Effects of Liraglutide on β-Cell-Specific Glucokinase-Deficient Neonatal Mice

Jun Shirakawa, Ritsuko Tanami, Yu Togashi, Kazuki Tajima, Kazuki Orime, Naoto Kubota, Takashi Kadowaki, Yoshio Goshima, and Yasuo Terauchi

Departments of Endocrinology and Metabolism (J.S., R.T., Y.To., K.T., K.O., Y.Te.) and Molecular Pharmacology and Neurobiology (J.S., Y.G.), Graduate School of Medicine, Yokohama City University, Yokohama 236-0004, Japan; and Department of Diabetes and Metabolic Diseases (N.K., T.K.), Graduate School of Medicine, University of Tokyo, Tokyo 113-0033, Japan

The glucagon-like peptide-1 receptor agonist liraglutide is used to treat diabetes. A hallmark of liraglutide is the glucose-dependent facilitation of insulin secretion from pancreatic β-cells. In β-cells, the glycolytic enzyme glucokinase plays a pivotal role as a glucose sensor. However, the role of glucokinase in the glucose-dependent action of liraglutide remains unknown. We first examined the effects of liraglutide on glucokinase haploinsufficient (Gck/H9252-/-) mice. Single administration of liraglutide significantly improved glucose tolerance in Gck/H9252-/- mice without increase of insulin secretion. We also assessed the effects of liraglutide on the survival rates, metabolic parameters, and histology of liver or pancreas of β-cell-specific glucokinase-deficient (Gck/H9252-/-/-) newborn mice. Liraglutide reduced the blood glucose levels in Gck/H9252-/-/- neonates but failed to prolong survival, and all the mice died within 1 wk. Furthermore, liraglutide did not improve glucose-induced insulin secretion in isolated islets from Gck/H9252-/-/- neonates. Liraglutide initially prevented increases in alanine aminotransferase, free fatty acids, and triglycerides in Gck/H9252-/-/- neonates but not at 4 d after birth. Liraglutide transiently prevented liver steatosis, with reduced triglyceride contents and elevated glycogen contents in Gck/H9252-/-/- neonate livers at 2 d after birth. Liraglutide also protected against reductions in β-cells in Gck/H9252-/-/- neonates at 4 d after birth. Taken together, β-cell glucokinase appears to be essential for liraglutide-mediated insulin secretion, but liraglutide may improve glycemic control, steatosis, and β-cell death in a glucokinase-independent fashion. (Endocrinology 153: 3066–3075, 2012)
Glucokinase, a member of the hexokinase family, is the main glucose sensor in β-cells (19). Glucose-stimulated insulin secretion (GSIS) from pancreatic β-cells depends critically on glucokinase activity, and inactivating mutations cause either maturity onset diabetes of the young 2 or permanent neonatal diabetes (20). Whether glucokinase is involved in the potentiation of insulin secretion by GLP1R agonists is uncertain. The expression of glucokinase in β-cells is critical for the maintenance of glycemic control (21). We previously reported the characteristics of β-cell-specific glucokinase-deficient (Gck−/−) mice, in which the neuron and β-cell-specific exon was disrupted (22). Mice that were heterozygous for β-cell glucokinase (Gck+/−) exhibited impaired insulin secretion and manifested mild hyperglycemia, whereas mice that were homozygous for β-cell glucokinase (Gck−/−) exhibited severe hyperglycemia and died within 1 wk after birth as a result of defective insulin secretion in response to glucose (22).

In this study, we used Gck−/− neonatal mice to evaluate the role of β-cell glucokinase in the actions of liraglutide and the effects on the survival rate, metabolic parameters, and histology of the endocrine pancreas and liver.

Materials and Methods

Animals and animal care

We backcrossed Gck−/− mice with C57BL/6J mice more than 10 times. Both the adult wild-type (WT) and Gck−/− mice were fed standard chow (MF; Oriental Yeast, Tokyo, Japan). To generate the Gck−/− mice, we crossed Gck−/− mice with each other. The neonatal WT, Gck+/−, and Gck−/− mice were weaned normally, with most of the pups being preweaning period at 15 d after birth. All the experiments were conducted on male littersmates. All the animal procedures were performed in accordance with the institutional animal care guidelines and the guidelines of the Animal Care Committee of the Yokohama-City University. The animal housing rooms were maintained at a constant room temperature (25 C) and on a 12-h light (0700 h)/12-h dark (1900 h) cycle.

Drugs

Liraglutide, a long-acting human insulin analog (insulin detemir), and intermediate-acting human insulin [neutral protamine Hagedorn (NPH)] in premanufactured sc injection pens were obtained from Novo Nordisk (Bagsvaerd, Denmark) and stored at 4 C until use. Saline solution was used as the vehicle control. For single administration experiments, the animals received either the vehicle or liraglutide (0.3 mg/kg ip) before oral glucose loading (1.5 mg/g) or insulin injection (0.75 mU/g). The ganglionic blocker chlorisondamine (CS) chloride (Sigma, St. Louis, MO) (3 mg/kg) and methyl atropine (Atr) (0.5 mg/kg; Sigma), a peripherally acting muscarinic blocker, were administered ip before the liraglutide injections. For the chronic administration of liraglutide in neonatal mice, WT, Gck+/−, and Gck−/− pups received either the vehicle or liraglutide as twice daily sc injections (0900 and 2100 h) for 15 d after birth in the following manner: 0.1 mg/kg on
the first day (d 0), 0.2 mg/kg on the second day (d 1), and 0.3 mg/kg as the maintenance dose from the third day until the end of the experiment (d 2–15). For the double-dose administration of liraglutide in neonatal mice, the pups were treated with liraglutide using twice daily sc injections (0900 and 2100 h) for 15 d after birth in the following manner: 0.2 mg/kg on the first day (d 0), 0.2 mg/kg on the second day (d 1), and 0.3 mg/kg as the maintenance dose from the third day until the end of the experiment (P2–P15). For insulin detemir or NPH injection, the pups were treated with insulin detemir or NPH using twice daily sc injections (0900 and 2100 h) for 15 d after birth in the following manner: 0.2 mg/kg on the first day (P0), 0.4 mg/kg on the second day (P1), and 0.6 mg/kg as the maintenance dose from the third day until the end of the experiment (P2–P15). For insulin detemir or NPH injection, the pups were treated with insulin detemir or NPH using twice daily sc injections (0900 and 2100 h) for 15 d after birth in the following manner: 0.25 U/kg on the first day (d 0), 0.5 U/kg on the second day (d 1), and 0.75 U/kg as the maintenance dose from the third day until the end of the experiment (d 2–15).

Measurement of biochemical parameters and cytokines

The plasma glucose levels, blood insulin levels, triglyceride (TG) content in the liver, and glycogen content in the liver were determined using a Gluestest Neo Super (Sanwa Chemical Co., Tokyo, Japan), insulin kit (Morinaga, Tokyo, Japan), Determiner-L TG II kit, and Determiner-GL-2 kit (Wako Pure Chemical Industries, Osaka, Japan), respectively. The plasma alanine aminotransferase (ALT), free fatty acid (FFA), total cholesterol (TCho), low-density lipoprotein cholesterol, and TG levels were assayed using enzymatic methods (Wako Pure Chemical Industries).

Statistical analyses

All the data were reported as the mean ± SE and were analyzed using the Student’s t test or ANOVA. Differences were considered significant if the P value was less than 0.05 (*).

Results

Single administration of liraglutide significantly improved glucose tolerance in both WT and Gck+/− mice

We previously demonstrated that Gck+/− islets exhibited the potentiation of GSIS via exendin-4, another GLP-1R agonist, at high glucose concentrations (23). To confirm the effects of liraglutide on Gck+/− mice, liraglutide (0.3 mg/kg) was injected ip 30 min before an oral glucose tolerance test (GTT) or an insulin tolerance test (ITT). Liraglutide significantly improved glucose tolerance in both WT mice (Fig. 1A). The GSIS at 15 min after glucose loading was increased by liraglutide in WT mice but not in Gck+/− mice (Fig. 1B). Liraglutide did not affect insulin sensitivity after an insulin injection in both WT and Gck+/− mice (Fig. 1C). The insulinotropic actions of GLP-1 are reportedly affected by the blockade
of the autonomic nervous system (24). The effects of autonomic nerve blockade on the action of liraglutide were evaluated by the ip administration of a ganglionic blocker, CS, or a peripheral muscarinic blocker, methyl Atr. Neither Atr nor CS affected the glucose-lowering effects of liraglutide in either WT or Gck−/− mice (Fig. 2A), although the insulin secretion at 15 min after glucose administration was reduced by both Atr and CS (Fig. 2B).

Liraglutide failed to prolong the survival of Gck−/− neonates

To evaluate the effects of liraglutide on the survival of severely hyperglycemic Gck−/− pups, WT, Gck+/−, and Gck−/− pups were injected twice daily with either the vehicle or liraglutide, as described in Materials and Methods.

Liraglutide did not affect GSIS in Gck−/− neonatal islets

We first measured the casual serum insulin levels at P2 or P3 in WT, Gck+/−, and Gck−/− pups treated with twice daily injections of either vehicle or liraglutide, as described above. We previously reported that basal insulin secretion was preserved in Gck−/− pups (22). Consistent with this finding, the insulin levels were massively decreased, but not abolished, in these pups under casual nonfasting conditions (Fig. 4, A and B). Liraglutide failed to compensate for the striking reduction in the serum insulin levels at both P2 and P3 in Gck−/− mice (Fig. 4, A and B).

We next examined GSIS by islets isolated from WT, Gck+/−, and Gck−/− pups at 3 d after birth (P3) in the presence or absence of liraglutide. As shown in Fig. 4C, the
P3 Gck−/− islets exhibited impaired insulin secretion at both low and high glucose concentrations, and their responses to glucose were diminished. Although insulin secretion at a glucose concentration of 22.2 mM showed a mild increment in the presence of liraglutide in islets from P3 WT and Gck−/− mice, liraglutide had no effect on insulin secretion in both low and high glucose concentrations, and their responses to glucose were diminished. Although insulin secretion at a glucose concentration of 22.2 mM showed a mild increment in the presence of liraglutide in islets from P3 WT and Gck−/− mice, liraglutide had no effect on insulin secretion in islets from WT and Gck−/− mice (Fig. 4C). About 50–70% of the insulin secretion in the presence of potassium chloride was detected in P3 Gck−/− islets, compared with P3 WT and Gck−/− islets (data not shown).

These results implied that glucokinase plays a critical role in liraglutide-induced insulin secretion from β-cells.

Elevations of plasma ALT, FFA, and TG were transiently prevented by liraglutide in Gck−/− mice

A previous report demonstrated that both TChol and TG levels were elevated in neonatal systemic glucokinase-deficient mice, and glucokinase expression in β-cells restored these levels (21). We determined the plasma ALT, FFA, and TG levels at P2 (Fig. 5A), but not at P4 (Fig. 5B), in Gck−/− mice. Liraglutide also reduced the plasma levels of FFA, TChol, and TG in both WT and Gck−/− mice at P15 (Supplemental Fig. 3). In adult mice, glucokinase haploinsufficiency in the β-cells did not affect the plasma ALT level or the lipid profiles (Supplemental Fig. 4).

Liraglutide transiently protected against hepatic steatosis in Gck−/− mice

Systemic glucokinase-deficient mice exhibited a significant reduction in liver glycogen stores and microvesicular steatosis, with an increase in vacuolization and lipid accumulation (21). We examined the liver TG content and the liver glycogen content in WT, Gck+/+, and Gck−/− pups. Gck−/− mice manifested a significant increase in liver TG and a decrease in liver glycogen at P2 (Fig. 6, A and E, and Supplemental Fig. 5). Gross observation and histological analysis revealed fatty changes in the liver and steatosis with vacuolization in Gck−/− mice at P2 (Fig. 6, B and C). In agreement with the transient amelioration of the increases in ALT, FFA, and TG induced by liraglutide in Gck−/− mice, liraglutide restored the increase in liver TG and the decrease in liver glycogen at P2 (Fig. 6A), but not at P4 (Fig. 6E), in Gck−/− mice. Liver steatosis was also improved by liraglutide in P2 in Gck−/− mice (Fig. 6, B and C). Thus, liraglutide improved circulating lipids and hepatic steatosis in a β-cell glucokinase-independent fashion. Because liver glucokinase plays a key role in glycogen synthesis, we assessed the expression of glucokinase in the liver. P2Gck−/− mice exhibited a reduced expression of glucokinase in the liver, and liraglutide restored the expression level (Fig. 6D). The amelioration of the histological changes and the glucokinase expression level in the liver by liraglutide also disappeared at P4 in Gck−/− mice (data not shown).

Liraglutide delayed β-cell loss in Gck−/− mice

We next investigated the histological analysis of endocrine pancreas in WT, Gck+/+, and Gck−/− pups. Although all genotypes exhibited similar islet morphologies at P2 (Fig. 7, A and B), a decrease in the β-cell mass and the β-cell ratio in the islet cells were observed in P4 Gck−/− mice, compared with WT and Gck+/+ mice (Fig. 7, C and D). Liraglutide and NPH increased the β-cell mass and the β-cell ratio in islet cells in P4 Gck−/− mice (Fig. 7, C and D).
Glucokinase plays a central role as a glucose sensor in insulin-producing pancreatic islet β-cells and regulates GSIS (25, 26). β Cell-specific glucokinase-deficient (Gck<sup>-/-</sup>) mice suffer from lethal hyperglycemia as a result of the lack of insulin secretion (22). In this study, we investigated the effects of liraglutide on β-cell-specific glucokinase-deficient Gck<sup>-/-</sup> neonatal mice.

Insulin detemir, but not liraglutide, prolonged the survival of Gck<sup>-/-</sup> neonatal mice. However, liraglutide decreased the blood glucose levels in Gck<sup>-/-</sup> pups and prevented body weight gain in all genotypes. Furthermore, a single administration of liraglutide reduced the blood glucose levels in an insulin concentration-independent manner in both WT and Gck<sup>-/-</sup> mice in the presence or absence of Atr and CS. These data suggest that the glucose-lowering effect of liraglutide is mediated, at least in part, by extrapancreatic effects (e.g. gastrointestinal motility), and liraglutide failed to compensate for the severe impairment of insulin secretion in Gck<sup>-/-</sup> neonatal mice. It is possible that the attainment of blood glucose levels by liraglutide is insufficient to affect survivability in Gck<sup>-/-</sup> pups, and more intense treatments may be required to enhance survivorship.

We demonstrated that liraglutide had no effects on insulin secretion in islets from Gck<sup>-/-</sup> neonatal mice. This result suggested that glucokinase is required for liraglutide action in insulin secretion from β-cells. In mice deficient in Kir6.2, an ATP-sensitive K<sup>+</sup> channel, pretreatment with GLP-1 potentiated insulin secretion and prevented an elevation in the blood glucose level after a GTT (27), whereas niflumic acid-sensitive ion channels are reportedly involved in the induction of GSIS by cAMP elevation after treatment with GLP-1 (28). Therefore, glucokinase may contribute to pathways other than Kir6.2-mediated depolarization in liraglutide-induced insulin secretion. The assessment of cAMP levels before and after treatment with liraglutide in Gck<sup>-/-</sup> islets is needed to clarify the mechanisms that are involved.

In human trials, liraglutide has been shown to induce significant reductions in T-Chol, low-density lipoprotein-cholesterol, and TG (6, 7, 9, 11). Liraglutide also lowered the TG levels in type 2 diabetes rats (17). We demonstrated the protective effects of liraglutide on elevations in ALT, FFA, and TG, and liraglutide improved hepatic steatosis in Gck<sup>-/-</sup> mice. However, the significance of aspartate aminotransferase (AAT or AST), ALT, or AAT/ALT ratio in fatty liver of neonatal mice remains unclear. Measurement of AAT may contribute to unravel the complexity of this mechanism of action in these neonates. In previous studies, GLP1R-mediated signals ameliorated fatty liver (29, 30), and DPP-4 inhibition protected against steatosis in a glucose-independent manner (4). Reductions in body weight and energy intake resulting from liraglutide administration have
been previously reported (31, 32). The reduction in body weight gain observed in this study may have contributed to the improvements in the serum lipid parameters and the liver steatosis. One of the limitations of this study was its lack of weight-matched control groups to assess the influence of body weight changes on metabolic differences.

In human hepatocytes, the expressions of GLP1R and GLP1R internalization by GLP-1 or exendin-4 have been reported (33). Additionally, GLP-1 reportedly suppresses hepatic lipogenesis via the activation of the AMP-activated protein kinase pathway in rats (34), raising the possibility that the prevention of liver steatosis by liraglutide was due to a glucokinase-independent direct action of GLP-1 on the liver. Liraglutide may be beneficial for the treatment of fatty liver even in patients in whom treatment with liraglutide does not lead to glycemic control.

Because glucokinase expression in the liver is dependent on insulin, hepatic glucokinase expression was diminished in Gck<sup>−/−</sup> mice, regardless of the intact regulation of gene expression in the liver. Our data, however, indicated that the restoration of glucokinase expression in the liver by liraglutide is not induced by the elevation of serum insulin levels. Furthermore, a single administration of liraglutide by ip injection did not affect glucokinase expression in the liver, although a single administration of insulin significantly increased glucokinase expression (data not shown). Sterol regulatory element binding protein-1c, peroxisome proliferator-activated receptor-γ, liver X receptor-α, small heterodimer partner, and PPAR<sub>γ</sub> coactivator-1α are also involved in the regulation of glucokinase gene transcription in the liver (35, 36). However, glucokinase gene expression induced by hormones other than insulin has been less well characterized. The reduction of serum glucagon levels by liraglutide may have contributed to the suppression of cAMP elevation in hepatocytes, resulting in an increase in glucokinase expression. Because the amelioration of glucokinase expression in the liver by liraglutide was also transient, glucokinase expression might have been regulated by the serum FFA and TG levels through genes controlling lipid metabolism in the liver. Further study is required to unravel the complex interactions involved in the regulation of glucokinase expression in the liver.

Liraglutide also protected against β-cell loss without increasing insulin secretion in Gck<sup>−/−</sup> mice. Numerous reports have suggested that GLP1R activation has protective effects against β-cell damage (37). Our results suggest that liraglutide may also protect β-cells in the absence of glucokinase or irrespective of insulin secretion. The reduction in serum lipid levels may contribute to the avoidance of lipotoxicity in β-cells.

In summary, we demonstrated that β-cell glucokinase is required for liraglutide-induced increments in insulin secre-
tion. However, the serum lipid parameters, liver steatosis, and β-cell loss were improved by liraglutide in a glucokinase- and insulin-independent manner. Because GLP-1 is involved in many biological activities in the brain, endothelial cells, peripheral nerves, and blood cells, GLP1R activation may have multiple pleiotropic effects. The results of the current study suggest a novel therapeutic potential of liraglutide for noninsulinotropic effects in diabetic patients, but further research is needed to clarify the mechanisms that are involved.

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Address all correspondence and requests for reprints to: Yasuo Terauchi, M.D., Ph.D., Department of Endocrinology and Metabolism, Graduate School of Medicine, Yokohama-City University, 3-9 Fuku-ura, Kanazawa-ku, Yokohama 236-0004, Japan. E-mail: terauchi-tyk@umin.ac.jp.

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