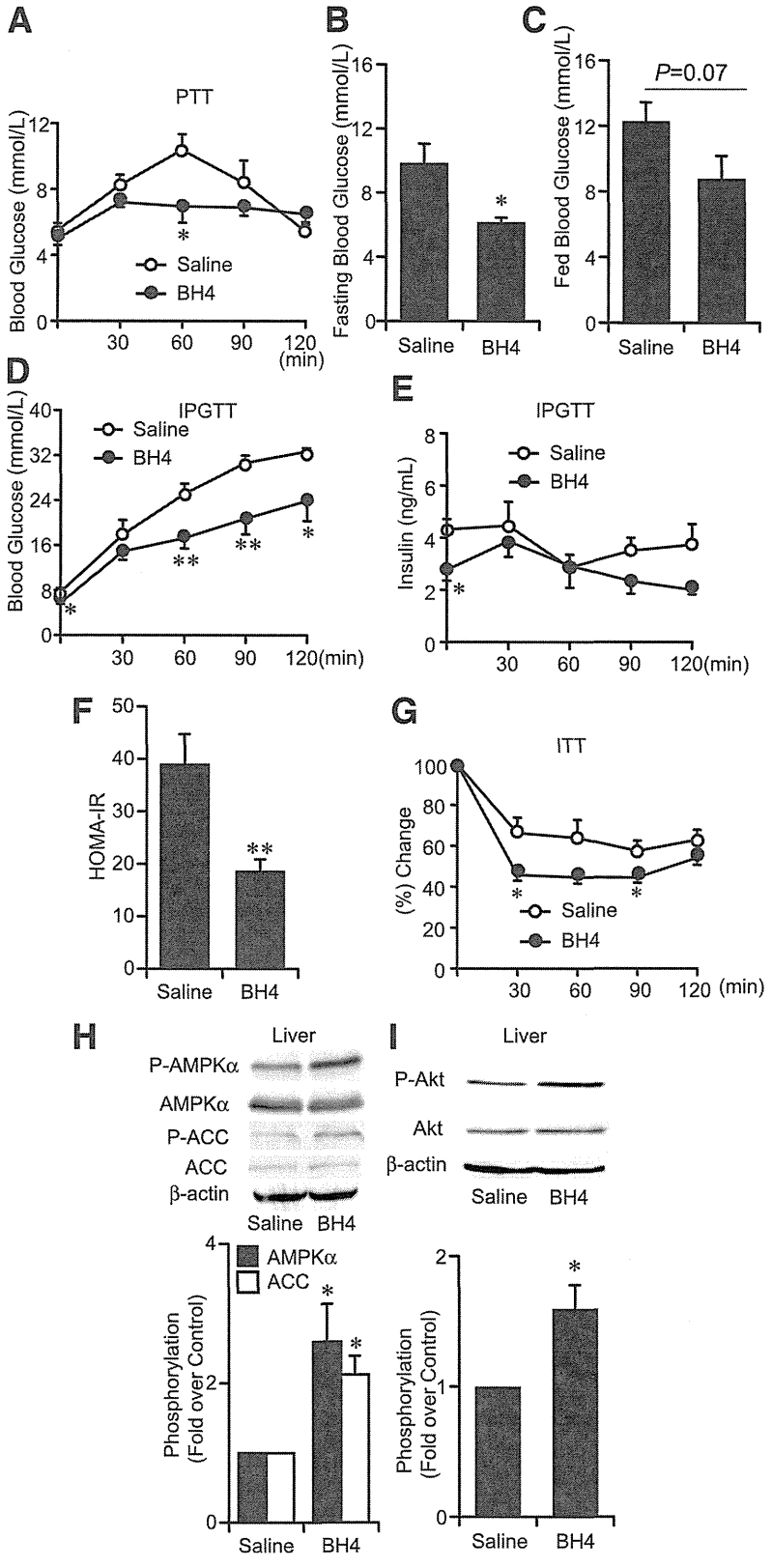

FIG. 7. Effects of BH_4 in *ob/ob* mice. *A*: PTT to *ob/ob* mice with or without single administration of BH_4 (20 mg/kg). Values are means ± SE (n = 6). *P < 0.05 vs. the value of saline. *B*: Fasting blood glucose levels of *ob/ob* mice treated with BH_4 (20 mg/kg/day) for 10 days were significantly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

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

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

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

ACKNOWLEDGMENTS

This study was supported by Scientific Research grants; a grant for Leading Project for Biosimulation from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; a grant from Core Research for Evolutional Science and Technology (CREST) of Japan Science and Technology Cooperation; a grant from the Ministry of Health, Labor, and Welfare, Japan; and a grant from Kyoto University Global Center of Excellence (COE) Program "Center for Frontier Medicine."

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

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

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.

The authors thank Ryo Tanaka and Miho Nishimura for their efforts in collaboration in the Student Research Program, Department of Biosciences, Teikyo University of Science and Technology.

REFERENCES

1. Triggle CR, Ding H. A review of endothelial dysfunction in diabetes: a focus on the contribution of a dysfunctional eNOS. J Am Soc Hypertens 2010;4:102–115
2. Huang PL. eNOS, metabolic syndrome and cardiovascular disease. Trends Endocrinol Metab 2009;20:295–302
3. Kim JA, Montagnani M, Koh KK, Quon MJ. Reciprocal relationships between insulin resistance and endothelial dysfunction: molecular and pathophysiological mechanisms. Circulation 2006;113:1888–1904
4. Duplain H, Burcelin R, Sartori C, et al. Insulin resistance, hyperlipidemia, and hypertension in mice lacking endothelial nitric oxide synthase. Circulation 2001;104:342–345
5. Shankar RR, Wu Y, Shen HQ, et al. Mice with gene disruption of both endothelial and neuronal nitric oxide synthase exhibit insulin resistance. Diabetes 2000;49:684–687
6. Stuehr D, Pou S, Rosen GM. Oxygen reduction by nitric-oxide synthases. J Biol Chem 2001;276:14533–14536
7. Vásquez-Vivar J, Kalyanaraman B, Martásek P, et al. Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. Proc Natl Acad Sci USA 1998;95:9220–9225
8. Crabtree MJ, Channon KM. Synthesis and recycling of tetrahydrobiopterin in endothelial function and vascular disease. Nitric Oxide 2011;25:81–88
9. Landmesser U, Dikalov S, Price SR, et al. Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. J Clin Invest 2003;111:1201–1209
10. Meininger CJ, Marinos RS, Hatakeyama K, et al. Impaired nitric oxide production in coronary endothelial cells of the spontaneously diabetic BB rat is due to tetrahydrobiopterin deficiency. Biochem J 2000;349:353–356
11. Xu J, Wu Y, Song P, et al. Proteasome-dependent degradation of guanosine 5′-triphosphate cyclohydrolase I causes tetrahydrobiopterin deficiency in diabetes mellitus. Circulation 2007;116:944–953
12. Meininger CJ, Cai S, Parker JL, et al. GTP cyclohydrolase I gene transfer reverses tetrahydrobiopterin deficiency and increases nitric oxide synthesis in endothelial cells and isolated vessels from diabetic rats. FASEB J 2004;18:1900–1902
13. Ding QF, Hayashi T, Packiasamy AR, et al. The effect of high glucose on NO and O2- through endothelial GTPCH1 and NADPH oxidase. Life Sci 2004;75:3185–3194
14. Kietadisorn R, Juni RP, Moens AL. Tackling endothelial dysfunction by modulating NOS uncoupling: new insights into its pathogenesis and therapeutic possibilities. Am J Physiol Endocrinol Metab 2012;302:E481–E495
15. Katusic ZS, d'Uscio LV, Nath KA. Vascular protection by tetrahydrobiopterin: progress and therapeutic prospects. Trends Pharmacol Sci 2009;30:48–54
16. Wajngot A, Chandramouli V, Schumann WC, et al. Quantitative contributions of gluconeogenesis to glucose production during fasting in type 2 diabetes mellitus. Metabolism 2001;50:47–52
17. Lin HV, Accili D. Hormonal regulation of hepatic glucose production in health and disease. Cell Metab 2011;14:9–19
18. Oyadomari S, Koizumi A, Takeda K, et al. Targeted disruption of the Chop gene delays endoplasmic reticulum stress-mediated diabetes. J Clin Invest 2002;109:525–532
19. Fujita Y, Hosokawa M, Fujimoto S, et al. Metformin suppresses hepatic gluconeogenesis and lowers fasting blood glucose levels through reactive nitrogen species in mice. Diabetologia 2010;53:1472–1481
20. Fujimoto S, Mukai E, Hamamoto Y, et al. Prior exposure to high glucose augments depolarization-induced insulin release by mitigating the decline of ATP level in rat islets. Endocrinology 2002;143:213–221
21. Ogura M, Nakamura Y, Tanaka D, et al. Overexpression of SIRT5 confirms its involvement in deacetylation and activation of carbamoyl phosphate synthetase 1. Biochem Biophys Res Commun 2010;393:73–78
22. Sawabe K, Wakasugi KO, Hasegawa H. Tetrahydrobiopterin uptake in supplemental administration: elevation of tissue tetrahydrobiopterin in mice following uptake of the exogenously oxidized product 7,8-dihydrobiopterin and subsequent reduction by an anti-folate-sensitive process. J Pharmacol Sci 2004;96:124–133
23. Sawabe K, Suetake Y, Nakanishi N, et al. Cellular accumulation of tetrahydrobiopterin following its administration is mediated by two different processes; direct uptake and indirect uptake mediated by a methotrexate-sensitive process. Mol Genet Metab 2005;86(Suppl. 1):S133–S138
24. Hoshiga M, Hatakeyama K, Watanabe M, et al. Autoradiographic distribution of [^{14}C]tetrahydrobiopterin and its developmental change in mice. J Pharmacol Exp Ther 1993;267:971–978
25. Matei V, Rodríguez-Vilarrupla A, Deulofeu R, et al. Three-day tetrahydrobiopterin therapy increases in vivo hepatic NOS activity and reduces portal pressure in CCl4 cirrhotic rats. J Hepatol 2008;49:192–197

26. Elrod JW, Duranski MR, Langston W, et al. eNOS gene therapy exacerbates hepatic ischemia-reperfusion injury in diabetes: a role for eNOS uncoupling. Circ Res 2006;99:78–85
27. Shah V, Cao S, Hendrickson H, et al. Regulation of hepatic eNOS by caveolin and calmodulin after bile duct ligation in rats. Am J Physiol Gastrointest Liver Physiol 2001;280:G1209–G1216
28. Wei CL, Khoo HE, Lee KH, et al. Differential expression and localization of nitric oxide synthases in cirrhotic livers of bile duct-ligated rats. Nitric Oxide 2002;7:91–102
29. McNaughton L, Puttagunta L, Martinez-Cuesta MA, et al. Distribution of nitric oxide synthase in normal and cirrhotic human liver. Proc Natl Acad Sci USA 2002;99:17161–17166
30. Mei Y, Thevananther S. Endothelial nitric oxide synthase is a key mediator of hepatocyte proliferation in response to partial hepatectomy in mice. Hepatology 2011;54:1777–1789
31. Zhang BB, Zhou G, Li C. AMPK: an emerging drug target for diabetes and the metabolic syndrome. Cell Metab 2009;9:407–416
32. Hardie DG. The AMP-activated protein kinase pathway—new players upstream and downstream. J Cell Sci 2004;117:5479–5487
33. Carabaza A, Ricart MD, Mor A, et al. Role of AMP on the activation of glycogen synthase and phosphorylase by adenosine, fructose, and glutamine in rat hepatocytes. J Biol Chem 1990;265:2724–2732
34. Ouyang J, Parakhia RA, Ochs RS. Metformin activates AMP kinase through inhibition of AMP deaminase. J Biol Chem 2011;286:1–11
35. Fogarty S, Hardie DG. Development of protein kinase activators: AMPK as a target in metabolic disorders and cancer. Biochim Biophys Acta 2010; 1804:581–589
36. Kojima S, Ona S, Iizuka I, et al. Antioxidative activity of 5,6,7,8-tetrahydrobiopterin and its inhibitory effect on paraquat-induced cell toxicity in cultured rat hepatocytes. Free Radic Res 1995;23:419–430
37. Delgado-Esteban M, Almeida A, Medina JM. Tetrahydrobiopterin deficiency increases neuronal vulnerability to hypoxia. J Neurochem 2002;82:1148–1159
38. Kang KT, Sullivan JC, Spradley FT, et al. Antihypertensive therapy increases tetrahydrobiopterin levels and NO/cGMP signaling in small arteries of angiotensin II-infused hypertensive rats. Am J Physiol Heart Circ Physiol 2011;300:H718–H724
39. Higaki Y, Hirshman MF, Fujii N, et al. Nitric oxide increases glucose uptake through a mechanism that is distinct from the insulin and contraction pathways in rat skeletal muscle. Diabetes 2001;50:241–247
40. Zhang J, Xie Z, Dong Y, et al. Identification of nitric oxide as an endogenous activator of the AMP-activated protein kinase in vascular endothelial cells. J Biol Chem 2008;283:27452–27461
41. Viollet B, Guigas B, Leclerc J, et al. AMP-activated protein kinase in the regulation of hepatic energy metabolism: from physiology to therapeutic perspectives. Acta Physiol (Oxf) 2009;196:81–98
42. Cook S, Scherrer U. Insulin resistance, a new target for nitric oxide-delivery drugs. Fundam Clin Pharmacol 2002;16:441–453
43. Shaw RJ, Lamia KA, Vasquez D, et al. The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. Science 2005; 310:1642–1646
44. Inzucchi SE, Maggs DG, Spollett GR, et al. Efficacy and metabolic effects of metformin and troglitazone in type II diabetes mellitus. N Engl J Med 1998;338:867–872
45. Blau N. Defining tetrahydrobiopterin (BH4)-responsiveness in PKU. J Inherit Metab Dis 2008;31:2–3
46. Moens AL, Kietadisorn R, Lin JY, et al. Targeting endothelial and myocardial dysfunction with tetrahydrobiopterin. J Mol Cell Cardiol 2011;51:559–563
47. Heitzer T, Krohn K, Albers S, et al. Tetrahydrobiopterin improves endothelium-dependent vasodilation by increasing nitric oxide activity in patients with Type II diabetes mellitus. Diabetologia 2000;43:1435–1438

INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

Lack of Goal Attainment Regarding the Low-density Lipoprotein Cholesterol Level in the Management of Type 2 Diabetes Mellitus

Masaya Hosokawa[1,2], Akihiro Hamasaki[1], Kazuaki Nagashima[1], Shinichi Harashima[1], Kentaro Toyoda[1], Yoshihito Fujita[1], Norio Harada[1], Yuko Nakahigashi[1], Shimpei Fujimoto[1,3] and Nobuya Inagaki[1]

Abstract

Objective The management of diabetes mellitus includes controlling the blood glucose level, body weight, blood pressure and serum lipid level. The coexistence of diabetes and a high low-density lipoprotein cholesterol (LDL-C) level promotes atherosclerosis of the coronary arteries and increases the risk of coronary artery disease (CAD). We compared the rates of attainment of LDL-C goals in type 2 diabetes patients receiving primary and secondary prevention therapy, the former without a history of CAD and the latter with a history of CAD. Because patients receiving secondary prevention are at greater risk of coronary events, LDL-C management is especially important in this group. This study was designed to determine how frequently diabetic patients attain their LDL-C goals and identify the reasons for the lack of attainment.

Methods The groups were distinguished according to the patients' medical records. Contributory factors for the patients not achieving their goals were recorded in a questionnaire filled out by each patient's physician.

Results The overall attainment rate in both groups was 61%. The most frequent impediment in both groups was "an LDL-C level above or below the goal at every hospital visit" followed by "continuously sufficient effects of dietary therapy only" and the "management of LDL-C by other departments or hospitals," the latter reflecting the increasing problems of polydisease and polypharmacy in diabetes care.

Conclusion Polydisease and polypharmacy issues in diabetes patients with a history of CAD constitute a growing barrier to medication adherence and the attainment of treatment goals.

Key words: type 2 diabetes, LDL cholesterol, coronary artery disease, questionnaire

(Intern Med 52: 2409-2415, 2013)
(DOI: 10.2169/internalmedicine.52.0599)

Introduction

The coexistence of diabetes and a high low-density lipoprotein cholesterol (LDL-C) level promotes atherosclerosis of the coronary arteries and increases the risk of coronary artery disease (CAD) and its associated morbidity. Treatment for high LDL-C includes dietary intervention, exercise and pharmacological therapy. Representative LDL-C-lowering drugs, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins), reduce the incidence of coronary events and mortality in patients with and without a prior history of CAD (1-7). Recently, the Steno-2 study revealed that the frequency of CAD was markedly reduced by interventions for various risk factors in diabetic patients (8). In that study, the attainment rate for the HbA1c goal was 15% and that for blood pressure was 60%. Strikingly, the attainment rate for the LDL-C goal in the intensive therapy group was 80%.

[1]Department of Diabetes, Endocrinology and Nutrition, Graduate School of Medicine, Kyoto University, Japan, [2]Faculty of Human Sciences, Tezukayamagakuin University, Japan and [3]Department of Endocrinology, Metabolism, and Nephrology, Kochi Medical School, Kochi University, Japan

Received for publication March 18, 2013; Accepted for publication June 17, 2013

Correspondence to Dr. Nobuya Inagaki, inagaki@metab.kuhp.kyoto-u.ac.jp

Table 1A. Patients' Background in Each Group

	Primary prevention group	Secondary prevention group
Number	499	121
Gender (male / female)	219/280	77/44
Age	65.8 ± 0.5	69.0 ± 0.8
BMI (kg/m^2)	24.1 ± 0.2	24.5 ± 0.3
Systolic BP (mmHg)	127.5 ± 0.7	128.0 ± 1.4
Diastolic BP (mmHg)	73.9 ± 0.4	72.2 ± 1.0
HbA1c (%)	7.7 ± 0.1	7.8 ± 0.1
LDL-C (mg/dL)	112.7 ± 1.0	95.0 ± 2.6
HDL-C (mg/dL)	55.0 ± 0.6	51.9 ± 1.2
Triglyceride (mg/dL)	154.0 ± 5.0	146.5 ± 8.3
Therapeutic modes for hyperglycemia (number of patients)		
Diet alone	92	19
OHA	222	55
Insulin	129	31
Combination of OHA + insulin	56	16

BMI: body mass index, BP: blood pressure, OHA: oral hypoglycemic agent. Data are shown as means ± S.E.M.

Table 1B. Details of Medication of OHA

categories of OHA	number of patients			
	without insulin treatment		with insulin treatment	
	Primary prevention group	Secondary prevention group	Primary prevention group	Secondary prevention group
SU	105	23	15	5
GLN	17	6	4	0
BG	12	1	11	5
α-GI	11	3	11	1
TZD	1	0	1	0
SU+α-GI	11	9	4	2
SU+BG	33	5	4	1
SU+TZD	2	1	0	0
GLN+BG	4	0	0	0
α-GI+TZD	1	0	0	0
BG+TZD	1	0	0	1
GLN+α-GI	0	0	1	1
α-GI+BG	0	0	3	0
GLN+TZD	0	1	0	0
SU+α-GI+BG	16	3	2	0
SU+α-GI+TZD	3	1	0	0
SU+BG+TZD	1	2	0	0
α-GI+BG+TZD	1	0	0	0
SU+α-GI+TZD+BG	3	0	0	0

In the present study, diabetes patients with no prior history of CAD comprised the primary prevention group, while those with a history of CAD comprised the secondary prevention group, the latter of which is more likely to develop new coronary events. According to the guidelines for the prevention of atherosclerosis-associated disease issued by the Japan Atherosclerosis Society in 2007, an LDL-C level less than 120 mg/dL is the goal for diabetes patients treated with primary prevention and an LDL-C level less than 100 mg/dL is the goal for patients treated with secondary prevention (9). In addition, recent randomized trials have demonstrated that intensive statin therapy lowers the LDL-C level more effectively than standard statin therapy (10, 11).

Diabetes patients often have hypertension as well as a high LDL-C level, which can be effectively treated with oral hypoglycemic, antihypertensive and lipid-lowering medications. For secondary prevention, antiplatelet agents and vasodilator agents are additionally added. In such cases, the medication to treat each disease is frequently prescribed separately by different physicians. Such complex polydisease and polypharmacy issues are a growing barrier to medication adherence and the attainment of treatment goals in the US (12).

In Japan, no investigations have addressed the reasons for the lack of attainment of LDL-C goals.

In the present study, we surveyed the attainment rates for LDL-C goals in diabetes patients receiving primary and secondary prevention. In addition, a physician's questionnaire regarding the patients' lack of achievement of the goals was distributed and analyzed. The most frequent response regarding the lack of achievement in secondary prevention was the "management of LDL-C by other departments or hospitals," which reflects the increasing polydisease and polypharmacy issues in current diabetes care.

Table 2. Questionnaires Regarding the Lack of Achievement of Optimum LDL-C Level

	frequency of emergence of answer (%)	
	primary prevention group	secondary prevention group
1) The patient's LDL-C levels are over or under the LDL-C goal at every hospital visit.	47	49
2) Continuously sufficient effect by diet therapy only.	34	11
3) The patient does not accept the prescription of statin despite having its importance as explained by the physician.	6	0
4) The patient takes prescribed drugs contraindicated for co-administration with statins.	3	6
5) The patient has been previously treated with statins and consequently shows moderately elevated serum CPK.	1	0
6) Severe adverse effects of statins on laboratory data besides elevation of serum CPK have been noted in the patient's past history.	1	0
7) Rhabdomyolysis concomitant with severe serum CPK elevation due to treatment with statins has been noted in the patient's past history.	1	4
8) The patient has experienced an adverse effect of statin as a subjective symptom.	4	4
9) Severe chronic renal failure has prohibited prescription of statins for the patient.	1	4
10) Severe muscle disease has prohibited prescription of statins for the patient.	1	0
11) The maximum dose of statin has been prescribed for the patient.	1	2
12) The physician has judged that incremental increases in the dosage of statin would not be helpful for the patient.	3	4
13) Other hospitals or departments have prescribed statins for the patient.	4	45
14) Various other, less common reasons.	13	15

Materials and Methods

Subjects

All patients regularly visited the outpatient clinic of Kyoto University Hospital and received nutritional instruction by dietitians. Because a total cholesterol (TC) level of 200 mg/dL corresponds to an LDL-C level of 120 mg/dL (13), patients with a TC level higher than 200 mg/dL or those previously treated with statins were enrolled in December 2007 (Table 1A, B). The LDL-C level was measured at least once between January and July 2008. Subjects with familial hypercholesterolemia or secondary hypercholesterolemia, such as those with nephrotic syndrome or hypothyroidism, were excluded. Data for the period January 1 to July 31 2008 were prioritized nearest to March 15, 2008.

Methods

The primary and secondary prevention groups were determined according to the patients' medical records. Standard statin therapy included pravastatin, simvastatin or fluvastatin, and intensive statin therapy included atorvastatin, pitavastatin or rosuvastatin. The maximum dose of each statin based on the medical package insert was as follows: pravastatin: 20 mg, simvastatin: 20 mg, fluvastatin: 60 mg, atorvastatin: 20 mg, pitavastatin: 4 mg and rosuvastatin: 10 mg. For several enrolled patients, the following lipid-lowering drugs were additionally prescribed: ethyl icosapentate, probucol, colestimide, fibrate and nicotinate. The LDL-C level was measured according to the selective solubilization method (Determiner L LDL-C test kit, Kyowa Medex Co., Ltd., Tokyo, Japan). The HbA1c level was measured using HPLC (HA-8180; Arcray, Kyoto, Japan). The HbA1c value (%) was estimated as the National Glycohemoglobin Standardization Program equivalent (%) according to the following formula: HbA1c (%) = HbA1c (JDS) (%) + 0.4%, considering the relational expression of HbA1c (JDS) (%) measured according to the previous Japanese standard substance and measurement methods and HbA1c (National Glycohemoglobin Standardization Program) (14).

Questionnaire analysis

The questionnaire consisted of 14 items, as shown in Table 2. The factors contributing to why the patients did not achieve their goals were recorded in the questionnaire by each patient's physician.

Statistical analysis

The data are presented as the mean ± SE. The Chi-square test or Fisher's exact probability test were used to the evaluate results. p values of <0.05 were considered to be statistically significant.

Results

Characteristics of the enrolled patients

Table 1A shows the demographic characteristics of the enrolled patients. The primary prevention group comprised 499 patients (men: 219 and women: 280) 65.8±0.5 years of age, with a body mass index (BMI) of 24.1±0.2 kg/m^2 and a systolic and diastolic blood pressure of 127.5±0.7 mmHg and 73.9±0.4 mmHg, respectively. The HbA1c, LDL-C, high-density lipoprotein cholesterol (HDL-C) and triglyceride levels were 7.7±0.1%, 112.7±1.0 mg/dL, 55.0±0.6 mg/dL and 154.0±5.0 mg/dL, respectively. Regarding treatment for hyperglycemia, 92, 222, 129 and 56 patients were treated with diet alone, OHAs (oral hypoglycemic agents), insulin and insulin plus OHAs, respectively.

Table 3A. Rate of Attainment of LDL-C Goal

		Primary prevention group				Secondary prevention group			
		Total number	Number attaining goal	Number not attaining goal	Rate of attainment (%)	Total number	Number attaining goal	Number not attaining goal	Rate of attainment (%)
Standard statin therapy									
	All dose	116	85	31	73	24	14	10	58
	Max. dose	3	3	0	100	1	0	1	0
	Non-max. dose	113	82	31	73	23	14	9	61
Intensive statin therapy									
	All dose	144	115	29	80	70	52	18	74
	Max. dose	10	9	1	90	10	4	6	40
	Non-max. dose	134	106	28	79	60	48	12	80
No statin		239	100	139	42	27	8	19	30
Total		499	300	199	61	121	74	47	61

Max. dose: Maximum dose, Non-max. dose: Non- maximum dose

Table 3B. Details of Additional Lipid-lowering Medication

additional lipid-lowering drug	number of patients					
	Primary prevention group			Secondary prevention group		
	Standard statin therapy	Intensive statin therapy	No statin	Standard statin therapy	Intensive statin therapy	No statin
ethyl icosapentate	4	0	3	0	2	0
probucol	0	1	0	0	1	0
colestimide	0	1	2	0	0	0
fibrate	0	2	13	1	0	3
nicotinate	8	3	4	0	2	1
ethyl icosapentate + nicotinate	0	1	2	0	0	0
colestimide + fibrate	1	0	0	0	0	0
Total	13	8	24	1	5	4

Table 3C. Rate of Attainment of LDL-C Goal by Additional Lipid-lowering Drug

additional lipid-lowering drug	Rate of attainment of LDL-C goal					
	Primary prevention group			Secondary prevention group		
	Standard statin therapy	Intensive statin therapy	No statin	Standard statin therapy	Intensive statin therapy	No statin
ethyl icosapentate	75%	-	100%	-	50%	-
probucol	-	100%	-	-	0%	-
colestimide	-	100%	50%	-	-	-
fibrate	-	100%	54%	100%	-	0%
nicotinate	75%	100%	50%	-	50%	0%
ethyl icosapentate + nicotinate	-	100%	100%	-	-	-
colestimide + fibrate	100%	-	-	-	-	-
Total	77%	100%	63%	100%	40%	0%

The secondary prevention group consisted of 121 (men: 77 and women: 44) patients 69.0±0.8 years of age, with a BMI of 24.5±0.3 kg/m^2 and a systolic and diastolic blood pressure of 128.0±1.4 mmHg and 72.2±1.0 mmHg, respectively. The HbA1c, LDL-C, HDL-C and triglyceride (TG) levels were 7.8±0.1%, 95.0±2.6 mg/dL, 51.9±1.2 mg/dL and 146.5±8.3 mg/dL, respectively. Regarding treatment for hyperglycemia, 19, 55, 31 and 16 patients were treated with diet alone, OHAs, insulin and insulin plus OHAs, respectively. Table 1B shows the number of patients treated with SUs (sulfonylureas), GLNs (rapid-acting insulin secretagogues), BGs (biguanides), α-GIs (α-glucosidase inhibitors), TZD (thiazolidinedione) and a combination of these drugs in the presence and absence of insulin treatment. The

frequency of SU+α-GI therapy in the secondary prevention group was significantly higher than that observed in the primary prevention group ($p<0.01$). The frequency of other OHAs in the secondary prevention group was indistinguishable from that observed in the primary prevention group.

Attainment rates for the LDL-C goals

Table 3A summarizes the rates of attainment for the LDL-C goals. In the primary prevention group, 300 subjects achieved the LDL-C goal, an achievement of 61%. The patients received standard statin therapy (n=116), intensive statin therapy (n=144) or no statin therapy (n=239). The attainment rates in the patients treated with standard statin therapy, intensive statin therapy and no statin therapy were 73%, 80% and 42%, respectively. We further analyzed the following four subgroups: (1) the maximum dose of standard statins, (2) the non-maximum dose of standard statins, (3) the maximum dose of intensive statins and (4) the non-maximum dose of intensive statins.

In the secondary prevention group, 74 patients achieved the LDL-C goal, an achievement rate of 61% (Table 3A). These patients also received standard statin therapy (n=10), intensive statin therapy (n=18) or no statin therapy (n=19) with attainment rates of 58%, 74% and 30%, respectively. We further analyzed the above-described four subgroups in the secondary prevention group.

Table 3B shows the number of patients treated with other lipid-lowering drugs besides statins (ethyl icosapentate, probucol, colestimide, fibrate and nicotinate). Of those receiving combination therapy with a statin and other lipid-lowering drugs, 0% received the maximum dose of the statin. Table 3C shows the rate of attainment of the LDL-C goal in the patients receiving additional lipid-lowering drugs. In the primary prevention group, the patients treated with combination therapy with a statin and other lipid-lowering drugs showed only a statistically insignificant higher goal attainment rate than those treated with single statin therapy (standard statins: 73% vs. 77%; intensive statins: 79% vs. 100%).

Effectiveness of statin therapy

Table 4A summarizes the details of the statin administration. In the primary prevention group, of the patients attaining the LDL-C goal, 28% received standard statin therapy, 39% received intensive statin therapy and 33% received no statin therapy. Of those not attaining the goal, 16% received standard statin therapy, 14% received intensive statin therapy and 70% received no statin therapy. In the secondary prevention group, of the patients attaining the LDL-C goal, 19% received standard statin therapy, 70% received intensive statin therapy and 11% received no statin therapy. Of those not attaining the goal, 22% received standard statin therapy, 38% received intensive statin therapy and 40% received no statin therapy.

Table 4B summarizes the details of the statin administration associated with additional lipid-lowering drugs in the patients attaining and not attaining the LDL-C goal.

Questionnaire analysis

The background characteristics of the patients who did not achieve their goal were analyzed using a questionnaire completed by each patient's physician. Table 2 shows the questionnaires for the primary and secondary prevention groups.

In the primary prevention group, the three most frequent reasons for the lack of goal attainment were: (1) an LDL-C level above or below the goal at every hospital visit (47%), (2) continuously sufficient effects with dietary therapy only (34%) and (3) low compliance (6%). In the secondary prevention group, the reasons for the lack of goal attainment were: (1) an LDL-C level above or below the goal at every hospital visit (49%), (2) management of the LDL-C level by other departments or hospitals (45%) and (3) continuously sufficient effects with dietary therapy only (11%).

Discussion

In the present study, 61% of the patients in both the primary and secondary prevention groups achieved the LDL-C goal. A study in a university hospital setting in 2001 reported rates of achievement of 56% and 33% in primary and secondary prevention groups, respectively, with a considerably lower rate in the secondary prevention group (15). In the present study, more intensive therapy was associated with an increased LDL-C goal attainment rate; the earlier study did not include intensive statin therapy. Recent randomized controlled trials have demonstrated that intensive statin therapy more effectively lowers the LDL-C level than standard statin therapy (10, 11). In our follow-up study conducted in 2010, the attainment rates for the LDL-C goal reached 71% and 67% in the primary and secondary prevention groups, respectively (unpublished data).

Unexpectedly, 38% of the secondary prevention patients not attaining the LDL-C goal were treated with intensive statin therapy, which indicates a limitation of this treatment. Similarly, 43% of the secondary prevention patients treated with another lipid-lowering drug who did not attain the LDL-C goal also received intensive statin therapy. In fact, in one recent study, cholesterol absorption was reported to be elevated in the secondary prevention group (16), suggesting statin resistance.

Therefore, a high LDL-C level accompanied by statin resistance may be better treated with a combination of statins and cholesterol transporter inhibitors. Very recently, such combination therapy was found to be more effective than statin monotherapy in secondary prevention patients (17). Ezetimibe use was not considered in this study; therefore, further investigation is required.

This is the first study to analyze the background of diabetes patients in Japan who do not attain their LDL-C goal. This is also the first study to use a physician questionnaire to analyze the lack of achievement of LDL-C goals in indi-

Table 4A. Statin Administration in Patients Attaining and not Attaining LDL-C Goal

	Primary prevention group							Secondary prevention group						
	Standard statin therapy			Intensive statin therapy			No statin	Standard statin therapy			Intensive statin therapy			No statin
	All dose	Max. dose	Non-max. dose	All dose	Max. dose	Non-max. dose		All dose	Max. dose	Non-max. dose	All dose	Max. dose	Non-max. dose	
Patients attaining LDL-C goal	28%	1%	27%	39%	3%	36%	33%	19%	0%	19%	70%	5%	65%	11%
Patients not attaining LDL-C goal	16%	0%	16%	14%	0%	14%	70%	22%	2%	20%	38%	13%	25%	40%

Max. dose: Maximum dose, Non-max. dose: Non- maximum dose

Table 4B. Statin Administration with Additional Lipid-lowering Drug in Patients Attaining and not Attaining LDL-C Goal

	Primary prevention group			Secondary prevention group		
	Standard statin therapy with additional lipid-lowering drug	Intensive statin therapy with additional lipid-lowering drug	No statin with additional lipid-lowering drug	Standard statin therapy with additional lipid-lowering drug	Intensive statin therapy with additional lipid-lowering drug	No statin with additional lipid-lowering drug
Patients attaining LDL-C goal	30%	24%	46%	33%	67%	0%
Patients not attaining LDL-C goal	25%	0%	75%	0%	43%	57%

vidual patients. Recently, a physician questionnaire regarding barriers to lipid goal attainment in patients with type 2 diabetes was used in a web-based international survey (18). That study found patient compliance to be the most common impediment, followed by financial restrictions to access to the product, lack of efficacy of available drugs and drug intolerability. Another group reported that the limited amount of time available during clinic visits is a barrier to the ideal management of the LDL-C level in diabetic patients (19). In the present study, statin intolerance and adverse effects were observed in several patients, consistent with previous results. Our questionnaire regarding the lack of achievement of individual patients reflects the details of medical treatment not evaluated using other methods.

In the present study, we found the most frequent reason for the lack of goal attainment in both groups to be a "LDL-C level above or below the goal at every hospital visit." The data for the period January 1 to July 31 2008 were prioritized nearest to March 15, 2008. In several patients, the LDL-C level nearest to March 15 happened to be above the goal, while the levels observed in other months were below the goal. Such occasional fluctuation underlies the "lack of goal attainment of the LDL-C level." This suggests that a certain number of patients may have attained an LDL-C level on the borderline of the goal, indicating that more potent drug therapy may be required.

In the secondary prevention group, the "management of the LDL-C level by other departments or hospitals" was reported with a high frequency. In several patients, particularly those in the secondary prevention group, other departments or hospitals often managed the dyslipidemia therapy and prescribed statins; however, the goal was not attained. In general, the secondary prevention group exhibited several chronic diabetic complications, such as coronary artery disease, retinopathy, nephropathy and neuropathy. In such cases, the medications for each complication may be separately prescribed. Therefore, polydisease might well result in poorer medication adherence. Although the health care system in Japan differs considerably from that observed in the US and elsewhere, polydisease and polypharmacy issues in diabetes care may well become a growing barrier to medication adherence and the attainment of treatment goals. Because patients treated with secondary prevention are more likely to develop new coronary events than those treated with primary prevention, controlling the LDL-C level is especially important in secondary prevention patients. To improve medication adherence, providing team medical care, including the participation of pharmacists and physicians in other fields, is essential.

Several limitations of this study should be considered.

First, the TC level rather than the LDL-C level was used for screening because direct measurement of the LDL-C level was not performed in several patients. Calculating the LDL-C level using Friedewald's equation with the TC, HDL-C and fasting TG levels is another method of determining the LDL-C level. For a considerable number of patients, however, the appointment time in the outpatient department was in the afternoon, when it is difficult to measure the fasting TG level, in which case, Friedewald's equation cannot be used. Strictly speaking, patients administered no statin therapy who attain the LDL-C goal but have a TC level over 200 mg/dL are expected to have a normal LDL-C level. Very few patients with a TC level under 200 mg/dL are expected to have an LDL-C level over 120 mg/dL and not be included. Second, this study was performed in a single university hospital. Further studies with more representative samples are required. In addition, to examine the efficacy of statins, the baseline level of LDL-C should be considered, which was not performed in this study. Due to this limitation of our database, we are unable to show the reduction rate of the LDL-C level in order to evaluate the power of statins in detail. Furthermore, medication adherence was not examined. Further investigations are therefore required.

In conclusion, we surveyed the goal attainment rates for the LDL-C levels in type 2 diabetes mellitus patients. Our analysis of the lack of goal attainment in each prevention group provides useful suggestions for improving LDL-C management in patients with type 2 diabetes.

Author's disclosure of potential Conflicts of Interest (COI).
Nobuya Inagaki: Honoraria, MSD and Novartis Pharma; Research funding, MSD; Subsidies or Donations, MSD, Astellas Pharma, Daiichi-Sankyo and Novartis Pharma.

Acknowledgement

The authors thank the Department of Medical Information Technology, Kyoto University Hospital for downloading the medical data. This study was supported by Scientific Research Grants and a Grant for Leading Project for Biosimulation from the Ministry of Education, Culture, Sports, Science and Technology of Japan, a grant from CREST of the Japan Science and Technology Cooperation and a grant from the Ministry of Health, Labour and Welfare, Japan and the Kyoto University Global COE Program "Center for Frontier Medicine."

References

1. Scandinavian Simvastatin Survival Study Group. Randomised trial of cholesterol lowering in 4 444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). Lancet **344**: 1383-1389, 1994.
2. Sacks FM, Pfeffer MA, Moye LA, et al. The effect of pravastatin on coronary events after myocardial infarction in patients with average cholesterol levels. N Engl J Med **335**: 1001-1009, 1996.
3. The Long-Term Intervention with Pravastatin in Ischaemic Disease (LIPID) Study Group. Prevention of cardiovascular events and death with pravastatin in patients with coronary heart disease and a broad range of initial cholesterol levels. N Engl J Med **339**: 1349-1357, 1998.
4. Heart Protection Study Collaborative Group. MRC/BHF Heart Protection Study of cholesterol lowering with simvastatin in 20 536 high-risk individuals: a randomised placebo-controlled trial. Lancet **360**: 7-22, 2002.
5. Shepherd J, Cobbe SM, Ford I, et al. Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. N Engl J Med **333**: 1301-1307, 1995.
6. Downs JR, Clearfield M, Weis S, et al. Primary prevention of acute coronary events with lovastatin in men and women with average cholesterol levels: results of AFCAPS/TexCAPS: Air Force/Texas Coronary Atherosclerosis Prevention Study. JAMA **279**: 1615-1622, 1998.
7. Shepherd J, Blauw GJ, Murphy MB, et al; PROSPER study group. Pravastatin in elderly individuals at risk of vascular disease (PROSPER): a randomised controlled trial. Lancet **360**: 1623-1630, 2002.
8. Gaede P, Vedel P, Larsen N, Jensen GV, Parving HH, Pedersen O. Multifactorial intervention and cardiovascular disease in patients with type 2 diabetes. N Engl J Med **348**: 383-393, 2003.
9. Japan Atherosclerosis Society Guidelines for Diagnosis and Treatment of Atherosclerotic Cardiovascular Diseases. 2008: 29.
10. Cannon CP, Braunwald E, McCabe CH, et al; Pravastatin or Atorvastatin Evaluation and Infection Therapy-Thrombolysis in Myocardial Infarction 22 Investigators. Intensive versus moderate lipid lowering with statins after acute coronary syndromes. N Engl J Med **350**: 1495-1504, 2004.
11. Nissen SE, Tuzcu EM, Schoenhagen P, et al; REVERSAL Investigators. Effect of intensive compared with moderate lipid-lowering therapy on progression of coronary atherosclerosis: a randomized controlled trial. JAMA **291**: 1071-1080, 2004.
12. Parris ES, Lawrence DB, Mohn LA, Long LB. Adherence to statin therapy and LDL cholesterol goal attainment by patients with diabetes and dyslipidemia. Diabetes Care **28**: 595-599, 2005.
13. Japan Atherosclerosis Society Guidelines for Diagnosis and Treatment of Atherosclerotic Cardiovascular Diseases. 2002: 9.
14. The committee of Japan Diabetes Society on the diagnostic criteria of diabetes mellitus. Report of the Committee on the classification and diagnostic criteria of diabetes mellitus. J Diabetes Invest **1**: 212-228, 2010.
15. Tsurumaru M, Yamaguchi Y, Yamakawa K, et al. The control of cholesterol, HbA1c, blood pressure in diabetic patients accompanied with hypercholesterolemia. Journal of the Japan Diabetes Society **45**: 773-778, 2002 (in Japanese).
16. Matthan NR, Pencina M, LaRocque JM, et al. Alterations in cholesterol absorption/synthesis markers characterize Framingham offspring study participants with CHD. J Lipid Res **50**: 1927-1935, 2009.
17. Uemura Y, Watarai M, Ishii H, et al. Atorvastatin 10 mg plus ezetimibe 10 mg compared with atorvastatin 20 mg: impact on the lipid profile in Japanese patients with abnormal glucose tolerance and coronary artery disease. J Cardiol **59**: 50-56, 2012.
18. Leiter LA, Betteridge DJ, Chacra AR, et al. AUDIT study. Evidence of global undertreatment of dyslipidaemia in patients with type 2 diabetes mellitus. Achieving Best Practice **6**: 31-40, 2006.
19. Grant RW, Pirraglia PA, Meigs JB, Singer DE. Trends in complexity of diabetes care in the United States from 1991 to 2000. Arch Intern Med **164**: 1134-1139, 2004.

JDI Journal of Diabetes Investigation Open access
Official Journal of the Asian Association for the Study of Diabetes
ORIGINAL ARTICLE

Palmitate induces reactive oxygen species production and β-cell dysfunction by activating nicotinamide adenine dinucleotide phosphate oxidase through Src signaling

Yuichi Sato[1], Shimpei Fujimoto[1,2], Eri Mukai[1], Hiroki Sato[1], Yumiko Tahara[1], Kasane Ogura[1], Gen Yamano[1], Masahito Ogura[1], Kazuaki Nagashima[1], Nobuya Inagaki[1]*

[1]Department of Diabetes and Clinical Nutrition, Graduate School of Medicine, Kyoto University, Kyoto, and [2]Department of Endocrinology, Metabolism and Nephrology, Kochi Medical School, Kochi University, Nankoku, Japan

Keywords
Lipotoxicity, Pancreatic β-cells, Reactive oxygen species

***Correspondence**
Nobuya Inagaki Tel.: +81-75-751-3560
Fax: +81-75-751-4244
E-mail address: inagaki@metab.kuhp.kyoto-u.ac.jp

J Diabetes Invest 2014; 5: 19–26

doi: 10.1111/jdi.12124

ABSTRACT

Aims/Introduction: Chronic hyperlipidemia impairs pancreatic β-cell function, referred to as lipotoxicity. We have reported an important role of endogenous reactive oxygen species (ROS) overproduction by activation of Src, a non-receptor tyrosine kinase, in impaired glucose-induced insulin secretion (GIIS) from diabetic rat islets. In the present study, we investigated the role of ROS production by Src signaling in palmitate-induced dysfunction of β-cells.

Materials and Methods: After rat insulinoma INS-1D cells were exposed to 0.6 mmol/L palmitate for 24 h (palmitate exposure); GIIS, ROS production and nicotinamide adenine dinucleotide phosphate oxidase (NOX) activity were examined with or without exposure to10 μmol/L 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2), a Src inhibitior, for 30 or 60 min.

Results: Exposure to PP2 recovered impaired GIIS and decreased ROS overproduction as a result of palmitate exposure. Palmitate exposure increased activity of NOX and protein levels of NOX2, a pathological ROS source in β-cells. Palmitate exposure increased the protein level of $p47^{phox}$, a regulatory protein of NOX2, in membrane fraction compared with control, which was reduced by PP2. Transfection of small interfering ribonucleic acid of $p47^{phox}$ suppressed the augmented $p47^{phox}$ protein level in membrane fraction, decreased augmented ROS production and increased impaired GIIS by palmitate exposure. In addition, exposure to PP2 ameliorated impaired GIIS and decreased ROS production in isolated islets of KK-A^y mice, an obese diabetic model with hyperlipidemia.

Conclusions: Activation of NOX through Src signaling plays an important role in ROS overproduction and impaired GIIS caused by chronic exposure to palmitate, suggesting a lipotoxic mechanism of β-cell dysfunction of obese mice.

INTRODUCTION

In pancreatic β-cells, glucose metabolism regulates exocytosis of insulin granules through metabolism-secretion coupling[1]. Reactive oxygen species (ROS) is one of the most important factors that impair glucose-induced insulin secretion (GIIS) in β-cells.

Exposure to exogenous hydrogen peroxide (H_2O_2), the most abundant ROS, reduces glucose-induced insulin secretion by impairing mitochondrial metabolism in β-cells[2,3]. ROS are normal byproducts of glucose metabolism, including glycolysis and mitochondrial oxidative phosphorylation[4]. In β-cells, ROS

Received 1 March 2013; revised 20 May 2013; accepted 28 May 2013

production through non-mitochondrial and mitochondrial pathways has been proposed. In the mitochondrial pathway, ROS is generated in the electron transport chain associated with the mitochondrial membrane potential[5]. However, in pathophysiological conditions, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX), an important non-mitochondrial ROS source, could play an important role in ROS generation in β-cells[6].

Deleterious effects of chronic hyperlipidemia on β-cell function are referred to as lipotoxicity[7]. Chronic exposure to palmitate, a long-chain saturated fatty acid, impairs GIIS with an increase in production of ROS in β-cells[8,9]. Recently, an important role of NOX in ROS production in β-cells by palmitate exposure has been proposed[10]. In addition, both oxidative stress markers and NOX expression are increased in islets of obese diabetic rodents with hyperlipidemia[11,12]. We have proposed that endogenous overproduction of ROS involving activation of Src, a non-receptor tyrosine kinase, plays an important role in impaired metabolism-secretion coupling in islets of diabetic Goto–Kakizaki (GK) rats[13–15]. An important role of Src in activation of NOX has been reported in various cells[16,17]. In the present study, to elucidate the mechanism of lipotoxicity in β-cells more precisely, we investigated involvement of Src in ROS production derived from NOX and impaired GIIS caused by chronic exposure to palmitate.

MATERIALS AND METHODS

Materials

Palmitate obtained from Nacalai (Kyoto, Japan) was dissolved in 95% ethanol at stock concentration of 100 mmol/L. The specific Src inhibitor, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2), was purchased from Tocris (Ellisville, MO, USA).

Cell Culture and Mouse Islet Isolation

Rat insulinoma cell line INS-1D cells were cultured as previously described[18] with or without palmitate in the presence of 0.5% bovine serum albumin (BSA) for 24 h.

Male KK-A^y mice and control C57/BL6 mice (Clea Japan, Tokyo, Japan) were maintained and used in accordance with the guidelines of the animal care committee of Kyoto University. All experiments were carried out with mice aged 8–10 weeks. Pancreatic islets were isolated as previously described[19].

Insulin Secretion

Insulin secretion from INS-1D cells was determined as previously described[18]. INS-1D cells cultured on 24-well plates coated with 0.001% poly-L-ornithine were washed with Krebs–Ringer bicarbonate HEPES (KRBH) buffer composed of (in mmol/L) 140 NaCl, 3.6 KCl, 0.5 $MgSO_4$, 0.5 NaH_2PO_4, 1.5 $CaCl_2$, 2 $NaHCO_3$ and 10 HEPES (pH 7.4) with 0.1% BSA and 2 mmol/L glucose, pre-incubated at 37°C for 30 min in KRBH with 2 mmol/L glucose, and then incubated at 37°C for 30 min in KRBH with 2 mmol/L glucose and 10 mmol/L glucose. Insulin release from intact islets was measured using batch incubation using KRBH supplemented with 0.2% BSA as previously described[19].

ROS Measurements

ROS was measured according to the method previously described[20]. INS-1D cells and isolated islets were incubated in KRBH medium containing 2 mmol/L glucose and 10 μmol/L 5-(and 6-) chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA; Invitrogen, Carisbad, CA, USA) for 60 min at 37°C, and then were rapidly frozen, stored at −80°C and thawed. Fluorescence of the supernatant was quantified using a reader (Powerscan HT; DS Pharma Biomedical, Suita, Japan) with excitation wavelength at 485 nm and emission at 530 nm, which was corrected by subtracting parallel blanks.

Isolation of Total Ribonucleic Acid and Quantitative Reverse Transcription Polymerase Chain Reaction

Total ribonucleic acid (RNA) was isolated from INS-1D cells using RNeasy mini kit (Qiagen, Hilden, Germany). cDNA was prepared by reverse transcriptase (Superscript II; Invitrogen) with an oligo (dT) primer. The rat sequences of forward and reverse primers to *NOX2/gp91^{phox}* and *β-actin* (as an inner control) were as follows: (*NOX2/gp91^{phox}*: 5′-TGA CTC GGT TGG CTG GCA TC-3′, 5′-CGC AAA GGT ACA GGA ACA TGG G -3′, *β-actin*: 5′- CAA TGA GCG GTT CCG ATG CC -3′, 5′- AAT GCC TGG GTA CAT GGT GG -3′). AmpliTaq Gold (Applied Biosystems, Foster, CA, USA) was used as a DNA polymerase for reverse transcription polymerase chain reaction (RT–PCR). SYBR Green PCR Master Mix (Applied Biosystems) was prepared for quantitative RT–PCR run. The thermal cycling conditions were denaturation at 95°C for 10 min followed by 40 cycles at 95°C for 30 s and 60°C for 30 s.

Immunoblot Analysis

For immunoblotting, cells were washed with phosphate-buffered saline (PBS) containing protease inhibitor cocktail (Complete; Roche, Mannheim, Germany) and phosphatase inhibitor cocktail (Calbiochem, Darmstadt, Germany), suspended in 1 mL of PBS containing protease inhibitor and phosphatase inhibitor, and homogenized as previously described[18]. Membrane fraction was prepared as described previously[21]. INS-1D cells were washed three times with PBS, suspended in buffer A consisting of 50 mmol/L Tris (pH 7.5), 1 mmol/L ethylenediaminetetraacetic acid, and containing protease inhibitor cocktail, phosphatase inhibitor cocktail and 5 mol/L sodium pyrophosphate, homogenized and then centrifuged at 10,000 *g* at 4°C for 1 h. The pellets were resuspended in 500 μL of buffer A and stored at −80°C until immunoblot analysis or NOX activity assay. Protein (20 μg per sample) was separated on a 15% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. After blocking with Tris-buffered saline

(10 mmol/L Tris/HCl and 100 mmol/L NaCl, pH 7.5) containing 0.1% Tween 20 and 5% BSA (blocking buffer) at room temperature (25°C) for 2 h, blotted membranes were incubated overnight at 4°C with anti-p418 Src antibody (Biosource, Camarillo, CA, USA) at 1:1000 dilution, anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (Millipore, Billerica, MA, USA) at 1:1000 dilution, anti-NCF1 ($p47^{phox}$) antibody (Abcam, Cambridge, UK) at 1:1000 dilution, anti-NOX2/$gp91^{phox}$ antibody (Abcam) at 1:1000 dilution, anti-flotillin-1 antibody (Cell Signaling Technology, Danvers, MA, USA) at 1:1000 dilution in blocking buffer and subsequently with anti-rabbit or anti-mouse immunoglobulin G horseradish peroxidase-conjugated secondary antibody (GE Healthcare, Little Chalfont, UK) diluted at 1:5000 at room temperature for 2 h before detection using ECL prime™ (GE Healthcare). Band intensities were quantified with Multi Gauge software (Fujifilm, Tokyo, Japan).

NOX Activity Assay

NOX activity was measured by a luminescence assay in a cuvette containing 50 mmol/L phosphate buffer (pH 7.0), 1 mmol/L ethylene glycol tetraacetic acid, 150 mmol/L sucrose, 500 μmol/L lucigenin as the electron acceptor and 100 μmol/L NADPH as the substrate (total volume 900 μL) as previously described[22]. No activity was measured in the absence of NADPH. In some experiments, PP2 (final concentration 10 μmol/L) was added to the cuvette 10 min before readings. The reaction was started by the addition of 100 μL of membrane fraction (50–300 μg protein). Photon emission was measured every 15 s for 15 min in a luminometer (GloMax 20/20n

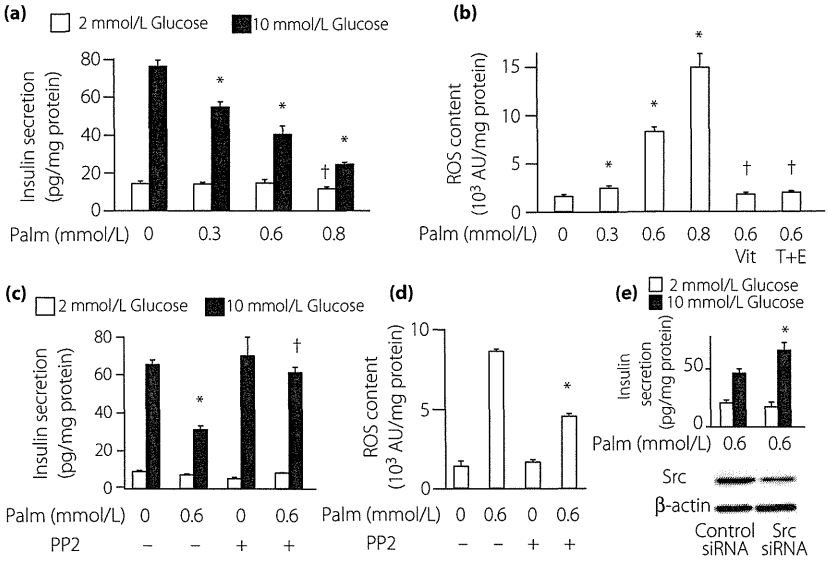

Figure 1 | Effect of palmitate exposure on glucose-induced insulin secretion (GIIS) and reactive oxygen species (ROS) production in INS-1D cells. Values are mean ± standard error of the mean (n = 4 in each bar). After INS-1D cells were cultured with or without various concentrations of palmitate (Palm) for 24 h, GIIS and ROS production were measured. (a) Effect of palmitate exposure on GIIS. GIIS was examined in the presence of 2 mmol/L (white bar) and 10 mmol/L glucose (black bar) for 30 min (n = 4 in each bar). *P < 0.01 vs 10 mmol/L glucose, culture without palmitate; †P < 0.01 vs 2 mmol/L glucose, culture without palmitate. (b) Effect of palmitate exposure on ROS production. After INS-1D cells were incubated in medium containing 2 mmol/L glucose and 10 μmol/L CM-H_2DCFDA for 60 min, ROS production was measured. ROS production was also measured using INS-1D cells cultured with 0.6 mmol/L palmitate plus ROS scavengers (0.1 mmol/L vitamin E + 0.2 mmol/L vitamin C [Vit]) or anti-oxidant mimics (10 mmol/L tempol [superoxide dismutase mimic] + 10 μmol/L ebselen [glutathione peroxidase mimic] [T + E]) for 24 h. Culturing with these agents suppressed enhanced ROS production by exposure to 0.6 mmol/L palmitate (n = 5 in each bar). *P < 0.01 vs culture without palmitate; †P < 0.01 vs culture with 0.6 mmol/L palmitate. (c) Effect of Src inhibitor on impaired GIIS by 0.6 mmol/L palmitate exposure. GIIS was measured in the presence of 2 mmol/L (white bar) and 10 mmol/L glucose (black bar) with or without 10 μmol/L 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) for 30 min (n = 4 in each bar). *P < 0.01 vs 10 mmol/L glucose without PP2, culture without palmitate; †P < 0.01 vs 10 mmol/L glucose without PP2, culture with 0.6 mmol/L palmitate. (d) Effect of Src inhibitor on augmented ROS production by 0.6 mmol/L palmitate exposure. After INS-1D cells were incubated in medium containing 2 mmol/L glucose and 10 μmol/L CM-H_2DCFDA with or without 10 μmol/L PP2 for 60 min, ROS production was measured (n = 5 in each bar). *P < 0.01 vs without PP2, culture with 0.6 mmol/L palmitate. (e) Effect of *Src* small interfering ribonucleic acid (siRNA) on protein expression and GIIS in INS-1D cells cultured with palmitate. After INS-1D cells transfected with control and *Src* siRNA were cultured with 0.6 mmol/L palmitate for 24 h, protein levels and GIIS were measured. Representative immunoblots were presented.

Luminometer; Promega, Fitchburg, WI, USA), which was corrected by a subtracting blank.

Small Interfering RNA Transfection

Stealth™ small interfering (si)RNAs were synthesized by Invitrogen. The sequences of siRNAs specific for rat *NCF1* (*p47^{phox}*) were as follows: 5′-GGU GAA GCC AUC GAG GUC AUU CAU A-3′, 5′-UAU GAA UGA CCU CGA UGG CUU CAC C-3′. The sequences of siRNAs specific for rat Src were as follows: 5′-GGG AGC GGC UGC AGA UUG UCA AUA A-3′, 5′-UUA UUG ACA AUC UGC AGC CGC UCC C-3′. The sequences of control siRNAs were as follows: 5′-ACC AAC AAC AGU UUG GGA AUA GGG A-3′, 5′-U CCC UAU UCC CAA ACU GUU GUU GGU -3′. Cultured INS-1D cells were trypsinized, suspended with RPMI 1640 medium without antibiotics, mixed with Opti-MEM (Invitrogen) containing siRNA and Lipofectamine 2000 (Invitrogen), plated on dishes or wells and then incubated at 37°C. The final contents of INS-1D cell, RPMI 1640, Opti-MEM, siRNA and Lipofectamine 2000 were 1 × 106 cells/mL, 75% v/v, 25% v/v, 80 nmol/L and 0.3% v/v, respectively. The medium was changed to RPMI 1640 3–4 h after transfection. All experiments using siRNA-transfected INS-1D cells were carried out 72 h after transfection.

Statistical Analysis

The data are expressed as mean ± standard error of the mean. Statistical significance was calculated by the unpaired Student's *t*-test. $P < 0.05$ was considered significant.

RESULTS

Src Inhibition Recovers Impaired Glucose-Induced Insulin Secretion and Decreases Augmented ROS Production as a Result of Exposure to Palmitate

Exposure to palmitate (C16:0) concentration dependently decreased GIIS and increased ROS production (Figure 1a,b). Oleic acid (C18:1) slightly increased ROS production, but arachidonic acid (C20:4 n-6) did not increase ROS production

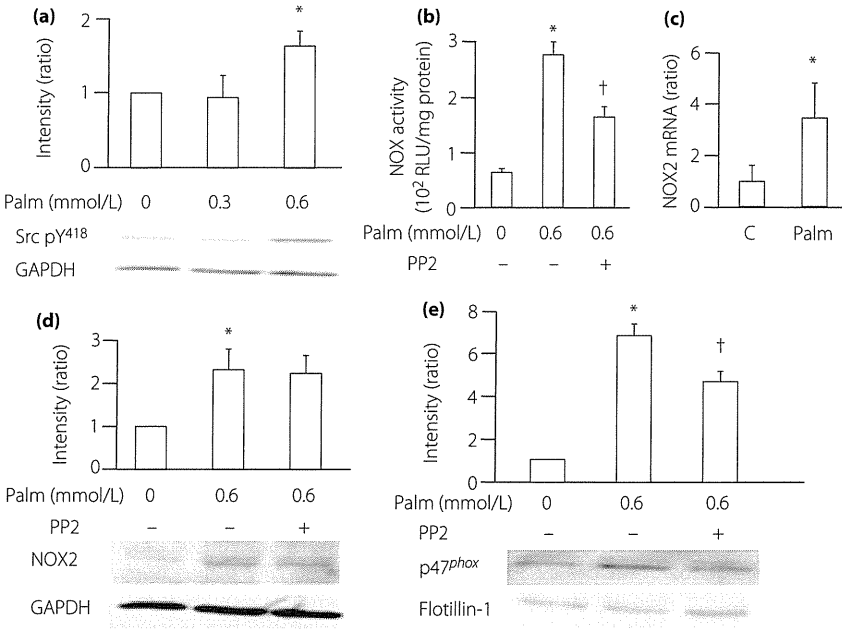

Figure 2 | Effect of palmitate exposure on Src and nicotinamide adenine dinucleotide phosphate oxidase (NOX). Values are mean ± standard error of the mean. After INS-1D cells were cultured with or without palmitate (Palm) for 24 h, and incubated with Krebs–Ringer bicarbonate HEPES with or without 10 μmol/L 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) for 30 min, messenger ribonucleic acid (mRNA), protein levels and NOX activity were measured. Immunoblot was carried out using (a,d) whole cell and (e) membrane fraction. (a,d,e) Representative immunoblots are presented. *$P < 0.01$ vs cultured without palmitate; †$P < 0.01$ vs without PP2, culture with 0.6 mmol/L palmitate. (a) Effect of palmitate exposure on Src activation. Src activation was detected by Tyr^{418}-phosphorylated Src. Data are expressed relative to control values without palmitate corrected by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) level ($n = 5$ in each bar). (b) Effect of palmitate exposure on NOX activity. NOX activity was measured using membrane fraction ($n = 5$ in each bar). (c) Effect of palmiate exposure on expression of *NOX2/gp91^{phox}* mRNA. Data were normalized by the expression of *β-actin* ($n = 4$ in each bar). C, control without palmitate; Palm, 0.6 mmol/L palmitate. (d) Effect of palmiate exposure on expression of NOX2/gp91^{phox} protein. Data are expressed relative to control values without palmitate corrected by GAPDH level ($n = 5$ in each bar). (e) Effect of palmiate exposure on the level of p47^{phox} protein. Data are expressed relative to control values without palmitate corrected by flotillin-1 level ($n = 4$ in each bar).