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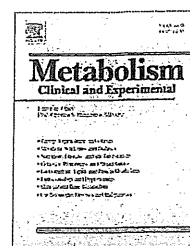
Basic Science

Dipeptidyl peptidase-4 inhibitor anagliptin ameliorates diabetes in mice with haploinsufficiency of glucokinase on a high-fat diet

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ABSTRACT

Objective. Type 2 diabetes is a chronic metabolic disorder characterized by hyperglycemia with insulin resistance and impaired insulin secretion. DPP-4 inhibitors have attracted attention as a new class of anti-diabetic agents for the treatment of type 2 diabetes. We investigated the effects of anagliptin, a highly selective DPP-4 inhibitor, on insulin secretion and insulin resistance in high-fat diet-fed mice with haploinsufficiency of glucokinase (GckKO) as animal models of type 2 diabetes.

Materials/Methods. Wild-type and GckKO mice were administered two doses of anagliptin by dietary admixture (0.05% and 0.3%) for 10 weeks.

Results. Both doses of anagliptin significantly inhibited the plasma DPP-4 activity and increased the plasma active GLP-1 levels in both the wild-type and GckKO mice to a similar degree. After 10 weeks of treatment with 0.3% anagliptin, body weight gain and food intake were significantly suppressed in both wild-type and GckKO mice. In addition, 0.3% anagliptin ameliorated insulin resistance and glucose intolerance in both genotypes of mice. On the other hand, treatment with 0.05% anagliptin was not associated with any significant change of the body weight, food intake or insulin sensitivity in either genotype of mice, but it did improve the glucose tolerance by enhancing insulin secretion and increasing the β-cell mass in both genotypes of mice.

Conclusions. High-dose anagliptin treatment improved glucose tolerance by suppression of body weight gain and amelioration of insulin resistance, whereas low-dose anagliptin treatment improved glucose tolerance by enhancing insulin secretion.

1. Introduction

Type 2 diabetes is a chronic metabolic disorder characterized by hyperglycemia with insulin resistance and impaired insulin secretion. Progression to type 2 diabetes is influenced by genetic and environmental or acquired factors, such as a sedentary lifestyle and dietary habits that promote obesity. Most patients with type 2 diabetes are obese, and obesity is associated with insulin resistance. β-cell mass in adults exhibits plasticity, and adjustments in β-cell growth and survival maintain the balance between insulin supply and the metabolic demand. For example, obese individuals who do not develop diabetes exhibit an increase of the β-cell mass that appears to compensate for the increased metabolic load and obesity-associated insulin resistance. However, this β-cell adaptation eventually fails in the subset of obese individuals who develop type 2 diabetes [1–3]. In fact, most individuals with type 2 diabetes show a net decrease of the β-cell mass [1,4,5]. Thus, type 2 diabetes is a disease of relative insulin deficiency.

Glucagon-like peptide-1 (GLP-1), which is a gut-derived incretin hormone, stimulates glucose-dependent insulin secretion via the cAMP/PKA pathway. In addition, GLP-1 exerts multiple actions, including decrease of the body weight through suppression of appetite, stimulation of β-cell proliferation, and inhibition of β-cell apoptosis [6]. However, GLP-1 is rapidly converted to a bioinactive form by dipeptidyl peptidase-4 (DPP-4), the key enzyme responsible for cleaving and inactivating at the penultimate alanine residue [7–9]. Thus, DPP-4 inhibitors to block the enzymatic inactivation of GLP-1 have emerged as a new class of anti-diabetic agents for the treatment of type 2 diabetes.

Glucokinase (Gck) is the key rate-limiting enzyme in glucose metabolism in the β-cells. Gck catalyzes the conversion of glucose to glucose 6-phosphate, which is a critical process in glucose sensing for insulin secretion by the pancreatic β-cells. It has been shown that maturity-onset diabetes of the young type 2 (MODY2) can be caused by mutation in a single Gck gene allele [10,11]. Moreover, in type 2 diabetes, the mRNA expression and activity of Gck are significantly reduced, which is associated with impaired glucose-stimulated insulin release [12,13]. Mice with haploinsufficiency of Gck (GckKO mice) also exhibit glucose intolerance associated with a reduction in the insulin secretion in response to glucose [14]. In addition, GckKO mice show insufficient β-cell growth in response to high-fat diet-induced obesity-linked insulin resistance, leading to the development of diabetes [15]. Thus, GckKO mice fed a high-fat diet are considered as a useful animal model of diabetes, which show a time course of the disease similar to that seen in patients with type 2 diabetes.

In the present study, we investigated whether anagliptin, a highly selective DPP-4 inhibitor, might ameliorate glucose intolerance in high-fat diet-fed GckKO mice. Treatment with 0.3% anagliptin ameliorated the insulin resistance by suppression of body weight gain, which resulted in a decrease of the fasting plasma glucose and improvement of the glucose tolerance. On the other hand, treatment with 0.05% anagliptin improved glucose tolerance by enhancing insulin secretion, which was attributed to an increase of the β-cell mass, but did not suppress the body weight gain or ameliorate the insulin resistance. Taken together, both low and high doses of anagliptin improved glucose tolerance in the high-fat diet-fed GckKO diabetic mice. These findings suggest that anagliptin could be a potentially efficacious agent for the treatment of type 2 diabetic patients.

2. Materials and methods

2.1. Animals and genotyping

GckKO mice were generated as described previously [14]. Then, the original GckKO mice were back-crossed more than seven times with the C57BL/6 mice. The mice were housed under a 12-h light/dark cycle and fed standard chow (CE-2; CLEA) until 8 weeks of age and then allocated to either an HF diet alone or an HF diet containing a DPP-4 inhibitor. All of the experiments in this study were conducted on 8-week-old male littermates. The animal care and experimental procedures were approved by the Animal Care Committee of the University of Tokyo.

2.2. DPP-4 inhibitor treatment study

The composition of the HF diet (High Fat Diet 32; Clea Japan) was as described previously [15]. DPP-4 inhibitor was admixed with the HF diet at 0.05% or 0.3% (wt/wt). The DPP-4 inhibitor used in this study was anagliptin [16], prepared by Sanwa Kagaku Kenkyusho, Ltd.

2.3. Measurement of the plasma DPP-4 activity

Plasma DPP-4 activity was measured using a fluorometric assay with Gly-Pro-MCA (Peptide Institute, Osaka, Japan), modified from a previously published method [17]. In brief, 10 µL of a plasma sample was mixed with 90 µL of the reaction buffer (0.2 mmol/L Gly-Pro-MCA, 0.1 mg/mL BSA, 25 mol/L HEPES, 140 mol/L NaCl, pH 7.8). The mixture was incubated for 20 min at room temperature in the dark, and the reaction was stopped by the addition of 100 µL of 25% acetic acid. The fluorescence intensity of the liberated 7-amino-4-methylcoumarin (AMC) was measured with a 96-well plate fluorometer (1420 ARVOsx, PerkinElmer) at an excitation wavelength of 390 nm and emission wavelength of 460 nm. Plasma DPP-4 activity was calculated as nmol AMC/min/mL plasma, and the result in the treated samples was expressed as a percentage of that in the control.

2.4. Measurement of the plasma parameters

Plasma adiponectin levels were determined with a mouse adiponectin enzyme-linked immunosorbent assay kit (Otsuka

Pharmaceutical). Plasma leptin levels were determined with a mouse leptin ELISA kit (Morinaga Institute of Biological Science). Plasma levels of active GLP-1 were assayed with a Glucagon-Like Peptide-1 (Active) ELISA kit (Millipore).

2.5. Insulin tolerance test

Mice were given free access to food and then fasted during the study. They were intraperitoneally challenged with human insulin at 0.75 mU/g body weight (Humulin R), and venous blood samples were drawn at different time-points [18].

2.6. Hyperinsulinemic–euglycemic clamp study

Clamp studies were carried out as described previously [19], with slight modifications. In brief, 2 days before the study, an infusion catheter was inserted into the right jugular vein of the study animals under general anesthesia induced by sodium pentobarbital. Studies were performed on the mice under conscious and unstressed conditions after 6 h of fasting. A primed continuous infusion of insulin (Humulin R) was administered (7.5 mU/kg/min), and the blood glucose concentration, monitored every 5 min, was maintained at 100–130 mg/dL by administration of glucose (5 g of glucose/10 mL enriched to ~20% with [6,6-^{2}H$_2$]glucose (Sigma)) for 120 min. Blood was sampled via tail-tip bleeds at 90, 105 and 120 min for determination of the rate of glucose disappearance (Rd). Rd was calculated according to nonsteady-state equations, and endogenous glucose production was calculated as the difference between the Rd and the exogenous glucose infusion rate [19].

2.7. Glucose tolerance test

Mice were fasted for a sufficient period of time before the study, to eliminate the acute effects of anagliptin on glucose-stimulated insulin secretion and then orally loaded with glucose at 1.5 mg/g body weight. Blood samples were collected from the orbital sinus at different time-points, and the blood glucose was measured with an automatic glucometer (Glutest Ace, Sanwa Kagaku Kenkyusho) or the glucose CII-test Wako (Wako). Whole blood was collected and centrifuged in heparinized tubes, and the plasma samples were stored at –20 °C. Insulin levels were determined with an AlphaLISA insulin kit (PerkinElmer).

2.8. Histological and immunohistochemical analysis of the islets

Isolated pancreata were fixed with 4% paraformaldehyde at 4 °C overnight. Tissues were routinely processed for paraffin embedding, and 4-μm sections were cut and mounted on silanized slides. Pancreatic sections were stained with polyclonal guinea pig anti-swine insulin antibodies (diluted 1:100; DAKO). Images of the pancreatic tissue and islet β-cells were viewed on the monitor of a computer through a microscope connected to a camera with a charged-coupled device (Keyence). The areas of the pancreata and beta cells were traced manually and analyzed with WinROOF software (Mitani), as previously described [20]. The β-cell mass was calculated as the β-cell area, as assessed by immunostaining, relative to the area of the whole pancreas. More than 100 islets were analyzed

per mouse in each group. BrdU incorporation was analyzed as described previously [15]. In brief, BrdU (100 mg/kg in saline; Sigma) was injected intraperitoneally, and the pancreas was removed 6 h later. The sections were immunostained with BrdU labeling and detection kit II (Roche Diagnostics). BrdU-positive beta cells were quantitatively assessed as a percentage of the total number of beta cells. Apoptotic cells were also detected in deparaffinized pancreatic sections using an in situ cell death detection kit (Roche Diagnostics), in accordance with the manufacturer's recommendations.

2.9. Immunoblotting

Polyclonal anti-IRS-2 antibody was purchased from Upstate. Antibodies to phospho-CREB and phospho-Akt were purchased from Cell Signaling Technology. The islets were sonicated in ice-cold buffer A (25 mol/L Tris-HCl, pH 7.4, 10 mol/L Na$_3$VO$_4$, 10 mol/L NaPPi, 100 mol/L NaF, 10 mol/L EDTA, 10 mol/L EGTA, and 1 mol/L phenylmethylsulfonyl fluoride) with an ultrasonic sonicator. Samples were separated by SDS-polyacrylamide gel electrophoresis, and immunodetection was performed with an ECL kit (Amersham Biosciences). Protein was prepared from more than 100 islets pooled from several mice of identical genotype, and 15 μg samples of the proteins were applied to the gel.

3. Results

3.1. Treatment with 0.3% anagliptin decreased body weight gain and food intake

After 10 weeks of treatment, 0.3% anagliptin significantly inhibited the plasma DPP-4 activity by more than 80%, and significantly increased the plasma levels of ad libitum active GLP-1 in the wild-type mice (Fig. 1A); 0.3% anagliptin also significantly inhibited the plasma DPP-4 activity and increased the plasma levels of ad libitum active GLP-1 in the GckKO mice, to degrees equivalent to those seen in the wild-type mice (Fig. 1B). Treatment with 0.3% anagliptin significantly decreased the body weight gain and food intake in the wild-type mice (Fig. 1, C and D), and also in the GckKO mice (Fig. 1, E and F). In contrast, this treatment had no significant effect on the rectal temperature or UCP-1 expression levels in the BAT in either mouse genotype (Fig. 1, G and H). Treatment with 0.3% anagliptin significantly decreased the weights of the epididymal fat and liver in the wild-type mice, and to an equivalent degree in the GckKO mice (Fig. 1, I and J). Furthermore, 0.3% anagliptin treatment significantly decreased the plasma leptin levels and significantly increased the plasma adiponectin levels in both the wild-type and GckKO mice (Fig. 1, K and L). These findings indicate that 0.3% anagliptin decreased body weight gain through inducing a reduction of the food intake in both wild-type and GckKO mice.

3.2. Treatment with 0.3% anagliptin improved insulin resistance and glucose tolerance in wild-type and GckKO mice

The insulin tolerance test (ITT) revealed that the glucose-lowering effect of insulin was significantly increased in the
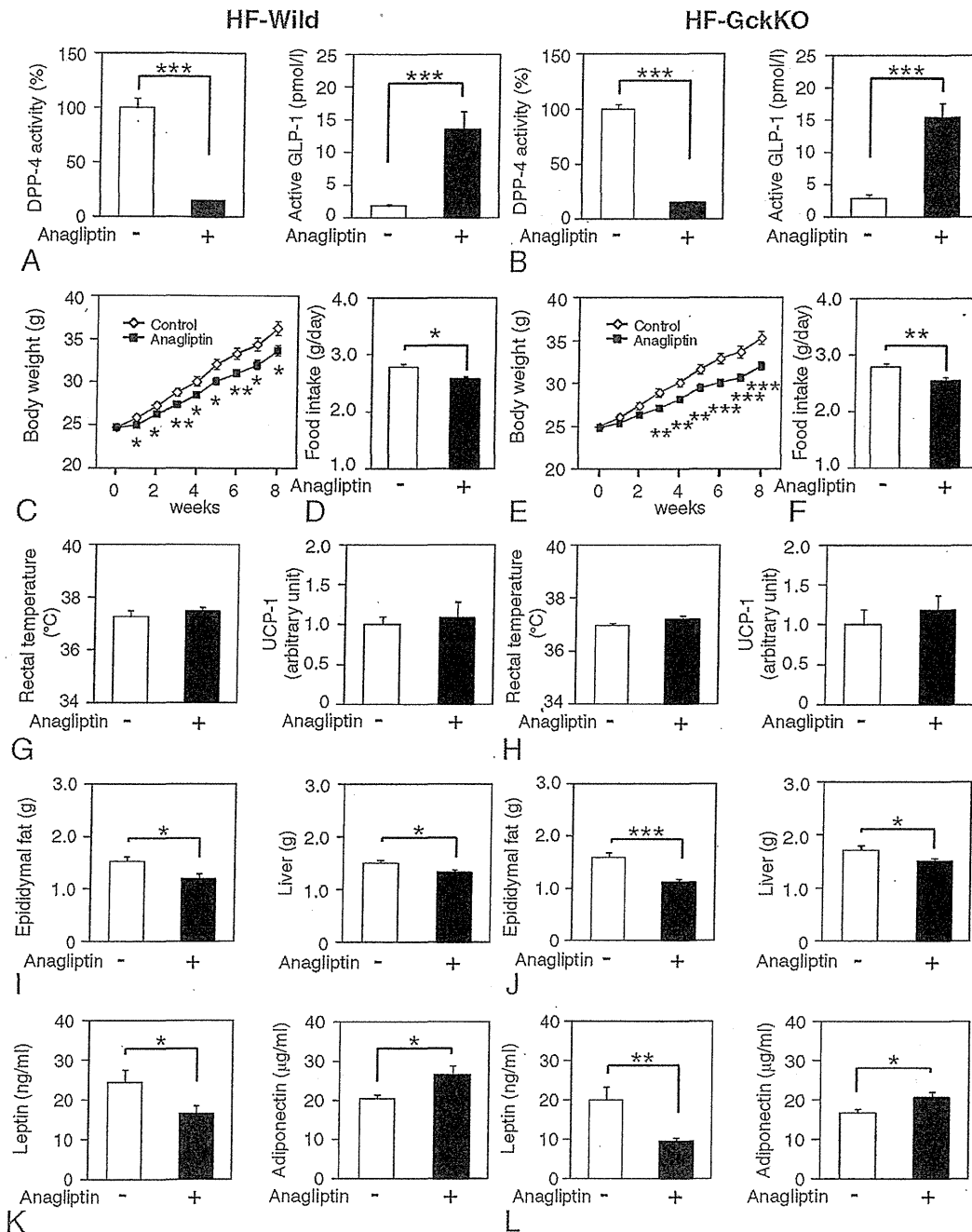
Fig. 1 – Treatment with 0.3% anagliptin decreased body weight gain and food intake. A and B, DPP-4 activities (left panels) and active GLP-1 levels (right panels) in wild-type (A) and GckKO (B) mice not treated (open bars) or treated (closed bars) with anagliptin (n = 11–12). C and D, body weights (left panel, n = 30) and food intake (right panel, n = 11–12) in wild-type mice not treated (open diamonds and open bar) or treated (closed squares and closed bar) with anagliptin. E and F, body weights (left panel, n = 23–24) and food intake (right panel, n = 11–12) of GckKO mice not treated (open diamonds and open bar) or treated (closed squares and closed bar) with anagliptin. G and H, rectal temperature (left panels, n = 23–24) and UCP-1 expression levels (right panels, n = 5–6) in wild-type (G) and GckKO (H) mice not treated (open bars) or treated (closed bars) with anagliptin. I and J, weights of epididymal fat (left panels) and liver (right panels) in wild-type (I) and GckKO (J) mice not treated (open bars) or treated (closed bars) with anagliptin (n = 22–24). K and L, leptin (left panels) and adiponectin (right panels) levels in wild-type (K) and GckKO (L) mice not treated (open bars) or treated (closed bars) with anagliptin (n = 12–14).Values are means ± S.E. of data obtained from the analysis of wild-type and GckKO mice. *, p < 0.05. **, p < 0.01. ***, p < 0.001.

0.3% anagliptin-treated wild-type mice as compared with that in the untreated wild-type mice (Fig. 2A). Although the GckKO mice showed hyperglycemia in the fed state before

insulin administration as compared with the wild-type mice, the glucose-lowering effect of insulin was also significantly more pronounced in the 0.3% anagliptin-
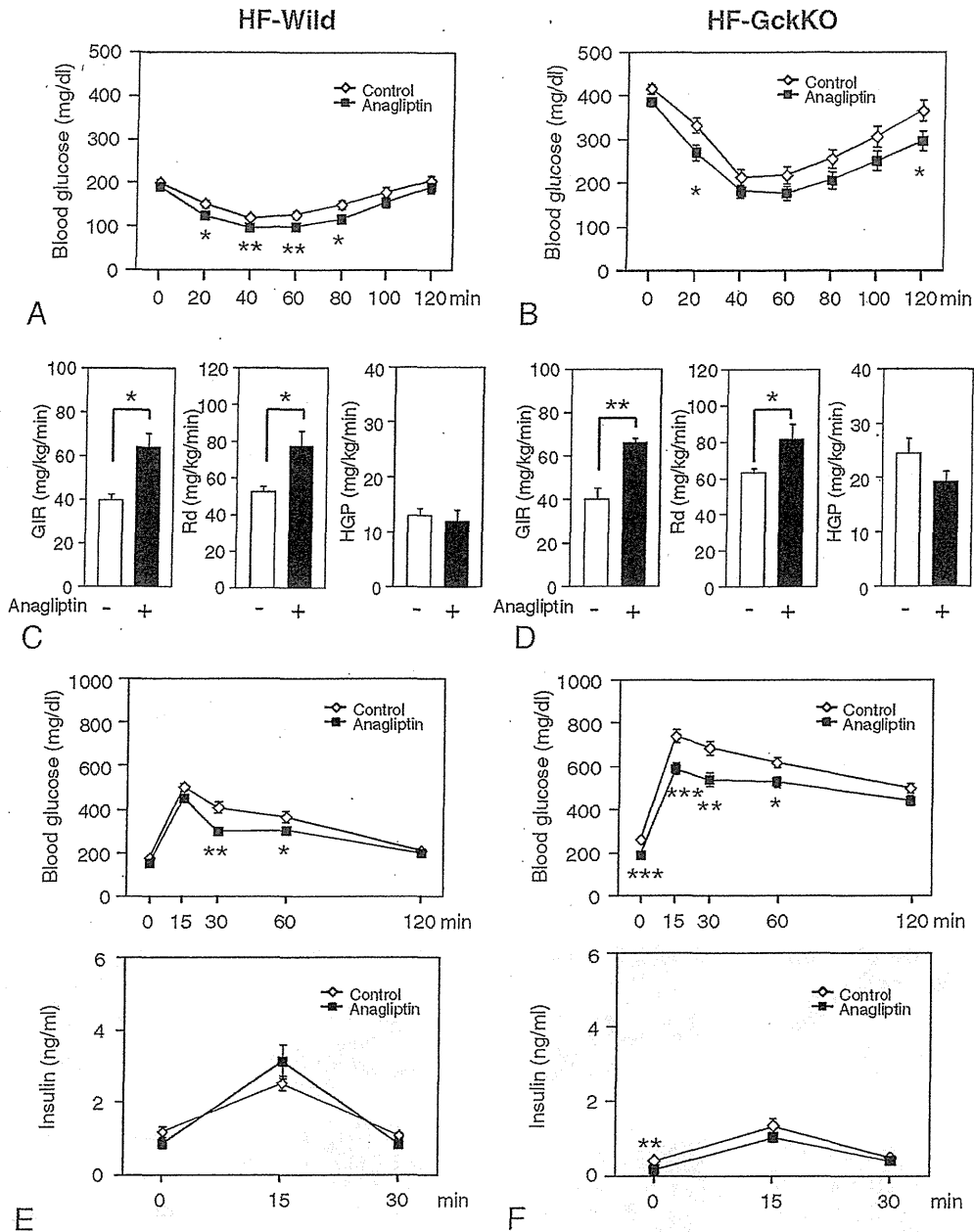
Fig. 2 – Treatment with 0.3% anagliptin improved insulin resistance and glucose tolerance in the wild-type and GckKO mice. A and B, blood glucose levels during the ITT in wild-type (A) and GckKO (B) mice not treated (open diamonds) or treated (closed squares) with anagliptin (n=30). C and D, GIR (left panels), Rd (middle panels) and HGP (right panels) in wild-type (C) and GckKO (D) mice not treated (open bars) or treated (closed bars) with anagliptin (n=5–6). E and F, blood glucose (upper panels) and plasma insulin (bottom panels) levels during OGTT in wild-type (E) and GckKO (F) mice not treated (open diamonds) or treated (closed squares) with anagliptin (n=23–24). Values are means±S.E. of data obtained from the analysis of wild-type and GckKO mice. *, p<0.05. **, p<0.01. ***, p<0.001.

treated GckKO mice as compared with that in the untreated GckKO mice (Fig. 2, A and B). Consistent with the results of the ITT, the glucose infusion rate (GIR) and rate of glucose disappearance (Rd) were significantly increased after 0.3% anagliptin treatment in both the wild-type and GckKO mice (Fig. 2, C and D). In contrast, the treatment had no effect on the endogenous glucose production (HGP) in either genotype of mice (Fig. 2, C and D). Blood glucose levels before and after glucose loading

were significantly higher in the untreated GckKO mice than in the untreated wild-type mice, along with impaired insulin secretion, as we previously reported (Fig. 2, E and F) [15]. In an oral glucose tolerance test (OGTT), the blood glucose levels at 30 min and 60 min after glucose loading were significantly lower in the 0.3% anagliptin-treated wild-type mice than in the untreated wild-type mice (Fig. 2E). The blood glucose levels in 0.3% anagliptin-treated GckKO mice before and after glucose loading were also

significantly lower than those in untreated GckKO mice (Fig. 2F). On the other hand, no significant increment of insulin secretion by anagliptin was observed in either genotype of mice (Fig. 2, E and F). These findings suggest that 0.3% anagliptin improves glucose tolerance predominantly by ameliorating insulin resistance rather than by increasing insulin secretion.

3.3. No increment of β-cell mass was observed following 0.3% anagliptin treatment in either genotype of mice

The β-cell mass tended to be lower in the untreated GckKO mice as compared with that in the untreated wild-type mice (P=0.06) (Fig. 3, A, B, E and F). The percentage of cells incorporating BrdU was significantly lower in the untreated GckKO mice than in the untreated wild-type mice (P<0.01) (Fig. 3, C and G). In contrast, the percentage of TUNEL-positive cells tended to be higher in the untreated GckKO mice than in the untreated wild-type mice (P=0.12) (Fig. 3, D and H). After 10 weeks of treatment with 0.3% anagliptin, the β-cell mass was indistinguishable from that in the untreated mice in both genotypes of mice (Fig. 3, A, B, E and F). Moreover, treatment with 0.3% anagliptin did not have any significant effect on the percentage of cells incorporating BrdU or the number of TUNEL-positive cells among the β-cells in either genotype of mice (Fig. 3, C, D, G and H).

3.4. Treatment with 0.05% anagliptin had no effect on the body weight or insulin sensitivity, but improved glucose tolerance by increasing insulin secretion

It was considered that the absence of any effect of 0.3% anagliptin on the β-cell mass was attributable to its effect of suppressing body weight gain and ameliorating insulin resistance. Therefore, we next investigated the dose-dependent effects of anagliptin on the active GLP-1 levels, the body weight, insulin resistance and glucose tolerance in wild-type mice. The active GLP-1 levels were significantly increased, in a dose-dependent manner, after anagliptin treatment (Fig. 4A). After 8 weeks of treatment, the body weight gain was indistinguishable between the untreated and 0.05% anagliptin-treated mice (Fig. 4B). In contrast, treatment with 0.3% anagliptin was associated with a significant reduction of the body weight gain (Fig. 4B) as seen in Fig. 1C. In the ITT, although the blood glucose levels after insulin administration were indistinguishable between untreated mice and the mice treated with 0.05% anagliptin, the blood glucose levels before and after insulin administration were significantly decreased in the 0.3% anagliptin-treated mice as compared with those in the untreated mice (Fig. 4C). In the OGTT, the blood glucose levels after glucose loading were significantly decreased, along with increased insulin secretion, in the 0.05% anagliptin-treated mice (Fig. 4D). On the other hand, whereas the blood
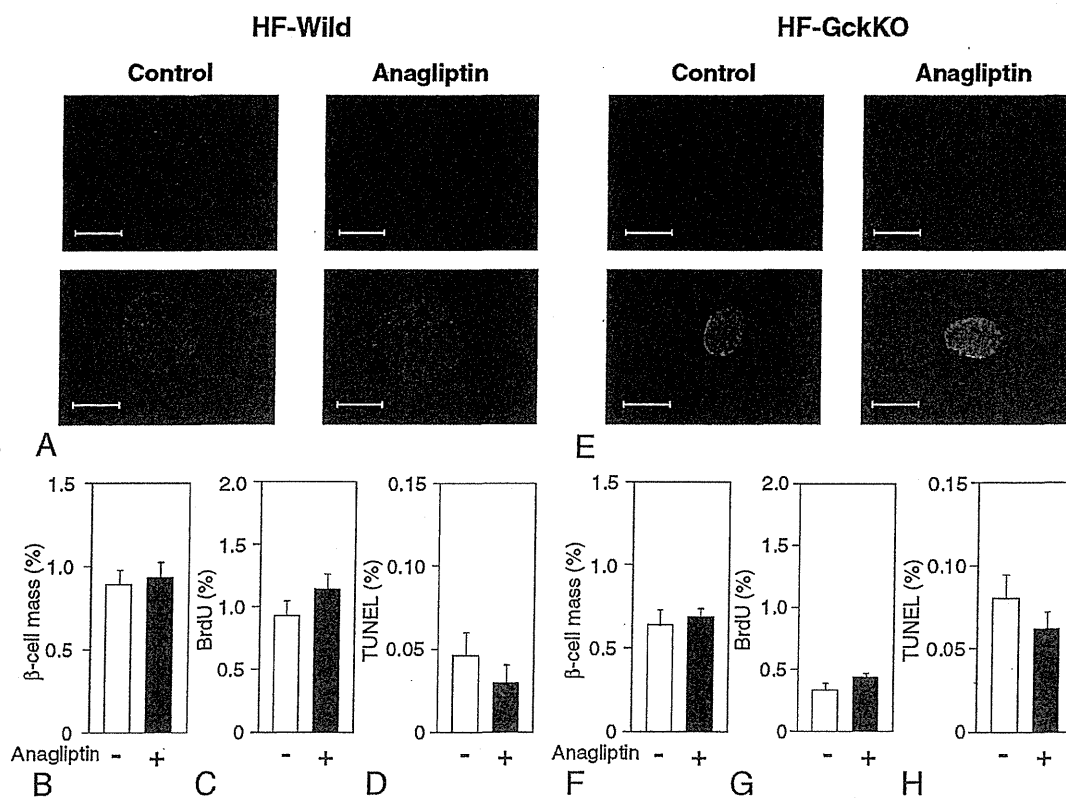

Fig. 3 – Effect of 0.3% anagliptin on the β-cell mass. Histological analysis of pancreatic β-cells (A and E) (upper panels; scale bar= 1000 μm, lower panels; scale bar=100 μm), quantitation of β-cell mass (B and F), BrdU incorporation (C and G) and TUNEL staining (D and H) in wild-type (A–D) and GckKO (E–H) mice not treated (open bars) or treated (closed bars) with anagliptin (n = 6–9). Sections were stained with anti-insulin antibody. Values are means ±S.E. of data obtained from the analysis of wild-type and GckKO mice.
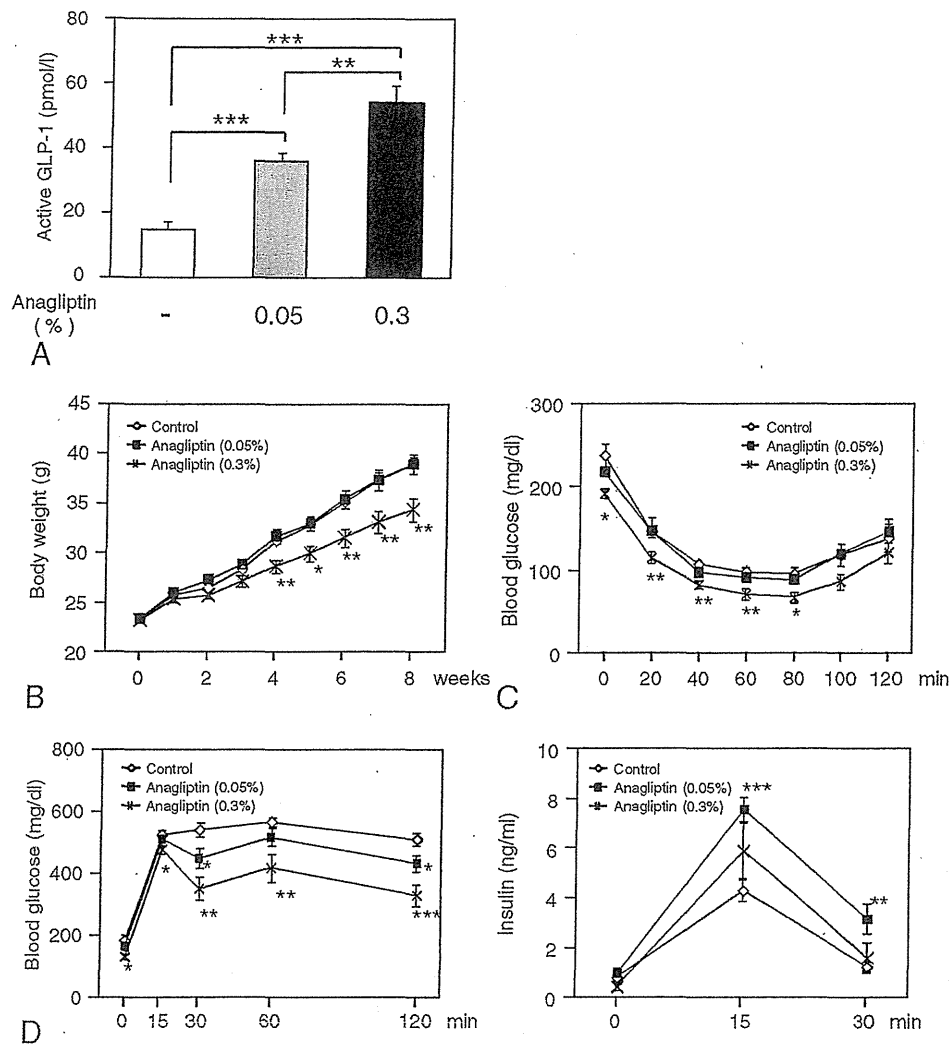
Fig. 4 – Treatment with 0.05% anagliptin had no effect on the body weight or insulin sensitivity, but improved glucose tolerance by increasing insulin secretion in the wild-type mice. A, plasma active GLP-1 levels in wild-type mice not treated (open bars) or treated (0.05%: gray bars, 0.3%: closed bars) with anagliptin. B, body weights of wild-type mice not treated (open diamonds) or treated (0.05%: closed squares and 0.3%: cross-lines) with anagliptin. C, blood glucose levels during the ITT in wild-type mice not treated (open diamonds) or treated (0.05%: closed squares and 0.3%: cross-lines) with anagliptin. D, blood glucose (left panel) and plasma insulin (right panel) levels during OGTT in wild-type mice not treated (open diamonds) or treated (0.05%: closed squares and 0.3%: cross-lines) with anagliptin (n=8). Values are means±S.E. of data obtained from the analysis of wild-type mice. *, p<0.05. **, p<0.01. ***, p<0.001.

glucose levels before and after glucose loading were significantly lower in the 0.3% anagliptin-treated mice than those in the untreated mice, the plasma levels of insulin before and after glucose loading were indistinguishable between untreated mice and the mice treated with 0.3% anagliptin (Fig. 4D). These results suggest that 0.05% anagliptin improved glucose tolerance by increasing insulin secretion, whereas 0.3% anagliptin improved glucose tolerance by ameliorating insulin resistance.

3.5.	0.05% anagliptin had no effect on the body weight or food intake of either wild-type or GckKO mice

After 10 weeks of treatment, 0.05% anagliptin significantly inhibited the plasma DPP-4 activity by more than 70%, and

significantly increased the plasma levels of ad libitum active GLP-1 in both the wild-type and GckKO mice (Fig. 5, A and B). Treatment with 0.05% anagliptin had little effect on the body weight gain or food intake in either the wild-type or the GckKO mice (Fig. 5, C, D, E and F). The rectal temperature and UCP-1 expression levels in the BAT were also indistinguishable between the untreated and 0.05% anagliptin-treated mice of either genotype (Fig. 5, G and H). Furthermore, treatment with 0.05% anagliptin also had no effect on the weights of epididymal fat or the liver in either genotype of mice (Fig. 5, I and J). The plasma leptin and adiponectin levels were indistinguishable between the untreated and 0.05% anagliptin-treated mice of either genotype (Fig. 5, K and L). These findings indicate that 0.05% anagliptin had no effect on the body weight or food intake in either the wild-type or the GckKO mice.
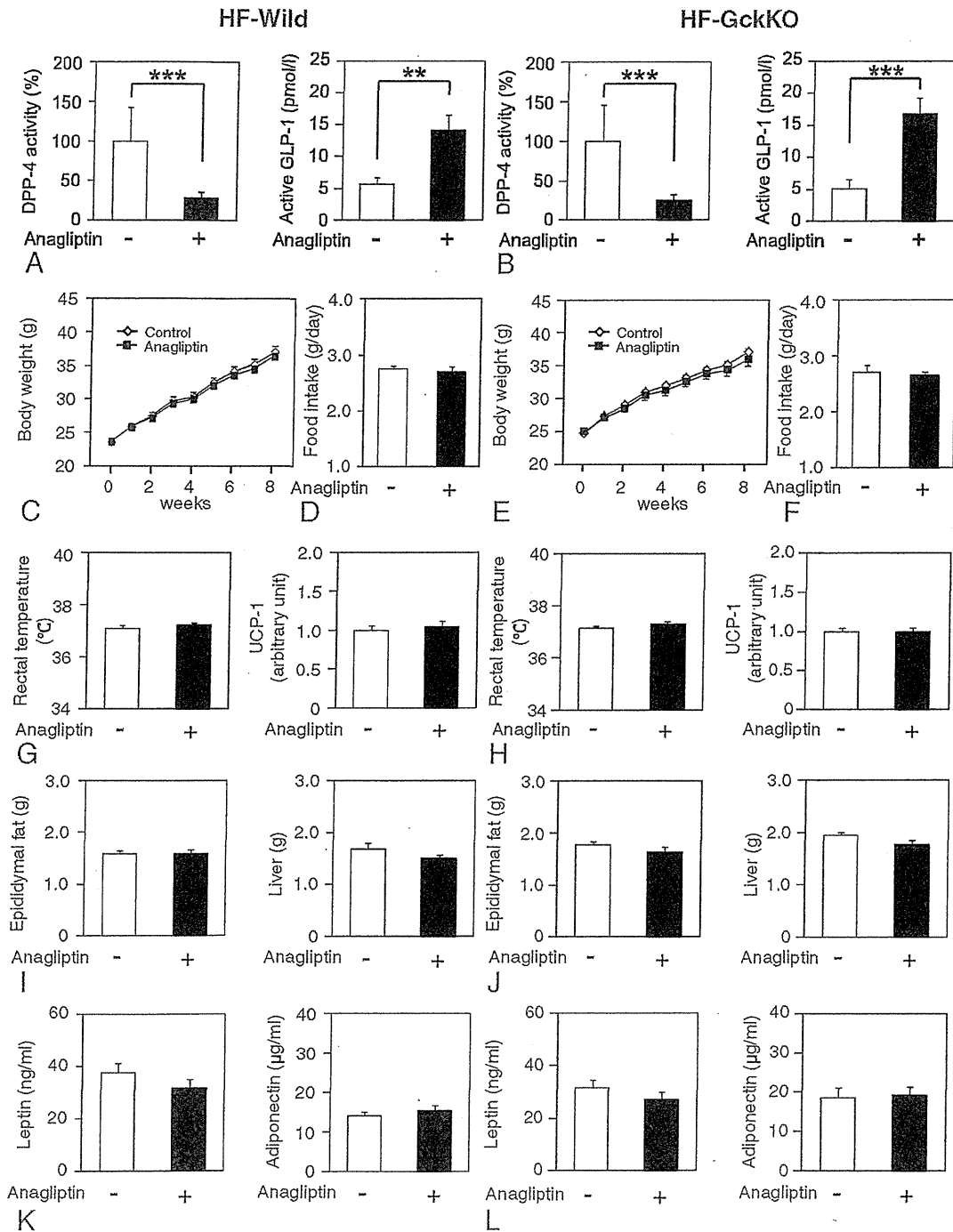
HF-Wild

HF-GckKO

Fig. 5 – Treatment with 0.05% anagliptin had no effect on the body weight or food intake in either the wild-type or GckKO mice. A and B, DPP-4 activities (left panels) and active GLP-1 levels (right panels) in wild-type (A, n=15–16) and GckKO (B, n=11–12) mice not treated (open bars) or treated (closed bars) with anagliptin. C and D, body weights (left panel, n=21) and food intake (right panel, n=8) in wild-type mice not treated (open diamonds and open bar) or treated (closed squares and closed bar) with anagliptin. E and F, body weights (left panel, n=18–21) and food intake (right panel, n=10) in GckKO mice not treated (open diamonds and open bar) or treated (closed squares and closed bar) with anagliptin. G and H, rectal temperature (left panels, n=18–21) and UCP-1 expression levels (right panels, n=5–6) in wild-type (G) and GckKO (H) mice not treated (open bars) or treated (closed bars) with anagliptin. I and J, weights of epididymal fat (left panels) and liver (right panels) in wild-type (I) and GckKO (J) mice not treated (open bars) or treated (closed bars) with anagliptin (n=14–16). K and L, leptin (left panels) and adiponectin (right panels) levels in wild-type (K) and GckKO (L) mice not treated (open bars) or treated (closed bars) with anagliptin (n=14–16). Values are means ± S.E. of data obtained from the analysis of wild-type and GckKO mice. **, p<0.01. ***, p<0.001.
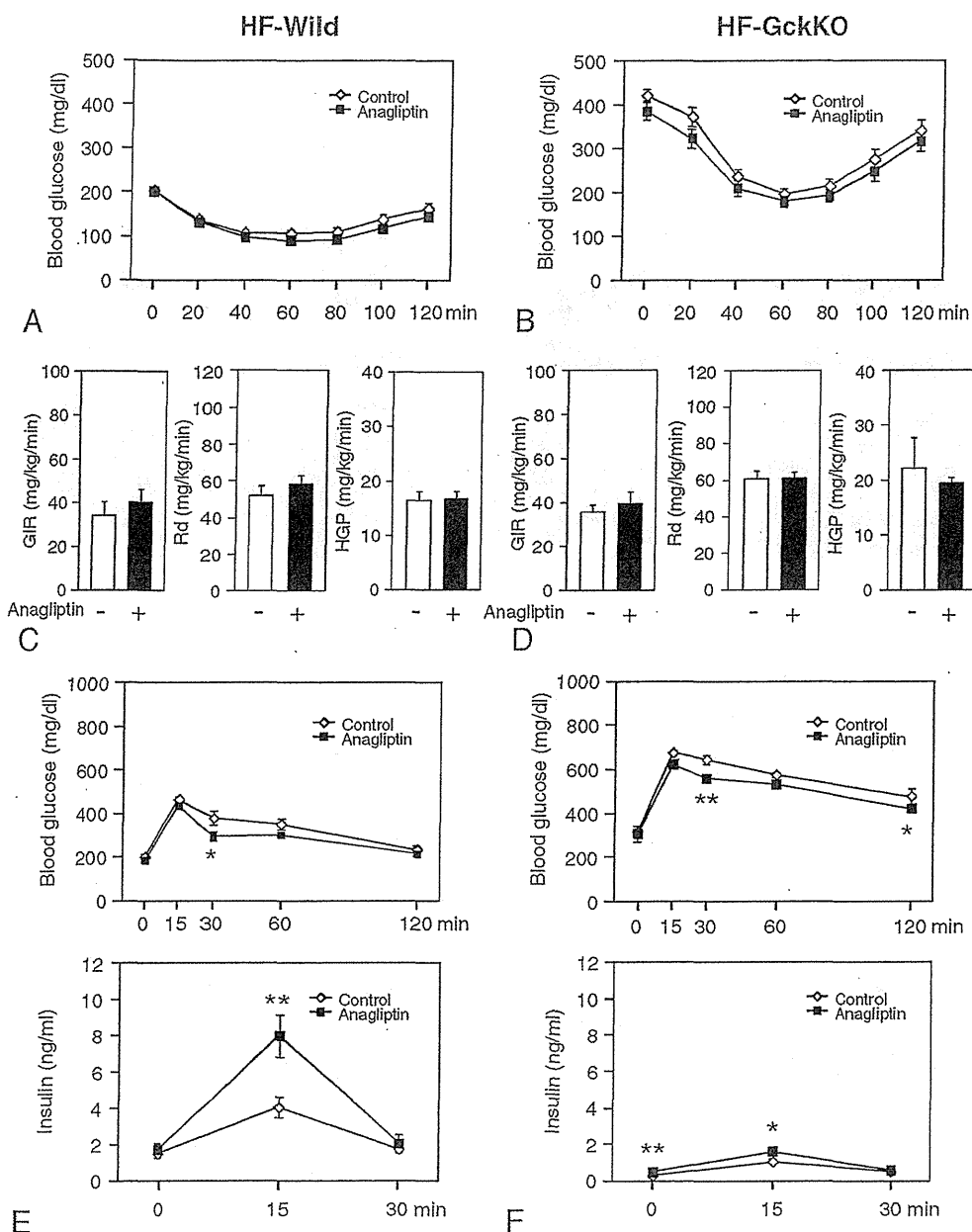
Fig. 6 – Treatment with 0.05% anagliptin improved glucose tolerance by increasing the insulin secretion in both wild-type and GckKO mice. A and B, blood glucose levels during the ITT in wild-type (A, n=15–16) and GckKO (B, n=18–21) mice not treated (open diamonds) or treated (closed squares) with anagliptin. C and D, GIR (left panels), Rd (middle panels) and HGP (right panels) in wild-type (C) and GckKO (D) mice not treated (open bars) or treated (closed bars) with anagliptin (n=4). E and F, blood glucose (upper panels) and plasma insulin (bottom panels) levels during OGTT in wild-type (E, n=12) and GckKO (F, n=23–24) mice not treated (open diamonds) or treated (closed squares) with anagliptin. Values are means ± S.E. of data obtained from the analysis of wild-type and GckKO mice. *, p<0.05. **, p<0.01.

3.6. 0.05% anagliptin improved glucose tolerance by enhancing insulin secretion in both wild-type and GckKO mice

Unlike 0.3% anagliptin treatment, the ITT showed that treatment with 0.05% anagliptin had no effect on the insulin sensitivity in the mice of either genotype (Fig. 6, A and B). Consistent with the results of the ITT, the GIR and Rd, as well as HGP, were indistinguishable between the untreated and 0.05% anagliptin-treated mice of either genotype (Fig. 6, C and D). These results indicate that the amelioration of insulin resistance by 0.3% anagliptin might be largely dependent on the suppression of body weight gain. In the OGTT, the blood glucose levels after glucose loading were significantly lower in the 0.05% anagliptin-treated wild-type mice than in the untreated wild-type mice, with increment of the insulin secretion (Fig. 6E). The blood glucose levels after glucose loading were also significantly lower in the anagliptin-treated GckKO mice than in the untreated GckKO mice (Fig. 6F). Treatment with 0.05% anagliptin significantly increased the

insulin secretion before and after glucose loading in the GckKO mice, unlike the case following treatment with 0.3% anagliptin (Fig. 6F). These data suggest that 0.05% anagliptin improved glucose tolerance with enhancement of insulin secretion in the GckKO mice as well as in the wild-type mice.
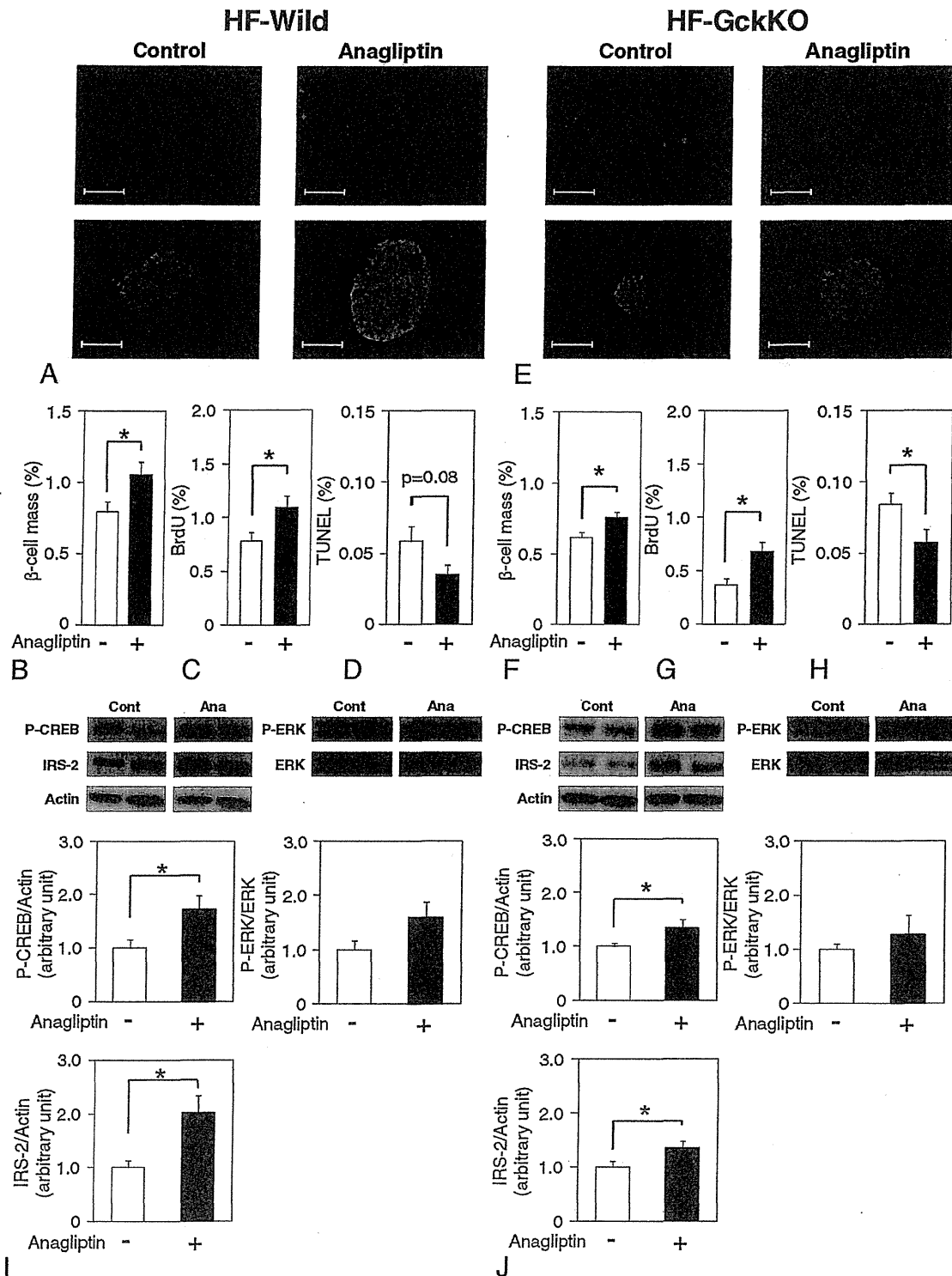

Fig. 7 – Treatment with 0.05% anagliptin increased the β-cell mass. Histological analysis of pancreatic β-cells (A and E) (upper panels; scale bar=1000 μm, lower panels; scale bar=100 μm), quantitation of β-cell mass (B and F), BrdU incorporation (C and G) and TUNEL staining (D and H) in wild-type (A-D) and GckKO (E-H) mice not treated (open bars) or treated (closed bars) with anagliptin (n =8-10). Sections were stained with anti-insulin antibody. I and J, protein levels of IRS-2, and phosphorylation level of CREB and ERK in the islets in wild-type (I) and GckKO (J) mice not treated (open bars) or treated (closed bars) with anagliptin (n =5-10). Values are means±S.E. of data obtained from the analysis of wild-type and GckKO mice. *, p<0.05.

3.7. Treatment with 0.05% anagliptin increased the β-cell mass in both wild-type and GckKO mice

We investigated the effects of 0.05% anagliptin on the β-cell mass in wild-type and GckKO mice, with no difference in the body weight or insulin sensitivity between the untreated and treated mice. After 10 weeks of treatment, 0.05% anagliptin significantly increased the β-cell mass in the wild-type mice (Fig. 7, A and B); the treatment also produced a slight, but significant increase of the β-cell mass in the GckKO mice (Fig. 7, E and F). However, the increment of β-cell mass tended to be lower in the GckKO mice than that of wild-type mice (0.26% in wild-type mice vs. 0.14% in GckKO mice) (Fig. 7, A, B, E and F), suggesting that the increment of β-cell mass by 0.05% anagliptin may be dependent, at least in part, on glucose metabolism via glucokinase. The percentage of cells incorporating BrdU was also significantly increased in both the 0.05% anagliptin-treated groups as compared with that in the untreated groups (Fig. 7, C and G). The percentage of TUNEL-positive cells tended to be decreased in the 0.05% anagliptin-treated wild-type mice as compared with that in the untreated wild-type mice, and the percentage of TUNEL-positive cells was significantly decreased in the 0.05% anagliptin-treated GckKO mice as compared with that in the untreated GckKO mice (Fig. 7, D and H). We next investigated phosphorylation of CREB and the protein level of IRS-2 in the islets of untreated and 0.05% anagliptin-treated mice. Phosphorylation of CREB and the protein level of IRS-2 were significantly increased in both the 0.05% anagliptin-treated groups as compared to the untreated groups (Fig. 7, I and J). However, the increment of CREB phosphorylation and up-regulation of IRS-2 tended to be less pronounced in the 0.05% anagliptin treated GckKO mice as compared with those in 0.05% anagliptin treated wild-type mice (CREB: 1.72%±0.07% in wild-type mice vs. 1.35%±0.14% in GckKO mice, IRS-2: 2.20%±0.10% in wild-type mice vs. 1.36%±0.12% in GckKO mice) (Fig. 7, I and J). On the other hand, the effect on ERK phosphorylation was not significantly different between the untreated and 0.05% anagliptin-treated mice of either genotype (Fig. 7, I and J).

4. Discussion

Decrease of the β-cell mass, as well as β-cell dysfunction and development of insulin resistance, has recently been reported to play crucial roles in the pathogenesis of type 2 diabetes [1,21]. In the present study, we investigated whether anagliptin might be capable of ameliorating insulin resistance and glucose intolerance in GckKO mice on a high-fat diet. High-dose (0.3%) anagliptin treatment decreased body weight gain via suppression of food intake, leading to improved glucose tolerance and amelioration of insulin resistance. On the other hand, low-dose (0.05%) anagliptin treatment, which had no apparent effect on the body weight or insulin sensitivity, improved glucose tolerance by enhancing insulin secretion and increasing the β-cell mass. These data suggest that high-dose anagliptin treatment improved glucose tolerance by suppression of body weight gain and amelioration of insulin

resistance, whereas low-dose anagliptin treatment improved glucose tolerance by enhancing insulin secretion.

However, the anagliptin-treated GckKO mice still showed sustained hyperglycemia, although anagliptin increased the active GLP-1 level in the GckKO mice, to degrees equivalent to those seen in the wild-type mice. Incretin effects may be, at least in part, dependent on the glucose metabolism. In fact, the effect of GLP-1 analogue on insulin secretion in response to glucose was attenuated in GckKO islets compared with WT islets [22]. Thus, impairment of glucose metabolism by haploinsufficiency of glucokinase is considered to be decreased GLP-1-induced insulin secretion, which may be the reason why anagliptin does not prevent the onset and development of diabetes. Considering that glucose metabolism in the β-cells is progressively impaired in type 2 diabetes [12], DPP-4 inhibitors and GLP-1 analogues may be more effective in the treatment of early-stage type 2 diabetes, in which milder abnormalities of glucose metabolism may be expected.

It has been reported from preclinical studies that GLP-1 analogues and DPP-4 inhibitors increase the β-cell mass through their effects of increasing β-cell proliferation and inhibiting β-cell apoptosis [23–26]. Shirakawa et al. reported that desfluorositagliptin (DFS), DPP-4 inhibitor, protected against β-cell apoptosis and restored the β-cell mass in GckKO mice fed with a diet containing sucrose and linoleic acid [22]. Low-dose anagliptin treatment indeed increased β-cell mass through stimulation of β-cell proliferation and inhibition of β-cell apoptosis in GckKO mice on a high-fat diet. GLP-1 analogues have been shown to increase the cAMP levels in human islets and MIN6 cells, which promote IRS-2 expression and stimulate Akt phosphorylation [27,28]. Moreover, GLP-1 analogue has been shown to increase the β-cell mass in wild-type mice, but not in IRS-2 KO mice, suggesting that insulin signaling via IRS-2 is essential for the effects of the GLP-1 analogue on the β-cell mass to be expressed [27]. Consistent with these results, low-dose anagliptin treatment increased β-cell mass along with the enhancement of CREB phosphorylation and IRS-2 expression.

Besides the CREB/IRS-2 pathway, ERK phosphorylation via cAMP/PKA has also been reported to be stimulated by the GLP-1 analogues, leading to increased expression of CyclinD1 and increased proliferation in pancreatic β-cell lines [29,30]. In the present study, however, the ERK phosphorylation level (Fig. 7, I and J) and CyclinD1 expression (data not shown) were not significantly different between the anagliptin-treated and untreated islets in either genotype of mice, although BrdU incorporation was elevated in the anagliptin-treated mice. These discrepancies may be caused by the difference in the GLP-1 activity between GLP-1 analogues and DPP-4 inhibitors. In the case of experiment using GLP-1, Gomez et al. treated MIN6 cells with 10 nmol/L GLP-1 to investigate GLP-1-stimulated ERK phosphorylation [29]. On the other hand, GLP-1 levels following treatment with anagliptin and other DPP-4 inhibitors were ~10 pmol/L [31]. Moreover, in the experiment conducted using INS-1 cells, GLP-1 produced short-term stimulation of ERK phosphorylation [30], suggesting that the ERK pathway may be involved in the short-term regulation of β-cell growth, while the CREB/IRS-2 pathway may be involved in the long-term regulation of β-cell growth.

Why did high-dose anagliptin fail to increase the β-cell mass or glucose-stimulated insulin secretion although the active GLP-1 levels were significantly increased? Expansion of the pancreatic β-cell mass and increase in insulin secretion are known to occur for maintaining normal glucose levels in the event of development of insulin resistance [21,32]; on the other hand, β-cell growth and insulin secretion are suppressed with the amelioration of insulin resistance. In fact, Gedulin et al. have reported absence of any increase of the β-cell mass or fasting plasma insulin levels in GLP-1 analogue-treated Zucker rats, which showed suppressed body weight gain and improved insulin sensitivity [33]; it is likely that the ameliorating effect of high-dose anagliptin on insulin resistance with suppression of body weight gain may have led to the absence of increase of the β-cell mass and insulin secretion. Instead, the elevated active GLP-1 might have obscured the suppression of insulin secretion due to amelioration of insulin resistance. In fact, the fasting plasma insulin and glucose levels tended to remain low in the high-dose anagliptin-treated mice.

Although both GLP-1 analogues and DPP-4 inhibitors have been shown to improve several indices of β-cell function, these two classes of drugs exert different effects on the body weight and appetite [34]. The differential effects on the body weight and appetite may be explained by the greater concentration of GLP-1 achieved with GLP-1 analogue treatment than with DPP-4 inhibition. In a clinical study comparing exenatide and sitagliptin, the mean 2-h plasma exenatide concentration was 64 pmol/L in patients treated with exenatide as compared to the mean 2-h postprandial plasma GLP-1 concentration of 15 pmol/L in patients treated with sitagliptin (baseline GLP-1 concentration 7.2 pmol/L) [35]. Considering that the plasma GLP-1 concentration was higher in the mice treated with high-dose anagliptin than in the mice treated with low-dose anagliptin in this study, the possibility that the difference in the plasma GLP-1 concentration between the two groups may account for the differential effects on the body weight and appetite cannot be excluded. However, unlike the effects of the GLP-1 receptor analogues, the effects of DPP-4 inhibitors on the body weight and appetite were small or absent in clinical studies [34]. Considering these findings, the low-dose, rather than high-dose, anagliptin used in this study may be more clinically relevant in the treatment of type 2 diabetes.

In conclusion, both low and high doses of anagliptin improved glucose tolerance in the high-fat diet-fed GckKO diabetic mice. These findings suggest that anagliptin could be a potentially efficacious agent for the treatment of type 2 diabetic patients.

Author contributions

N.K. researched the data, wrote the manuscript, contributed to the discussion, and reviewed and edited the manuscript. I.T., T.K., H.K., H.S. and K.T. researched the data and contributed to the discussion. S.H. contributed to the discussion. M.G. researched the data and contributed to the discussion. T.J. and K.U. contributed to the discussion. T.K. reviewed and edited the manuscript, and contributed to the discussion.

Conflicts of interest

The authors report no conflicts of interest.

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ORIGINAL ARTICLE

Combined treatment with low-dose pioglitazone and beraprost sodium improves glucose intolerance without causing body weight gain

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Abstract Pioglitazone (PIO) is widely used as an insulin-sensitizing agent in the treatment of type 2 diabetes, but it also causes an increase in body weight in a dose-dependent manner. Beraprost sodium (BPS), a stable prostaglandin I_2 analog, is used clinically for the treatment of peripheral arterial disease and primary pulmonary hypertension. BPS has recently been demonstrated to promote insulin-induced glucose uptake by skeletal muscle. In this study, we examined whether low-dose PIO (PIO-L; 3 mg/kg/day) plus BPS treatment might exert antidiabetic effects without causing body weight gain in obese/diabetic KKAy mice. Treatment with PIO-L plus BPS tended to improve the hepatic insulin resistance and significantly improved the skeletal muscle insulin resistance, yielding a similar degree of hyperglycemia improvement to that obtained with high-dose PIO (PIO-H; 30 mg/kg/day) treatment. Moreover, the increase in body weight observed following PIO-H treatment was not observed following PIO-L plus BPS treatment. An increase in plasma adiponectin levels, which have been shown to be negatively correlated with hepatic glucose production, but not with the glucose disappearance rate during hyperinsulinemic-euglycemic clamp, was observed in the animals treated with PIO-L, PIO-L plus BPS, and PIO-H. This suggests that the improvement in hepatic insulin resistance was associated, at least in part, with an increase in the plasma adiponectin levels observed during PIO treatment. In contrast, treatment with BPS, which failed to increase the plasma adiponectin levels, ameliorated skeletal, but not hepatic insulin resistance. PIO-L plus BPS treatment may represent efficacious treatment for insulin resistance and type 2 diabetes without causing body weight gain.

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Introduction

Obesity-linked insulin resistance is a key factor in the development of type 2 diabetes and metabolic syndrome, eventually leading to micro- and macrovascular complications. Thiazolidinediones (TZDs) have been shown to enhance the actions of insulin in the liver and skeletal muscle in animal models of obesity-linked insulin resistance and also in subjects with type 2 diabetes [1, 2]. TZDs bind to and activate the ligands of peroxisome proliferator-activated receptor-γ (PPAR-γ), thereby promoting adipose tissue differentiation, an increase in the number of small adipocytes, and an increase in the expression and secretion of adiponectin [3]. Increased production of adiponectin has been reported to improve insulin sensitivity, including glucose metabolism in the liver, where adiponectin decreases gluconeogenesis via activation of AMP-activated protein kinase (AMPK) [4, 5]. These beneficial effects of TZDs have made them attractive agents for the treatment of type 2 diabetes mellitus [6]. There has been growing recognition, however, that treatment with TZDs is associated with body weight gain and edema. The body weight gain and edema associated with the use of TZDs can be a cause for concern, as it may be a harbinger or sign of congestive heart failure (CHF), especially in subjects with type 2 diabetes who are at increased risk for cardiovascular diseases (CVD). These side effects of TZDs have limited their clinical use [7]. Recently, it has been reported that the body weight gain associated with low-dose TZD treatment may be significantly more modest than that associated with high-dose TZD treatment [8, 9]. On the other hand, low-dose TZDs may not produce adequate improvement in insulin resistance and glucose intolerance [10, 11].

Beraprost sodium (BPS), which is a stable prostaglandin I_2 (PGI_2) analog, binds to the PGI_2 receptor and increases cellular cyclic adenosine monophosphate (cAMP) levels in the platelets and smooth muscle cells [12, 13] and has been used clinically for the treatment of peripheral arterial disease and primary pulmonary hypertension [14, 15]. In addition, BPS increases the expression levels of eNOS mRNA and protein through cAMP-, protein kinase A (PKA)-, and cAMP-responsive element-mediated pathways in endothelial cells [16]. We recently reported that restoration of insulin-induced eNOS activation by BPS treatment improved the skeletal muscle glucose uptake in the HF diet-fed obese mice [17].

In the present study, we investigated whether combined treatment with low-dose pioglitazone (PIO-L) plus BPS might yield sufficient improvement of the insulin resistance and glucose intolerance in obese diabetic KKAy mice and also whether the use of this combination treatment might prevent PIO-induced body weight gain. The results revealed that combined PIO-L plus BPS treatment improved hyperglycemia and insulin resistance to a degree similar to that obtained with high-dose PIO (PIO-H) treatment; in addition, no significant change in the body weight of the animals was noted. These results suggest that combined use of PIO-L plus BPS may be one of the useful therapeutic strategies for patient with type 2 diabetes.

Materials and methods

Animal preparation and experimental protocol

Male KKAy mice (5–7 weeks old) were purchased from CLEA Japan (Tokyo, Japan) and housed individually under a 12-h light/dark cycle. The animals were allowed access to water ad libitum. Pioglitazone was suspended in 0.5 % methylcellulose (MC). Low-dose pioglitazone (PIO-L; 3 mg/kg/day), high-dose pioglitazone (PIO-H; 30 mg/kg/day), or vehicle (0.5 % MC) was administered orally once daily for 20 consecutive days. The total administration volume was 0.01 ml/g body weight of the mice. On day 6 after the start of PIO treatment, a mini-osmotic pump (model 1002, ALZA Corp.) filled with BPS (1 mg/kg/day) or saline was implanted subcutaneously in the KKAy mice for 14 days (Supplementary Figure S1). The animals' body weight was monitored daily. The rate of increase in body weight was calculated by dividing the difference in body weight between the first and final day by that on the first day. BPS and pioglitazone were provided by Toray Industries, Inc., Tokyo, Japan. The animal care and experimental procedures used in this study were approved by the Animal Care Committee of the University of Tokyo.

Hyperinsulinemic-euglycemic clamp

The hyperinsulinemic-euglycemic clamp study was carried out as described previously [18], with slight modifications. In brief, an infusion catheter was inserted into the right jugular vein of the test animals under sodium pentobarbital anesthesia. The clamp studies were performed under conscious and unstressed conditions after the animals had been denied access to food for 4 h. To measure the glucose infusion rate (GIR), a primed continuous infusion of insulin (Humulin R, Lilly) was administered (10 mU/kg/min), and the blood glucose concentration, monitored every 5 min, was maintained at approximately 120 mg/dl by administration of glucose [5 g of glucose per 10 ml enriched to about 20 % with [6,6-^{2}H$_2$]glucose (Sigma)] for 120 min. Blood samples (20 µl) were obtained via tail-tip bleeds at 90, 105, and 120 min for determination of the rate of glucose disappearance (Rd). Rd was calculated according to nonsteady-state equations, and hepatic glucose production (HGP) was calculated as the difference between the Rd and GIR.

Glucose tolerance test

After being denied access to food for 16 h, the mice were loaded orally with glucose at 1.5 mg/g body weight, as described previously [18]. Blood samples were collected at 15, 30, 60, and 120 min after glucose loading. Blood glucose measurements were performed using an automatic glucometer (Glutest Ace, Sanwa Chemical Co., Nagoya, Japan).

Blood sample assay

Plasma insulin and adiponectin levels were determined using the mouse insulin (Morinaga, Yokohama, Japan) and adiponectin (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) ELISA kits, respectively. Plasma triglyceride (TG), free fatty acid (FFA), and total cholesterol (T-chol) (Wako Pure Chemical Industries Ltd., Osaka, Japan) were assayed by enzymatic methods.

Statistical analysis

Values are expressed as mean $\pm$ SEM. The statistical significance of all possible pairwise differences of the means was determined by the Tukey-Kramer test. Statistical significance was set at $p \leq 0.05$.
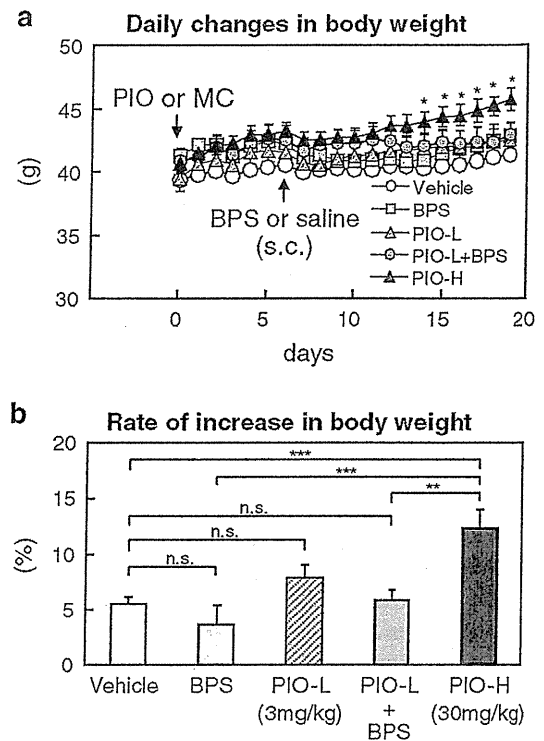
Results

Combined PIO-L plus BPS treatment did not produce any significant increase in body weight of KKAy mice

Although a significant increase in body weight was observed in KKAy mice treated with PIO-H, as compared with that in the vehicle-treated mice, from 14 days after the start of treatment, no such increase was observed in the animals treated with BPS, PIO-L, or PIO-L plus BPS (Fig. 1a). The rate of body weight increase was significantly higher in the animal group treated with PIO-H than in the groups treated with vehicle, BPS, or PIO-L plus BPS (Fig. 1b). The rate of body weight increase in the animals treated with BPS, PIO-L, or PIO-L plus BPS was not significantly different from that in the animals treated with vehicle (Fig. 1b).

Combined PIO-L plus BPS treatment tended to improve the hepatic insulin resistance and significantly improved the skeletal muscle insulin resistance in KKAy mice

Although the fasting blood glucose levels were not significantly different among the treatment groups (Fig. 2a), fasting plasma insulin levels in KKAy mice treated with

Fig. 1 Combined PIO-L plus BPS treatment produced no significant increase in body weight in KKAy mice. Daily changes (a) and rate of increase of the body weight (b) in KKAy mice treated with vehicle, BPS, PIO-L, PIO-L plus BPS, or PIO-H. Values are mean $\pm$ SEM ($n = 5$–12). $*p \leq 0.05$ (a), $**p \leq 0.01$, $***p \leq 0.001$ (b). n.s. not significant

BPS, PIO-L, PIO-L plus BPS, or PIO-H were significantly decreased as compared with the values in the vehicle-treated KKAy mice (Fig. 2b). These data suggest that both BPS and PIO treatment improved the insulin resistance in KKAy mice. In fact, in the hyperinsulinemic-euglycemic clamp studies carried out in these mice, the glucose infusion rate (GIR) in KKAy mice treated with BPS, PIO-L plus BPS, or PIO-H were significantly increased compared with the rate in the vehicle-treated KKAy mice (Fig. 2c). Consistent with results in respect to the fasting insulin levels, the GIR tended to be higher in the animals receiving PIO-L treatment than in those treated with vehicle. Although no increase in the glucose disappearance rate (Rd) was observed in either the animals treated with PIO-L or those treated with PIO-H, the Rd was significantly higher in the animals treated with BPS or PIO-L plus BPS than in those treated with vehicle (Fig. 2d). In contrast, hepatic glucose production (HGP) was significantly suppressed following PIO-H treatment and tended to be lower in the animals treated with PIO-L or PIO-L plus BPS than in those treated with vehicle. No such decrease in HGP was observed following BPS treatment. These data suggest that PIO and BPS improved the hepatic and skeletal muscle insulin resistance, respectively (Fig. 2d, e).

Fig. 2 Combined PIO-L plus BPS treatment ameliorated insulin resistance in KKAy mice. Fasting blood glucose (a) and plasma insulin (b) levels in KKAy mice treated with vehicle, BPS, PIO-L, PIO-L plus BPS, or PIO-H. Glucose infusion rates (GIR) (c), rates of glucose disappearance (Rd) (d), and hepatic glucose production (HGP) (e) in KKAy mice treated with vehicle, BPS, PIO-L, PIO-L plus BPS, or PIO-H during the hyperinsulinemic-euglycemic clamp study. Values are mean ± SEM ($n = 4$–9). $*p < 0.05$, $**p < 0.01$, $***p \le 0.001$. n.s. not significant
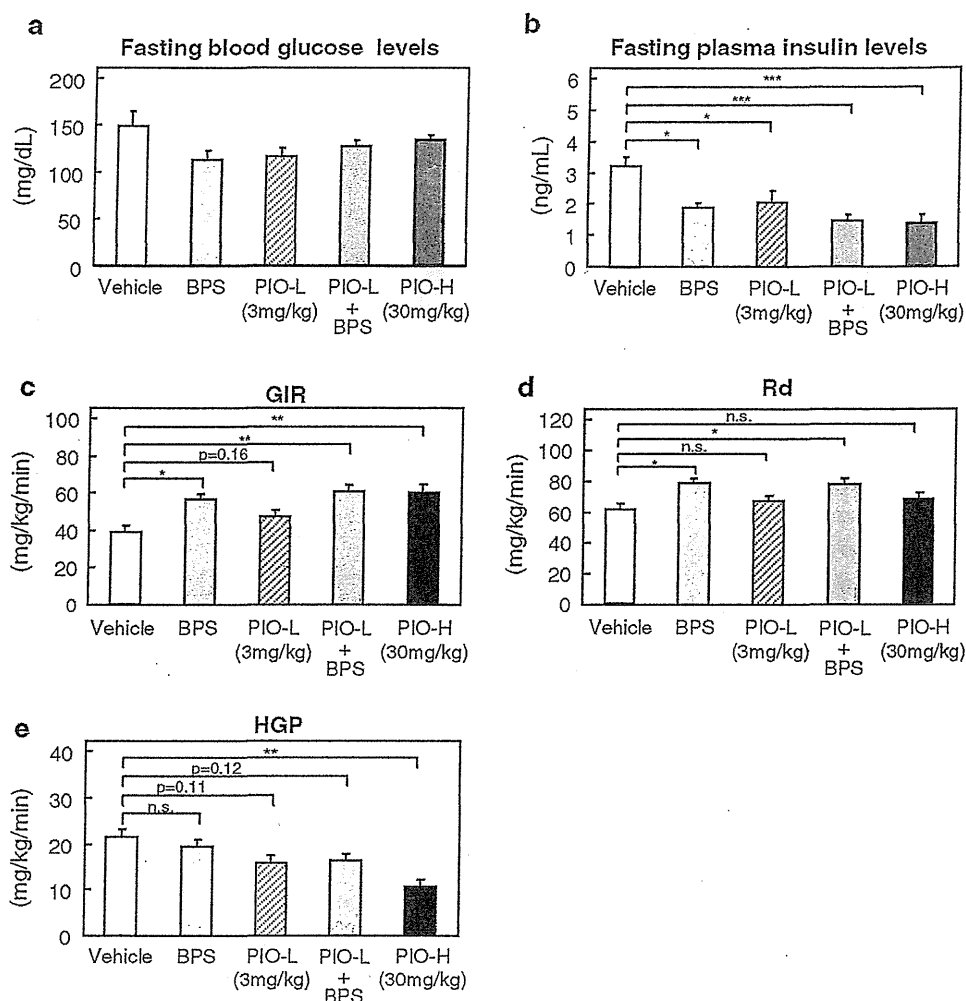
Combined PIO-L plus BPS treatment improved the glucose intolerance in KKAy mice

In the random-fed state, blood glucose levels in the PIO-H- or PIO-L plus BPS-treated KKAy mice were significantly lower as compared with the levels in the vehicle-treated KKAy mice, while the blood glucose levels in latter groups were not significantly different from the value in the vehicle-treated animals (Fig. 3a). Consistent with these data, a significant decrease in the blood glucose levels after glucose loading during the oral glucose tolerance test (OGTT) was observed in the animals treated with PIO-H or PIO-L plus BPS, whereas PIO-L or BPS treatment produced no significant lowering of the blood glucose (Fig. 3b).

Plasma adiponectin levels were significantly correlated with the hepatic glucose production

Since adiponectin production induced by TZD treatment has been reported to improve insulin resistance [4, 5], we next measured the plasma adiponectin levels. Plasma adiponectin levels in the PIO-L, PIO-L plus BPS, and PIO-H treatment

groups were significantly increased as compared with the values in the vehicle treatment group, but remained unchanged in the BPS treatment group (Fig. 4a). Furthermore, the plasma adiponectin levels in the PIO-H-treated KKAy mice were significantly higher than those in the BPS-, PIO-L-, or PIO-L plus BPS-treated KKAy mice (Fig. 4a). We next investigated whether the increased plasma adiponectin levels might be correlated with improvement of insulin resistance in KKAy mice. Plasma adiponectin levels and the HGP, but not the Rd, were negatively correlated, suggesting that the increase in plasma adiponectin levels induced by PIO treatment contributed to the improvement of the hepatic, but not the skeletal muscle insulin resistance (Fig. 4b, c).

Combined PIO-L plus BPS treatment decreased the plasma TG levels in KKAy mice

Plasma triglyceride (TG) levels were significantly reduced following PIO-L plus BPS treatment, although no such decrease was observed following treatment with BPS, PIO-L, or PIO-H (Fig. 5a). No significant differences in the

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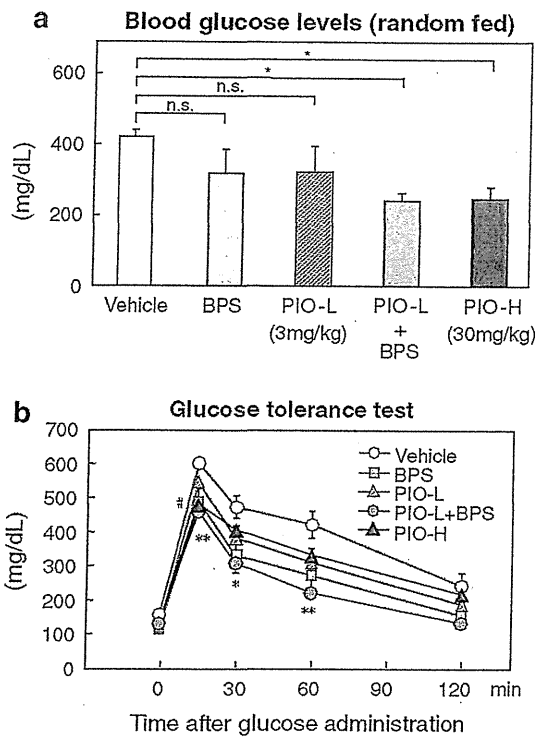
Fig. 3 Combined PIO-L plus BPS treatment ameliorated glycemic control in KKAy mice. a Blood glucose levels in random-fed KKAy mice treated with vehicle, BPS, PIO-L, PIO-L plus BPS, or PIO-H. b Glucose tolerance test results in KKAy mice treated with vehicle, BPS, PIO-L, PIO-L plus BPS, or PIO-H. Values are mean ± SEM (n = 5–9). *p ≤ 0.05, **p ≤ 0.01 (in PIO-L plus BPS), #p ≤ 0.05 (in PIO-H) compared to vehicle treatment. n.s. not significant

plasma-free fatty acid (FFA) or total cholesterol (T-chol) levels were noted among the five treatment groups in this study (Fig. 5b, c).

Discussion

In this study, we demonstrated that PIO-L plus BPS treatment tended to improve the hepatic insulin resistance and significantly improved the skeletal muscle insulin resistance, producing a similar degree of improvement of hyperglycemia as that observed following PIO-H treatment, but without the body weight gain observed in the PIO-H-treated mice. PIO treatment increased the plasma adiponectin levels in a dose-dependent manner, which may have contributed to improvement in the insulin resistance in the liver. On the other hand, BPS treatment ameliorated the skeletal muscle insulin resistance, but not the hepatic insulin resistance. These data suggest that combined PIO-L plus BPS treatment may be a safe and effective anti-diabetic treatment strategy in patients with type 2 diabetes.

In our previous study, low-dose PIO treatment significantly improved the insulin resistance and ameliorated
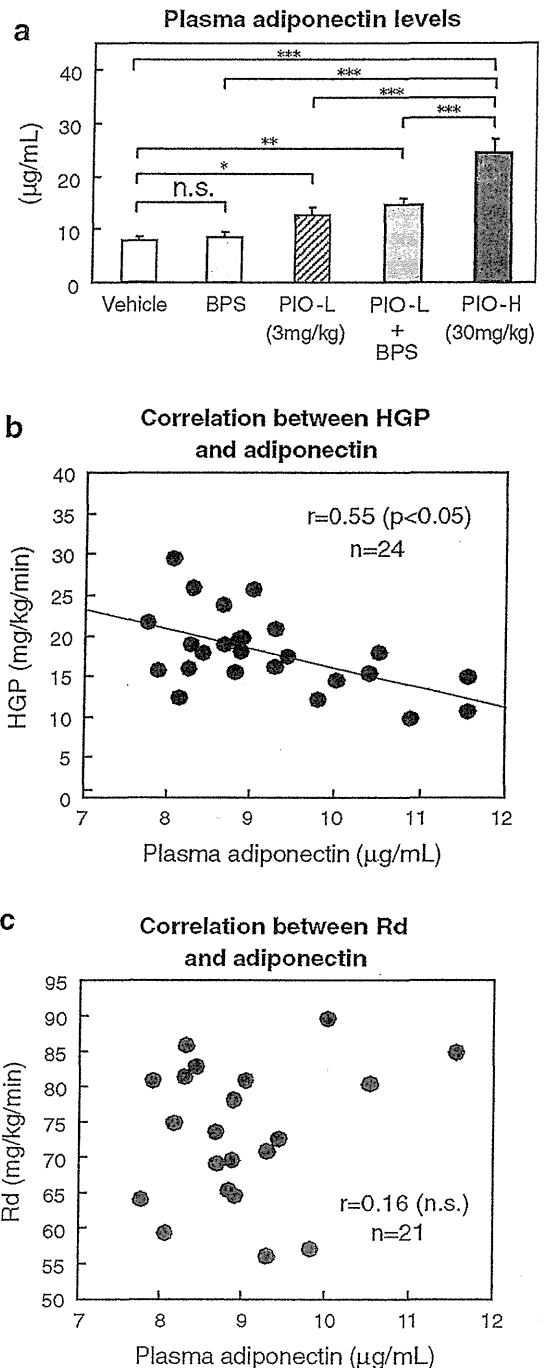
Fig. 4 Plasma adiponectin levels were significantly correlated with hepatic glucose production. a Plasma adiponectin levels in KKAy mice treated with vehicle, BPS, PIO-L, PIO-L plus BPS, or PIO-H. Correlation between the plasma adiponectin levels and hepatic glucose production (HGP) (b) and rates of glucose disappearance (Rd) (c). Values are mean ± SEM (n = 5–10). *p < 0.05, **p < 0.01, ***p < 0.001. n.s. not significant

diabetes in an adiponectin-dependent manner in ob/ob mice. The increase in the plasma adiponectin levels following low-dose PIO treatment was attributed to a decrease in glucose production and increase in AMP-activated
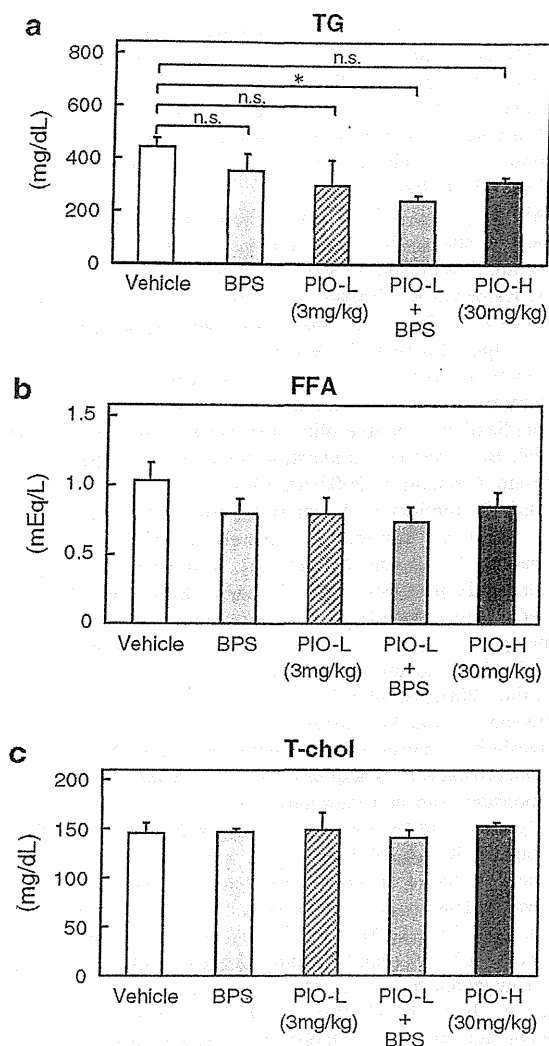
Fig. 5 Combined PIO-L plus BPS treatment decreased plasma TG in KKAy mice. Plasma triglyceride (TG) (a), free fatty acid (FFA) (b), and total cholesterol (T-chol) (c) levels in KKAy mice treated with vehicle, BPS, PIO-L, PIO-L plus BPS, or PIO-H. Values are mean ± SEM ($n = 5-9$). $*p < 0.05$. n.s. not significant

protein kinase (AMPK) activation in the liver [10]. Consistent with these data [10], PIO-L treatment in this study increased the plasma adiponectin levels and tended to decrease the HGP in KKAy mice. The plasma adiponectin concentrations were closely related to the HGP (Fig. 4b). These data suggest that the amelioration of hepatic insulin resistance induced by PIO treatment in KKAy mice was mediated, at least in part, via an increase in the plasma adiponectin levels. On the other hand, PIO-H treatment has been shown to ameliorate skeletal muscle insulin resistance in ob/ob mice in an adiponectin-independent manner, accompanied by reduced plasma TG and FFA levels [10]. Since KKAy mice are known to show greater severities of hyperlipidemia than ob/ob mice [19], it is speculated that the plasma TG and FFA levels in the PIO-H-treated KKAy mice were not sufficiently decreased, which may be the

reason why the PIO-H treatment failed to improve the skeletal muscle insulin resistance in the KKAy mice.

Skeletal muscle is one of the major target organs of insulin actions and plays an essential role in insulin-induced glucose uptake, giving it a central role in the development of insulin resistance. We recently demonstrated that impaired insulin signaling in the endothelial cells, with reduction of insulin-induced eNOS phosphorylation, reduces insulin-induced glucose uptake by the skeletal muscle, decreased capillary recruitment, and decreased interstitial insulin concentrations in the skeletal muscle [17]. We found that in the HF diet-fed mice, BPS treatment significantly restored the eNOS mRNA and protein expression levels and also the insulin-induced phosphorylation of eNOS to levels similar to those observed in the saline-treated normal chow-fed mice. Restoration of the insulin-induced eNOS phosphorylation in the endothelial cells by BPS treatment also restored the insulin-induced capillary recruitment and interstitial insulin concentrations, resulting in improvement of the skeletal muscle glucose uptake in the HF diet-fed obese mice [17]. Therefore, in the present study also, BPS treatment might have improved the skeletal muscle insulin resistance in KKAy mice via these same mechanisms. Similarly, a significant increase of the insulin-stimulated glucose uptake following infusion of iloprost, a PGI_2 analog, has been reported during a hyperinsulinemic-euglycemic clamp study in subjects with type 2 diabetes [20]. Thus, PGI_2 analogs, including BPS, may serve as novel agents for the treatment of patients with type 2 diabetes with skeletal muscle insulin resistance.

Currently, many patients with type 2 diabetes are receiving treatment with TZDs in combination with other antidiabetic agents. Roy et al. [21] showed that low-dose rosiglitazone (1 mg/kg/day) administered with a DPP4 inhibitor (vildagliptin) yielded similar efficacy to that of high-dose rosiglitazone (10 mg/kg/day) treatment in respect to lowering the blood glucose levels without producing any significant increase in the body weight in obese diabetic db/db mice. These data suggest that low-dose TZD administration in combination with other anti-diabetic agents may also be sufficiently effective for lowering the blood glucose levels, without producing any clinically relevant adverse events [22]. BPS may be one such suitable candidate for administration in combination with low-dose TZDs.

In conclusion, combined PIO-L plus BPS treatment tended to improve the hepatic insulin resistance and significantly improved the skeletal muscle insulin resistance, thereby improving glucose intolerance in KKAy mice to a degree similar to that observed following PIO-H treatment, without causing any body weight gain. This combination therapy therefore appears to be safe and effective for patients with type 2 diabetes mellitus.