indicate that insulin-induced capillary recruitment preceded the increase in total blood flow.

In our experimental results of normal mice, although capillary recruitment was observed 10 min after insulin infusion, no such increase was observed in the interstitial insulin concentration. An increase of the interstitial concentrations of insulin was observed 60 min after insulin infusion. Consistent with the results for the interstitial concentrations of insulin, the phosphorylation levels of Akt in the skeletal muscle were increased 60 min after insulin infusion. [38]. These data, along with careful studies by Miles [16], demonstrate that capillary recruitment increases slowly, followed by interstitial insulin concentration and tyrosine phosphorylation of the skeletal muscle insulin receptor, whereas plasma insulin levels rise immediately after the insulin infusion. However, 2 h or more is typically required to see the effects of insulin on blood flow.

5.2.1 Insulin signaling in the endothelial cells regulates capillary recruitment

Endothelium-derived NO is known to mediate insulin-induced microvascular perfusion in skeletal muscle in animals and humans [38, 64–66]. In fact, eNOS knockout mice showed impaired insulin-induced capillary recruitment in the skeletal muscle [38, 64]. N-monomethyl-L-arginine (L-NMMA), an NO synthase (NOS) inhibitor, blocked capillary recruitment in response to insulin in the skeletal muscle [65]. Moreover, inhibiting NOS diminished insulin-induced capillary recruitment in the skeletal muscle in humans [66]. This finding suggests that capillary recruitment is regulated in an NO-dependent manner. The correlations between insulin signaling in the endothelial cells and capillary recruitment in the skeletal muscle were elucidated in the endothelial-cell-specific IRS-2 knockout (ETIRS2KO) mice. The insulin-induced eNOS phosphorylation, capillary recruitment and increases of interstitial concentrations of insulin were impaired in the ETIRS2KO mice. Furthermore, decreased insulin-induced glucose uptake by the skeletal muscle was observed in the ETIRS2KO mice after insulin infusion. Consistent with these results, the phosphorylation levels of Irβ in the skeletal muscle were also decreased in the ETIRS2KO mice after insulin infusion [38]. These data suggest that IRS-2 deletion in the endothelial cells causes an insulin signaling defect in the cells that results in impairment of insulin-induced eNOS activation, capillary recruitment and increase of the interstitial concentrations of insulin, consequently impairing the skeletal muscle glucose uptake. Some evidence indicates that IRS-1, as well as IRS-2, may play an important role in the endothelial cells. The activation of Jun N-terminal protein kinase (JNK) by IL-6 increased IRS-1 serine phosphorylation resulting in impairment of the vasodilator effects of insulin [67]. Moreover, insulin-stimulated Akt and eNOS activation were impaired in cultured human endothelial cells carrying the IRS-1 gene G972R variant, which is associated with an impairment of the ability of IRS-1 to recruit the p85 regulatory subunit of PI3 kinase [68]. The role of IRS-1 expressed in the endothelial cells in the regulation of insulin-induced glucose uptake by the skeletal muscle was investigated using the endothelial-cell-specific IRS-1 knockout (ETIRS1KO) mice. The insulin-stimulated phosphorylation levels of Akt or eNOS in the ETIRS1KO mice remained unchanged. ETIRS1KO mice did not show skeletal muscle insulin resistance. Moreover, endothelial-cell-specific IRS-1/IRS-2 double-knockout (ETIRS1/2DKO) mice were used to address the role of IRS-1 and IRS-2 in the endothelial cells. The insulin-stimulated phosphorylation of Akt and eNOS were almost completely abolished in the ETIRS1/2DKO mice. Insulin-induced glucose uptake by the skeletal muscle in the ETIRS1/2DKO mice was reduced as compared with the values not only in the control mice, but also in the ETIRS2KO mice [38]. These data suggest that IRS-1 may play a significant role in endothelial cell insulin signaling. This becomes evident especially when IRS-2 expression is downregulated, such as in the ETIRS2KO and HF diet-fed mice.

5.2.2 eNOS activation increases insulin-induced capillary recruitment and glucose uptake by the skeletal muscle

To determine whether restoration of the insulin-induced eNOS phosphorylation in the endothelial cells might restore the glucose uptake by the skeletal muscle, beraprost sodium (BPS), a stable prostaglandin $(PGI)_2$ analogue [69], was administered to the ETIRS2KO mice. This agent has been reported to increase the expression levels of eNOS mRNA and protein through the cyclic adenosine monophosphate (cAMP)-, protein kinase A- and cAMP-responsive element-mediated pathways [70], and is clinically used for the treatment of peripheral artery disease and primary pulmonary hypertension [71, 72]. Indeed, this treatment increased the eNOS mRNA and protein expression levels in the endothelial cells. BPS treatment restored insulin-induced phosphorylation of eNOS in the ETIRS2KO mice. The insulin-induced capillary recruitment and increase of interstitial concentrations of insulin were restored in the BPS-treated ETIRS2KO mice. The restoration of the insulin-induced capillary recruitment by BPS treatment in the ETIRS2KO mice was completely blocked by administration of the NOS inhibitor, N^G-nitro-$_L$-arginine methyl ester ($_L$-NAME), suggesting that the restoration of the insulin-induced capillary recruitment by BPS treatment was NO-dependent. Moreover, glucose uptake by the skeletal muscle was completely restored in the BPS-treated ETIRS2KO mice. Consistent with the results for the interstitial insulin concentrations, the phosphorylation levels of Irβ in the skeletal muscle were also completely restored in the BPS-treated ETIRS2KO mice after insulin infusion [38].

NO-dependence has also been supported by the use of an agent other than BPS. Glucagon-like peptide 1(GLP-1),

which increased insulin secretion from the β cells and is clinically used for the treatment of type 2 diabetes [73], has been known to improve endothelial function [74–76]. Recently, Chai et al. reported that GLP-1 increased insulin-induced capillary recruitment and glucose uptake by the skeletal muscle in normal rats; these effects were abolished by L-NAME treatment [77]. These data suggest that eNOS activation in endothelial cells increases the insulin-induced capillary recruitment and interstitial concentrations of insulin, consequently promoting the insulin-induced glucose uptake by the skeletal muscle.

5.2.3 Insulin-induced capillary recruitment and glucose uptake by the skeletal muscle were impaired in obesity-related insulin resistance - effects restored by eNOS activation

Clark and colleagues reported that insulin resistance, generated either by infusion of free fatty acids [78], TNFα [79, 80], pharmacological blockade [81], or in obese Zucker rats [82], was accompanied by impairments in both insulin-induced capillary recruitment and glucose uptake by the skeletal muscle. There are also some data that indicate impairment of insulin-induced capillary recruitment in type 2 diabetes subjects [83–86]. In HF diet-fed mice, a series of changes in eNOS activation, capillary recruitment, interstitial concentrations of insulin and activation of the myocyte Akt after insulin infusion were carefully observed. Unlike in normal mice, eNOS activation and capillary recruitment were significantly impaired along with IRS-2 downregulation, presumably due to hyperinsulinemia and the significant decrease in the level of interstitial insulin in the HF diet-fed mice after insulin infusion. Consistent with the results for the interstitial concentrations of insulin, the phosphorylation levels of Akt and glucose uptake by the skeletal muscle also decreased in the HF diet-fed mice after insulin infusion [38]. These data suggest that the decreased eNOS activation appears to be involved along with the impaired insulin-induced capillary recruitment and increase

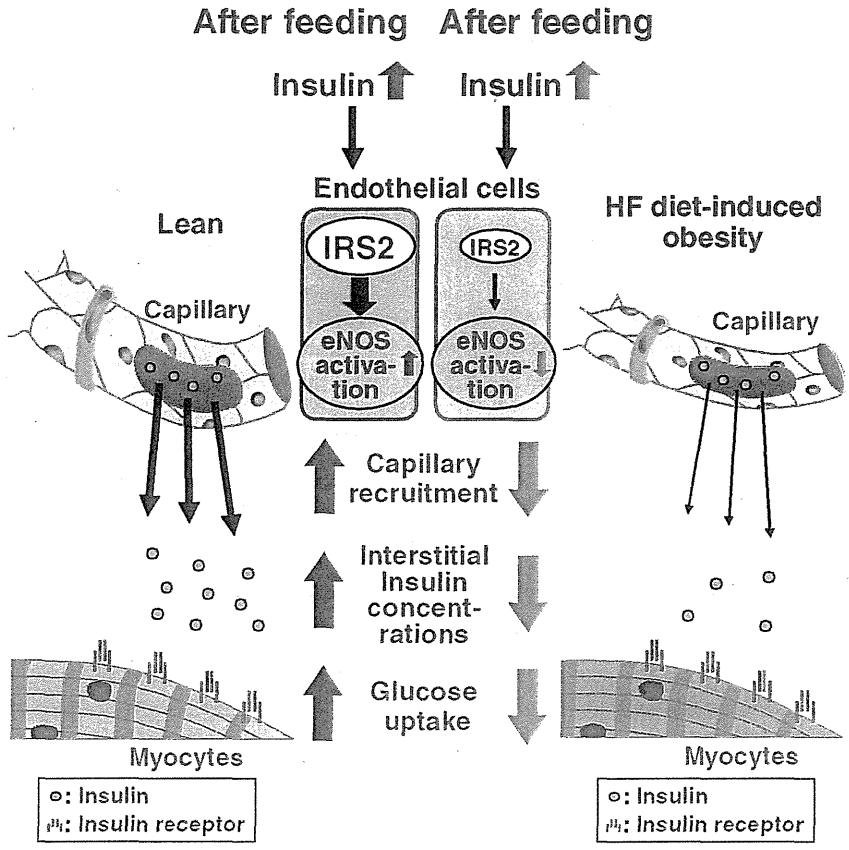

Fig. 4 Impaired insulin signaling in the endothelial cells reduces insulin-induced glucose uptake by the skeletal muscle in obese subjects. Since the plasma insulin levels of lean subjects are low, and the expression levels of IRS-2 in their endothelial cells are maintained under the fasting conditions, after feeding, insulin-mediated Akt and eNOS activations are induced optimally, resulting in insulin-induced capillary recruitment, increase of interstitial insulin concentrations, and glucose uptake by the skeletal muscle. By contrast, since downregulation of IRS-2 expression is induced by hyperinsulinemia in the endothelial cells of obese subjects, the insulin-mediated Akt and eNOS activations after feeding are inadequate, and as a result, insulin-induced capillary recruitment, increase of interstitial insulin concentrations, and glucose uptake by the skeletal muscle are impaired

of the interstitial concentrations of insulin in the impaired glucose uptake by the skeletal muscle in the HF diet-fed obese mice.

To investigate whether the restoration of insulin-induced eNOS phosphorylation in endothelial cells can equivalently ameliorate the impaired glucose uptake by the skeletal muscle in the HF diet-fed mice, BPS was administered to these mice. BPS treatment in the HF diet-fed mice restored insulin-induced phosphorylation of eNOS. The decreased capillary recruitment and interstitial concentrations of insulin observed in the HF diet-fed mice were restored after BPS treatment. NO-dependency was confirmed by the fact that the restoration of the capillary recruitment by BPS treatment in the HF diet-fed mice was completely blocked by L-NAME treatment. Consequently, the insulin-induced glucose uptake was significantly, but not completely, restored by BPS treatment. Consistent with the results for glucose uptake, the insulin-induced phosphorylation levels of Irβ in the skeletal muscle were significantly, but not completely, restored following BPS treatment in the HF diet-fed mice. The glucose uptake by the skeletal muscle isolated from HF diet-fed mice remained essentially unchanged after BPS treatment, indicating that BPS treatment does not mitigate the HF diet-induced impairment of glucose uptake by the skeletal muscle per se. This may explain why insulin-induced glucose uptake was not completely improved by BPS treatment, although both capillary recruitment and interstitial insulin concentration were completely restored by BPS treatment. Taken together, restoration of insulin-induced eNOS activation in endothelial cells restored the insulin-induced capillary recruitment and interstitial insulin concentrations, resulting in improvement of skeletal muscle glucose uptake in the HF diet-fed obese mice. Iloprost infusion, another PGI_2 analogue, improved insulin-stimulated whole-body glucose uptake in type 2 diabetes subject. Jansson et al. reported that the selective PDE-5 inhibitor tadalafil, which intensifies the effect of NO in smooth muscle cells [26, 87], improved capillary recruitment and forearm glucose uptake in insulin resistance with type 2 diabetes [88].

6 Conclusion

Recent studies have provided us with great insight into the mechanism of insulin resistance in skeletal muscle (Fig. 4). Since the plasma insulin levels of lean subjects are low, and the expression levels of IRS-2 in their endothelial cells are presumably maintained under the fasting condition, insulin-mediated Akt and eNOS activations are induced optimally after feeding, resulting in insulin-induced capillary recruitment, increase of interstitial insulin concentrations, and increase of glucose uptake by the skeletal muscle. In contrast, since downregulation of IRS-2 expression is probably induced by hyperinsulinemia in the endothelial cells of obese subjects, the insulin-mediated Akt and eNOS activations after feeding are inadequate, and as a result, insulin-induced capillary recruitment, increase of interstitial insulin concentrations, and increase of glucose uptake by the skeletal muscle are impaired in obese subjects. Restoration of the insulin-induced eNOS phosphorylation in the endothelial cells completely reversed the reduction in the capillary recruitment and insulin delivery, and as a result, significantly restored glucose uptake by the skeletal muscle. Taken together, treatment directed at improving insulin signaling in the endothelial cells as well as myocytes may serve as a novel therapeutic strategy for ameliorating skeletal muscle insulin resistance.

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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.

Abbreviations: DPP-4, dipeptidyl peptidase-4; GLP-1, glucagon-like peptide-1; IRS-2, insulin receptor substrate-2; CREB, cAMP response element-binding protein; Gck, glucokinase; GIR, glucose infusion rate(s); EGP, endogenous glucose production; R_d, rate of glucose disappearance; ITT, insulin tolerance test; OGTT, oral glucose tolerance test.

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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-$^2H_2$]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_3VO_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

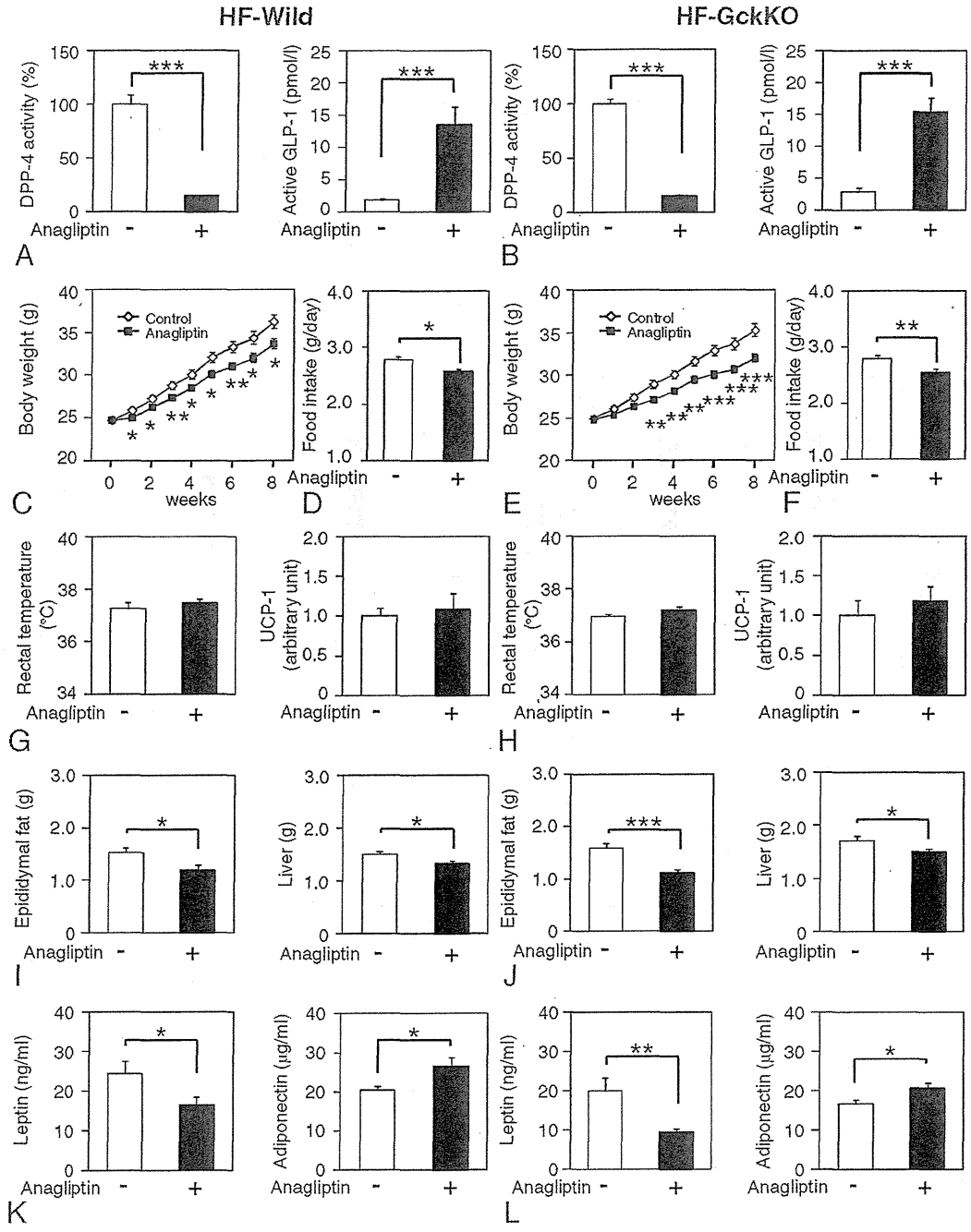

Fig. 1 – Treatment with 0.3% anagliptin decreased body weight gain and food intake. A and B, DPP-4 activities (left panels) and active GLP-1 levels (right panels) in wild-type (A) and GckKO (B) mice not treated (open bars) or treated (closed bars) with anagliptin (n = 11–12). C and D, body weights (left 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-

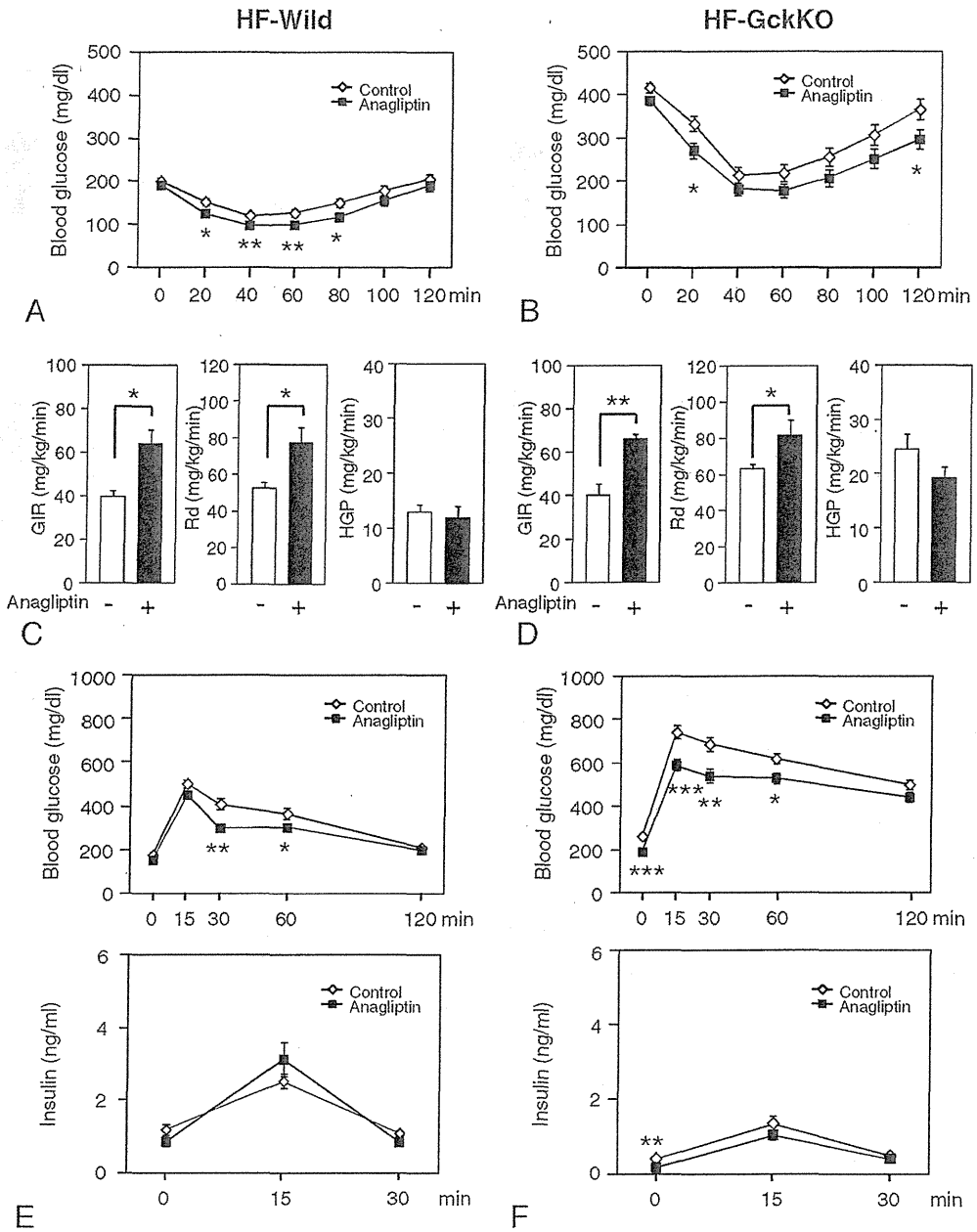

Fig. 2 – Treatment with 0.3% anagliptin improved insulin resistance and glucose tolerance in the wild-type and GckKO mice. A and B, blood glucose levels during the ITT in wild-type (A) and GckKO (B) mice not treated (open diamonds) or treated (closed squares) with anagliptin (n = 30). C and D, GIR (left 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

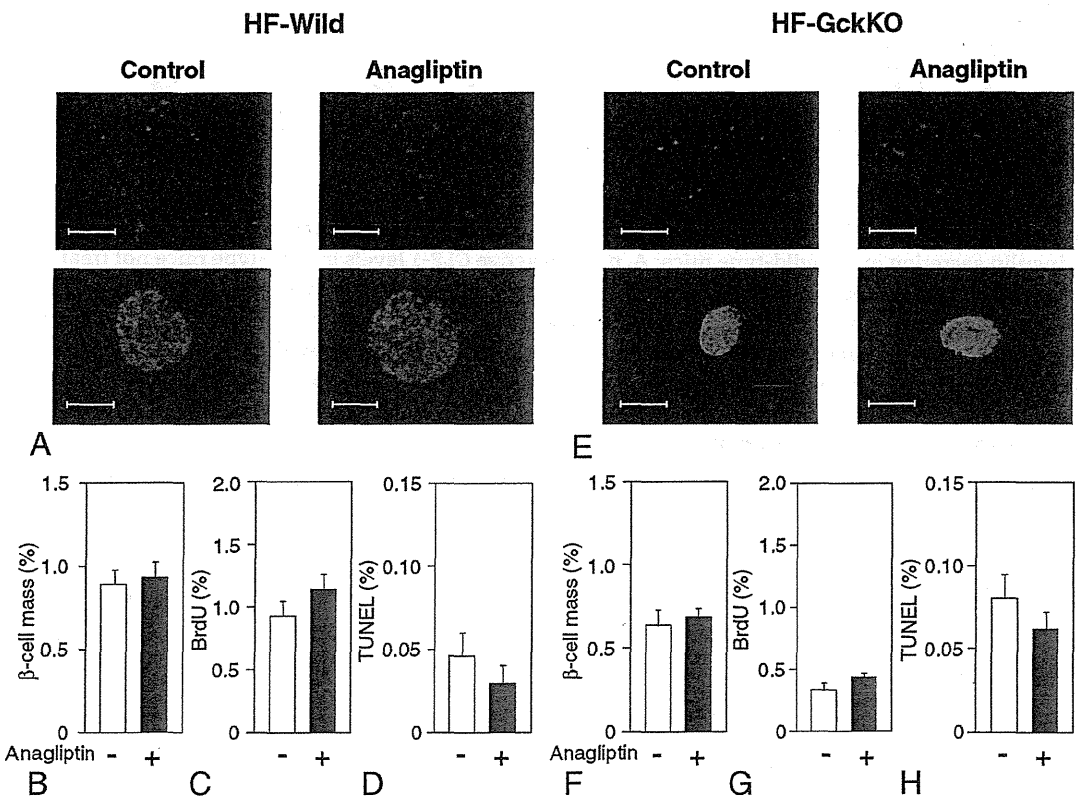

Fig. 3 – Effect of 0.3% anagliptin on the β-cell mass. Histological analysis of pancreatic β-cells (A and E) (upper panels; scale bar = 1000 μm, lower panels; scale bar = 100 μm), quantitation of β-cell mass (B and F), BrdU incorporation (C and G) and TUNEL staining (D and H) in wild-type (A–D) and GckKO (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.

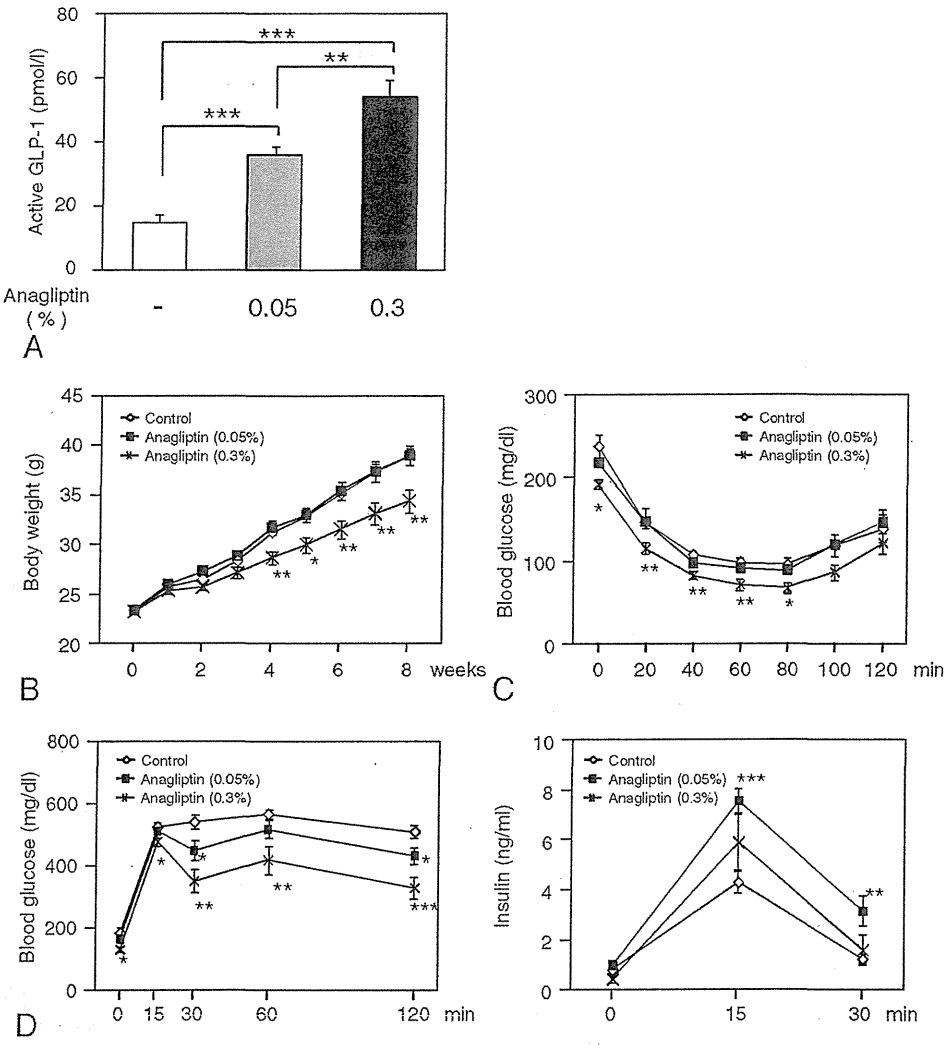

Fig. 4 – Treatment with 0.05% anagliptin had no effect on the body weight or insulin sensitivity, but improved glucose tolerance by increasing insulin secretion in the wild-type mice. A, plasma active GLP-1 levels in wild-type mice not treated (open bars) or treated (0.05%: gray bars, 0.3%: closed bars) 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.

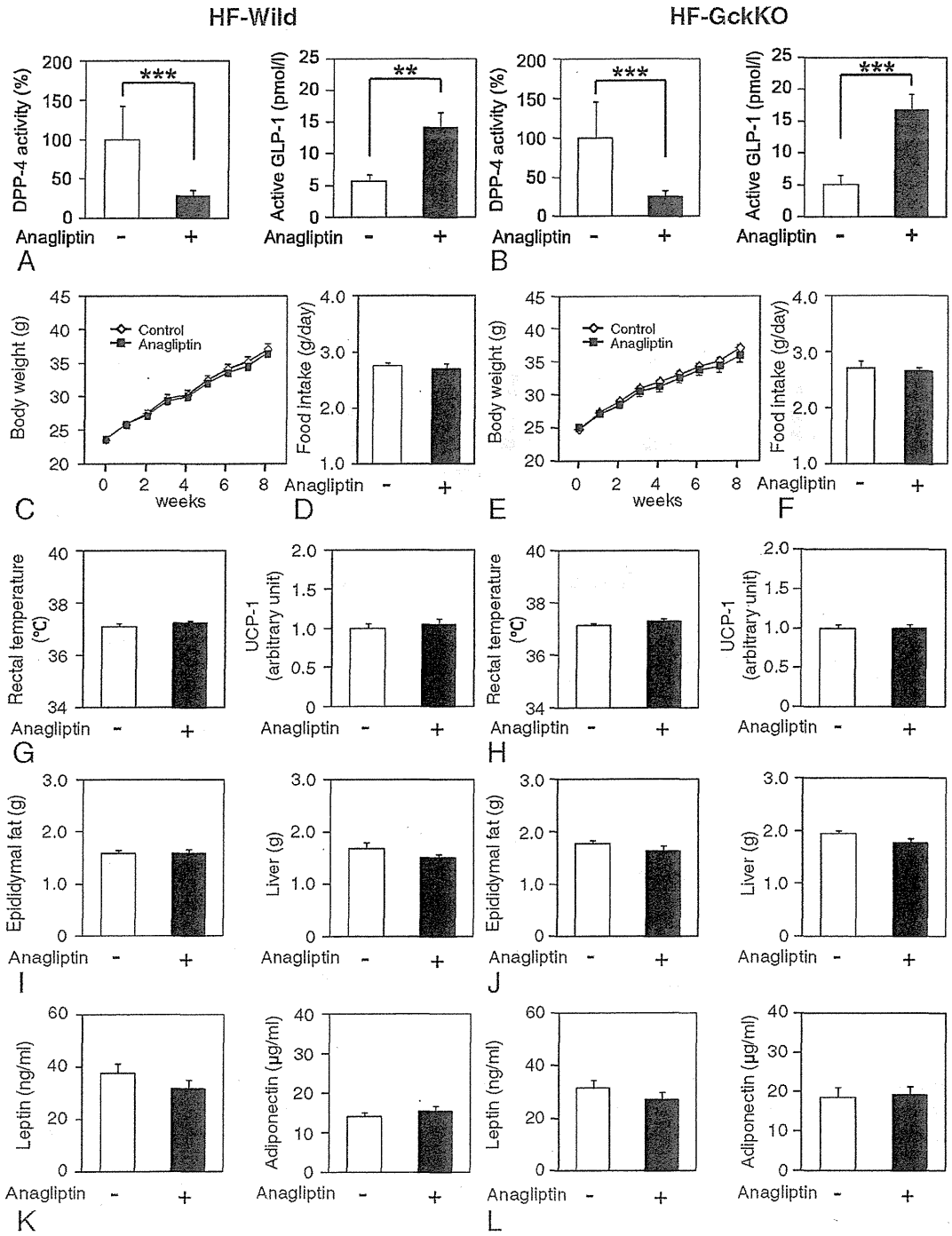

Fig. 5 – Treatment with 0.05% anagliptin had no effect on the body weight or food intake in either the wild-type or GckKO mice. A and B, DPP-4 activities (left panels) and active GLP-1 levels (right panels) in wild-type (A, n=15–16) and GckKO (B, n=11–12) mice not treated (open bars) or treated (closed 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. **, $p<0.01$. ***, $p<0.001$.

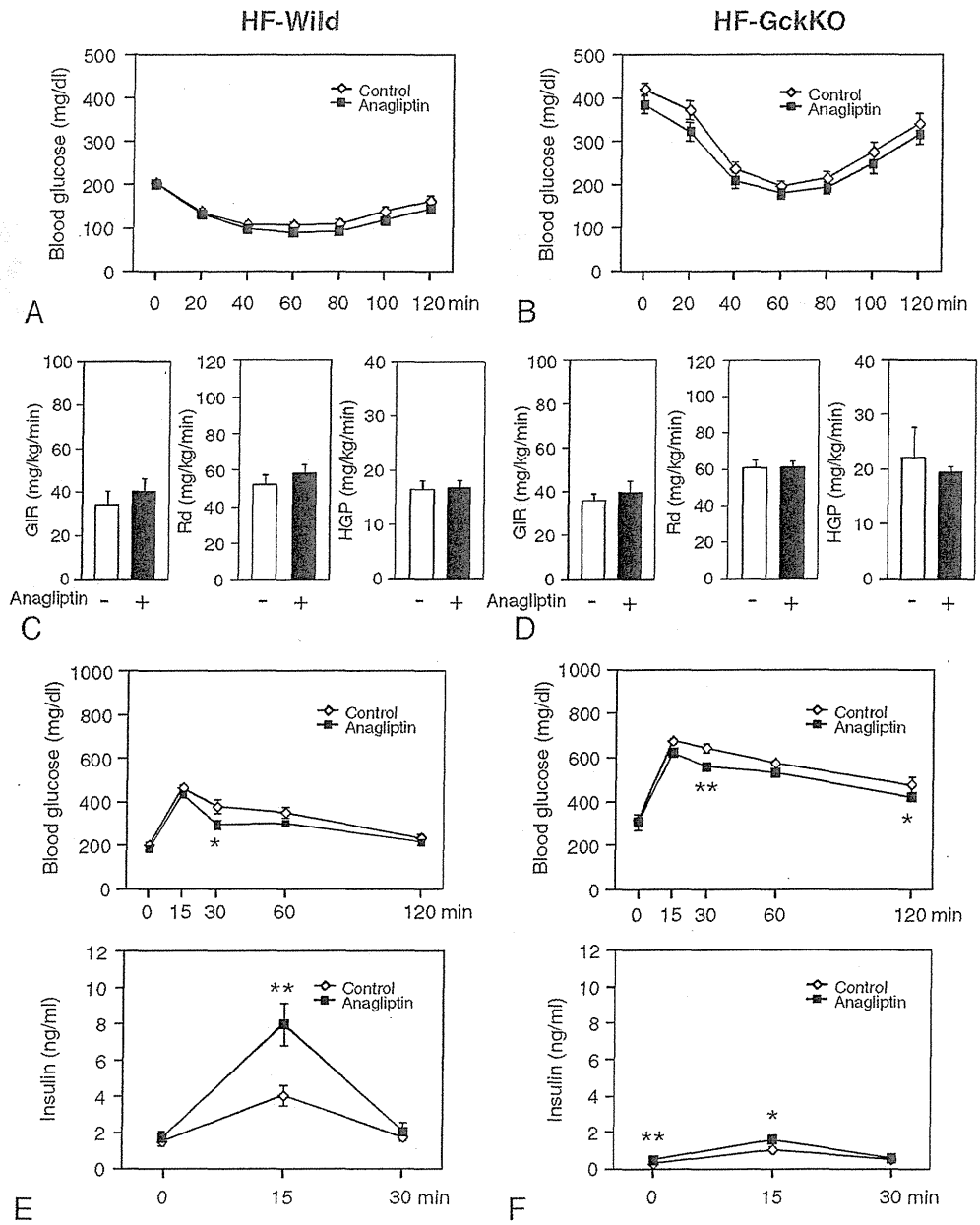

Fig. 6 – Treatment with 0.05% anagliptin improved glucose tolerance by increasing the insulin secretion in both wild-type and GckKO mice. A and B, blood glucose levels during the ITT in wild-type (A, n=15–16) and GckKO (B, n=18–21) mice not treated (open diamonds) or treated (closed squares) with anagliptin. 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.

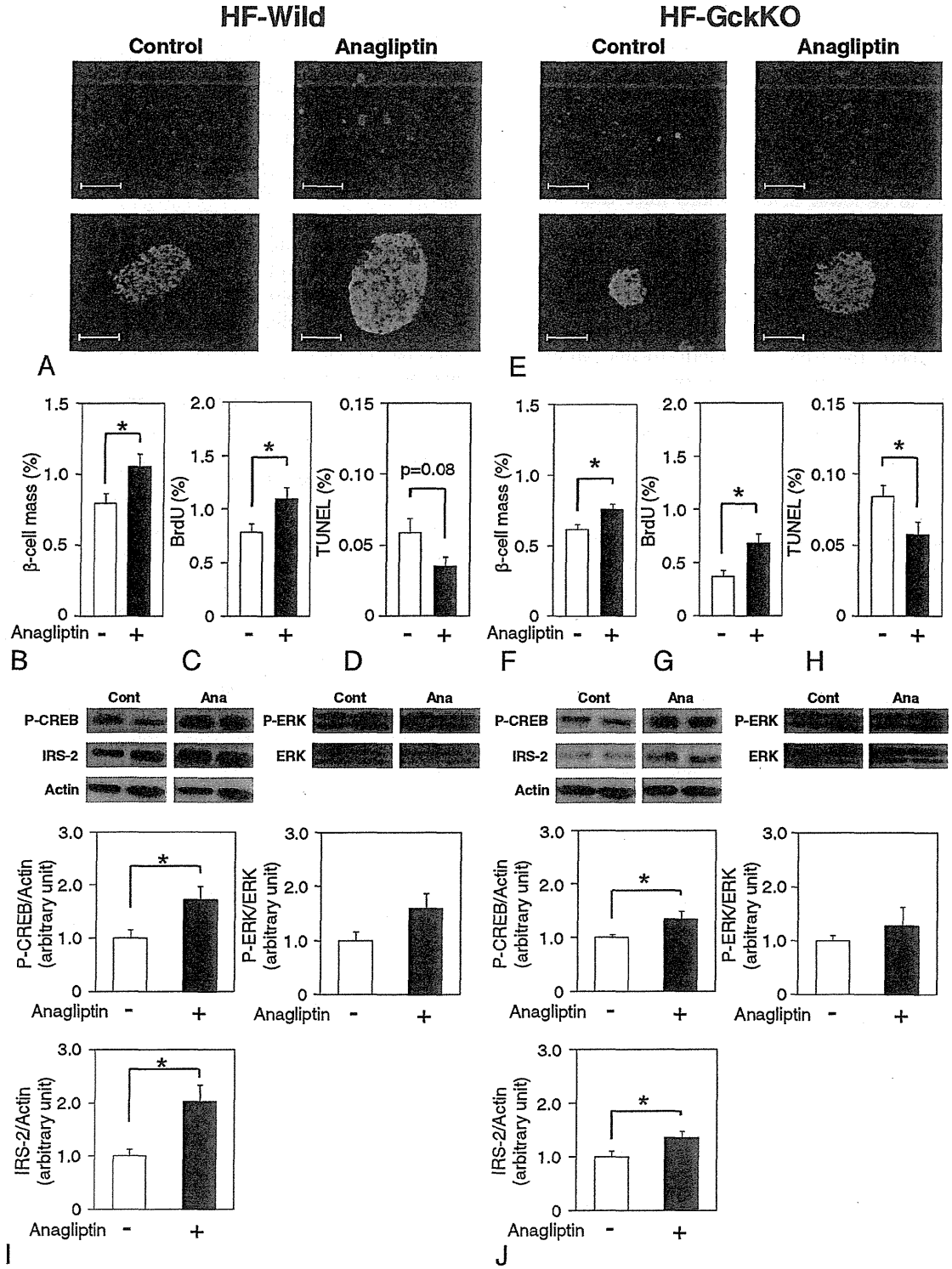

Fig. 7 – Treatment with 0.05% anagliptin increased the β-cell mass. Histological analysis of pancreatic β-cells (A and E) (upper panels; scale bar = 1000 μm, lower panels; scale bar = 100 μm), quantitation of β-cell mass (B and F), BrdU incorporation (C and G) and TUNEL staining (D and H) in wild-type (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.

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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.

Keywords Pioglitazone · Beraprost sodium · Body weight · Insulin resistance · Glucose intolerance

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