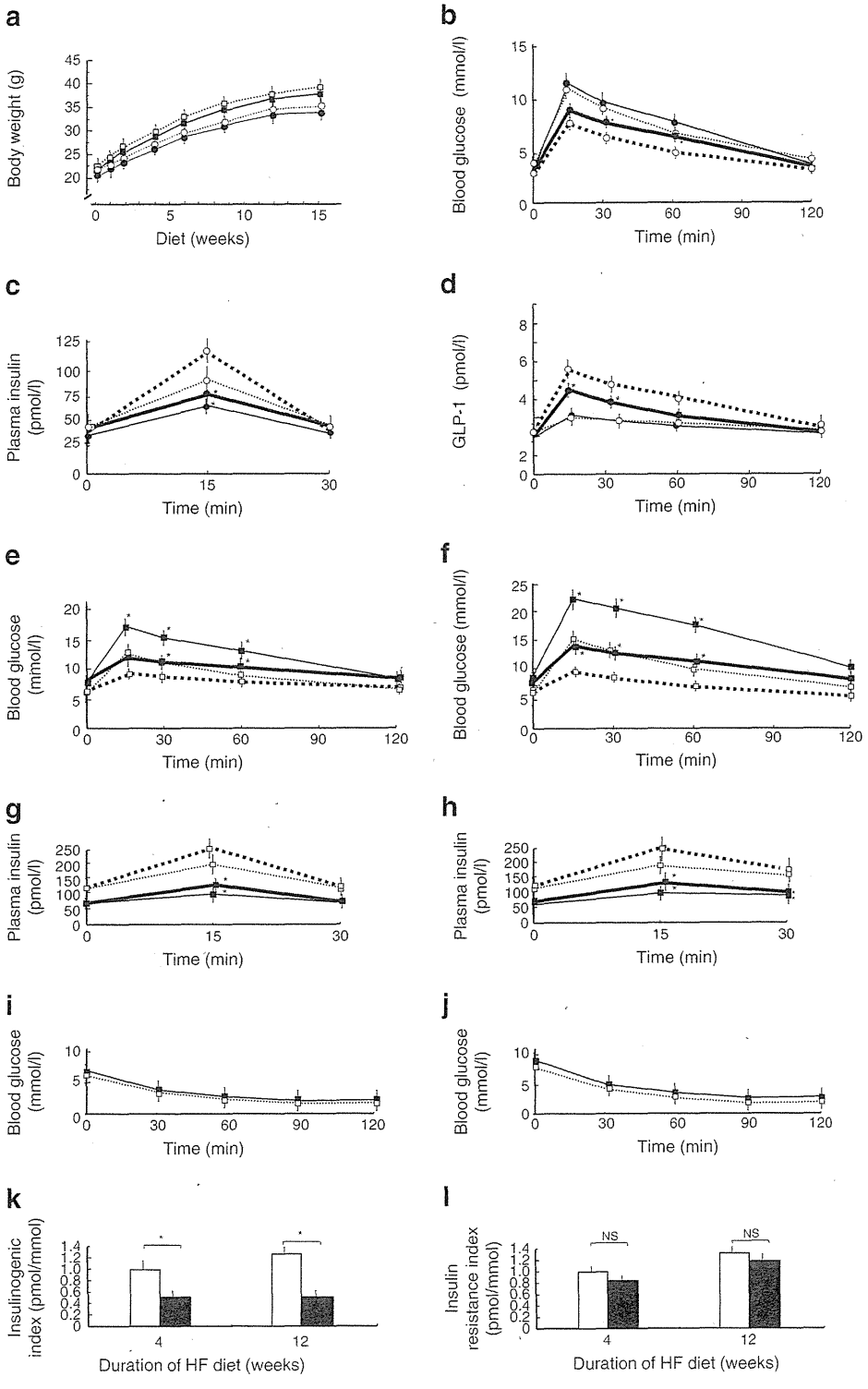

Fig. 3 Impaired glucose homeostasis and insulin secretion in *Hipk3*$^{-/-}$ mice. (**a**) The body weight of *Hipk3*$^{-/-}$ mice on a high-fat (black squares) and control (black circles) diet, and of wild-type mice on high-fat (white squares) and control (white circles) diet was similar. (**b**) Mice were loaded with 1.5 mg/g body weight glucose by oral (OGTT) (bold dotted line, wild-type; bold continuous line, *Hipk3*$^{-/-}$) or intraperitoneal (IPGTT) (thin dotted line, wild-type; thin continuous line, *Hipk3*$^{-/-}$) administration. The OGTT revealed impaired glucose homeostasis in *Hipk3*$^{-/-}$ mice (n=7). (**c**) The OGTT revealed impaired insulin secretion in *Hipk3*$^{-/-}$ mice. Insulin levels were determined using a mouse insulin ELISA kit. (**d**) Impaired GLP-1 secretion in *Hipk3*$^{-/-}$ mice. **e** *Hipk3*$^{-/-}$ mice fed a high-fat diet for 4 or (**f**) 12 weeks had significantly impaired glucose tolerance. Key, as above (**b**). (**g**) The glucose intolerance observed in *Hipk3*$^{-/-}$ mice fed a high-fat diet for 4 or (**h**) 12 weeks coincided with impaired insulin release. (**i**) The insulin tolerance test showed that wild-type (dotted line) and *Hipk3*$^{-/-}$ (continuous line) mice fed a high-fat diet for 4 or (**j**) 12 weeks had similar rates of glucose clearance. (**k**) Insulinogenic index, defined as the ratio of insulin to glucose at 30 min after an oral glucose load in *Hipk3*$^{-/-}$ (black bars) and wild-type (white bars) mice on a high-fat (HF) diet. (**l**) Values under the glucose curve and under the insulin curve during the OGTT were similar between *Hipk3*$^{-/-}$ and wild-type mice. The insulin resistance index was calculated as AUC of insulin/AUC of glucose in the OGTT. Data represent means±SEM; *p<0.05 compared with wild-type control mice

(Fig. 4c). The ATP content was significantly decreased in *Hipk3*$^{-/-}$ islets (Fig. 4d). The increase in intracellular calcium concentration in response to glucose was impaired in islets of *Hipk3*$^{-/-}$ mice (Fig. 4e).

Loss of Hipk3 decreases beta cell proliferation and increases apoptosis Although the insulin content of islets from *Hipk3*$^{-/-}$ mice was not decreased at week 4 of a high-fat diet, it was significantly decreased at week 12 of a high-

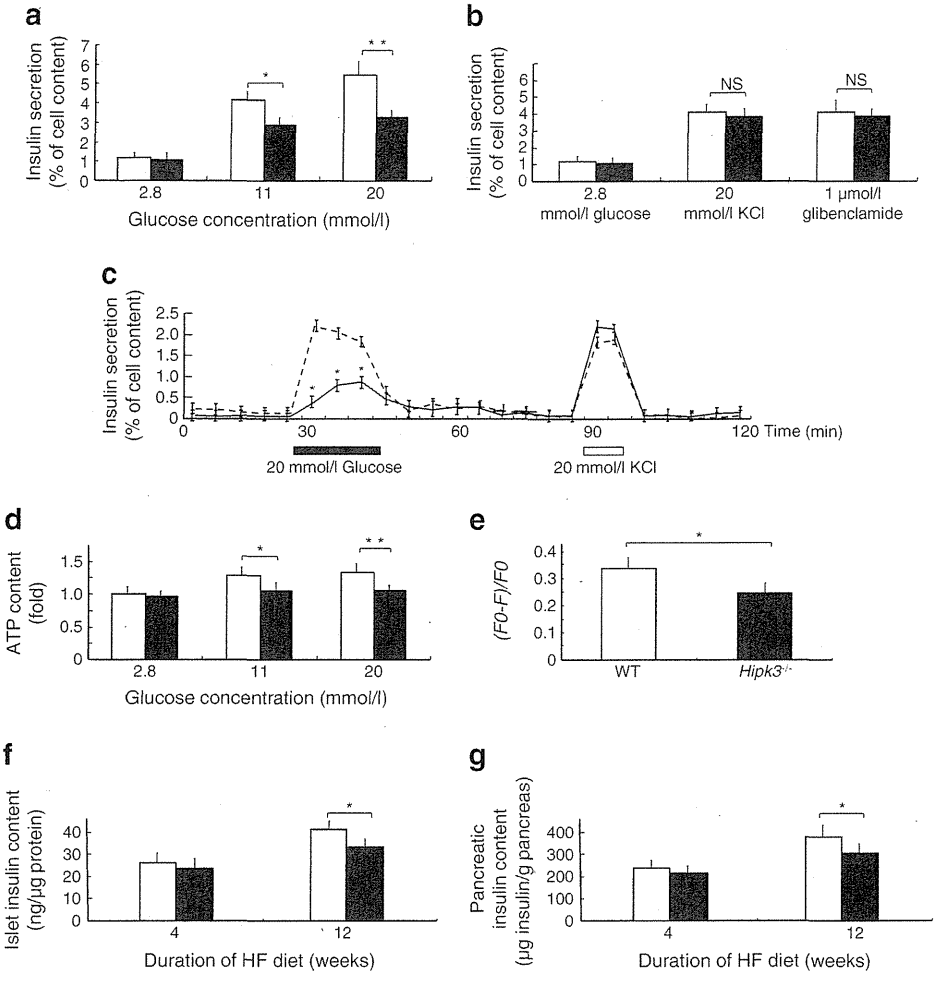

Fig. 4 Characteristics of islets from *Hipk3*$^{-/-}$ mice. (**a**) Insulin secretion by islets from *Hipk3*$^{-/-}$ mice (black bars) fed a high-fat diet for 4 weeks was significantly decreased at 11 and 20 mmol/l glucose vs wild-type mice (white bars) (n=4). (**b**) Insulin secretion of *Hipk3*$^{-/-}$ and wild-type islets in the presence of KCl and glibenclamide. (**c**) Islets from *Hipk3*$^{-/-}$ (continuous line) and wild-type (dashed line) mice were perifused with a medium containing 20 mmol/l glucose and 20 mmol/l KCl (n=4). (**d**) Changes in ATP content in islets from wild-type and *Hipk3*$^{-/-}$ mice after glucose stimulation for 1 h (n=4). (**e**) The increase in calcium concentration in response to glucose was impaired in *Hipk3*$^{-/-}$ islets (n=6). Glucose-stimulated Ca^{2+} influx in single beta cells was measured with the Ca^{2+} indicator fura-2. Fura-2 post-stimulation fluorescence (F) was normalised to resting fluorescence ($F0$). (**f**) Changes in beta cell mass during consumption of a high-fat (HF) diet. The insulin content of islets was decreased in *Hipk3*$^{-/-}$ vs wild-type mice at week 12 of an HF diet. (**g**) Insulin content per pancreas was compared between *Hipk3*$^{-/-}$ and wild-type mice (n=4). Data represent means±SEM: *p<0.05 and **p<0.01 compared with wild-type control mice

fat diet (Fig. 4f, g). Similarly, the proportion of islets to total area of dissected pancreas from *Hipk3*$^{-/-}$ mice was not decreased at week 4 of a high-fat diet, but was significantly decreased at week 12 of a high-fat diet (Fig. 5a, b). We estimated beta cell proliferation on the basis of PCNA staining. During high-fat diet conditions, significantly more PCNA-positive cells were observed in the islets of wild-type mice than in *Hipk3*$^{-/-}$ mice (Fig. 5a, c). We estimated islet cell apoptosis on the basis of TUNEL staining. During high-fat diet conditions, significantly more TUNEL-positive cells were observed in the islets of wild-type mice than in *Hipk3*$^{-/-}$ mice (Fig. 5a, d). In summary, HIPK3 has an effect on beta cell secretory function, beta cell proliferation and apoptosis.

Decreased PDX1 and GSK3β phosphorylation in the absence of Hipk3 To identify the mechanism underlying the loss of glucose-stimulated insulin secretion and beta cell proliferation, we evaluated the mediators of HIPK3-regulated genes relative to islet function. Expression of *Pdx1*, a beta cell-specific transcription factor, was significantly decreased in the islets of *Hipk3*$^{-/-}$ mice fed a high-fat diet for 12 weeks, falling by approximately 50% (Fig. 6a, m, n). Notably, we detected decreases of 34% and 30% in *Gck* and *Slc2a2* mRNA expression in *Hipk3*$^{-/-}$ mice, indicating that loss of HIPK3 negatively modulates the expression of these genes that encode glucose-sensing proteins (Fig. 6b, c). Moreover, we observed significantly reduced mRNA levels of *Hnf4a* and *Tcf7l2* in *Hipk3*$^{-/-}$

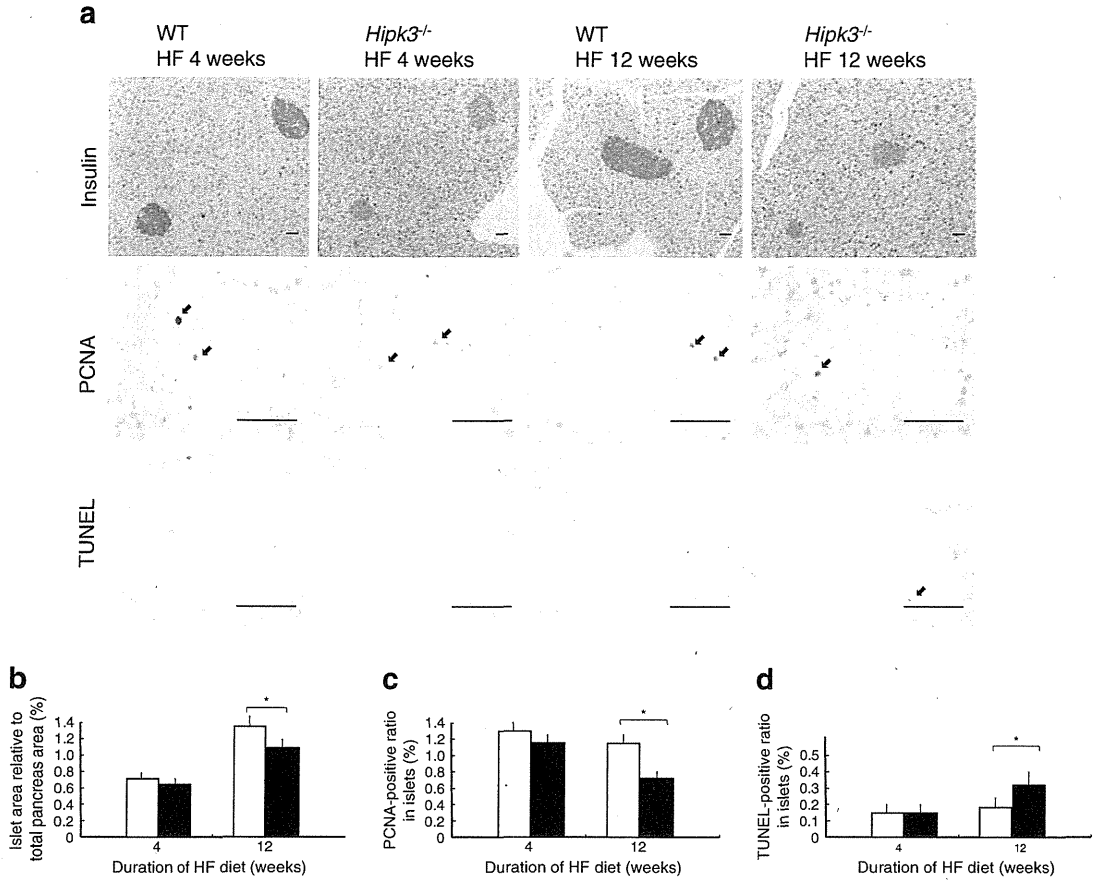

Fig. 5 (**a**) PCNA immune staining analysis was based on the counting of 1,662 and 1,879 cells, respectively, per islet sample from wild-type (WT) (n=4) and *Hipk3*$^{-/-}$ (n=4) mice fed a high-fat diet for 4 weeks, and (high-fat diet, 12 weeks) on the counting of 1,674 and 1,518 cells, respectively, per islet sample from wild-type (n=4) and *Hipk3*$^{-/-}$ (n=4) mice. Tissues were processed for paraffin embedding, and 4 μm sections were cut and mounted on silanised slides. Pancreatic sections were stained with anti-insulin and anti-PCNA antibodies as indicated. We estimated islet cell apoptosis on the basis of TUNEL staining. Scale bars, 100 μm. (**b**) The proportion of islet to total area was decreased in *Hipk3*$^{-/-}$ compared with wild-type mice fed a HF diet (n=4). (**c**) Replication rate of beta cells from *Hipk3*$^{-/-}$ and wild-type mice (n=4). (**d**) The apoptosis rate of islet cells from *Hipk3*$^{-/-}$ and wild-type mice (n=4). Data represent means±SEM: *p<0.05 compared with wild-type control mice

islets, suggesting that HIPK3 plays a role in influencing the expression of transcription factors. Additionally, cyclin D1 gene expression (Fig. 6d), GSK3β phosphorylation (Fig. 6m, o) and β-catenin abundance (Fig. 6m, p) were decreased in the islets of *Hipk3*$^{-/-}$ mice, indicating a role of HIPK3 in Wnt signalling. Histological analysis showed that nuclear PDX1 abundance was significantly decreased in the islets of *Hipk3*$^{-/-}$ mice fed a high-fat diet for 12 weeks (Fig. 6q, r). Histological analysis also showed that GSK3β phosphorylation was decreased in the islets of *Hipk3*$^{-/-}$ mice (Fig. 6s).

Wnt signalling lessens the decrease in insulin secretion induced by loss of Hipk3 We investigated whether Wnt signalling can also increase glucose-stimulated insulin secretion in *Hipk3*$^{-/-}$ islets. Wnt3a enhanced insulin secretion by 19% in *Hipk3*$^{-/-}$ islets (Fig. 7a) upon 20 mmol/l glucose stimulation. We also found that the GSK3 inhibitor LiCl and 1-azakenpaullone treatment enhanced insulin secretion in *Hipk3*$^{-/-}$ islets by 31% and 28%, respectively, upon 20 mmol/l glucose stimulation (Fig. 7b, c). These observations suggest that impaired Wnt signalling caused by *Hipk3* deficiency leads to impaired beta cell function.

Discussion

It is becoming increasingly clear that the diverse biological consequences of the loss of HIPK activity include the coordinated death of cells in earlier developmental stages and the dysregulation of proper cell number in diverse tissue types. Mice that are deficient for *Hipk1* and *Hipk2* singly have a normal overall appearance, a finding that is probably due to a functional redundancy between HIPK1 and HIPK2 [41]. However, *Hipk1*$^{-/-}$; *Hipk2*$^{-/-}$ mice are progressively lost in

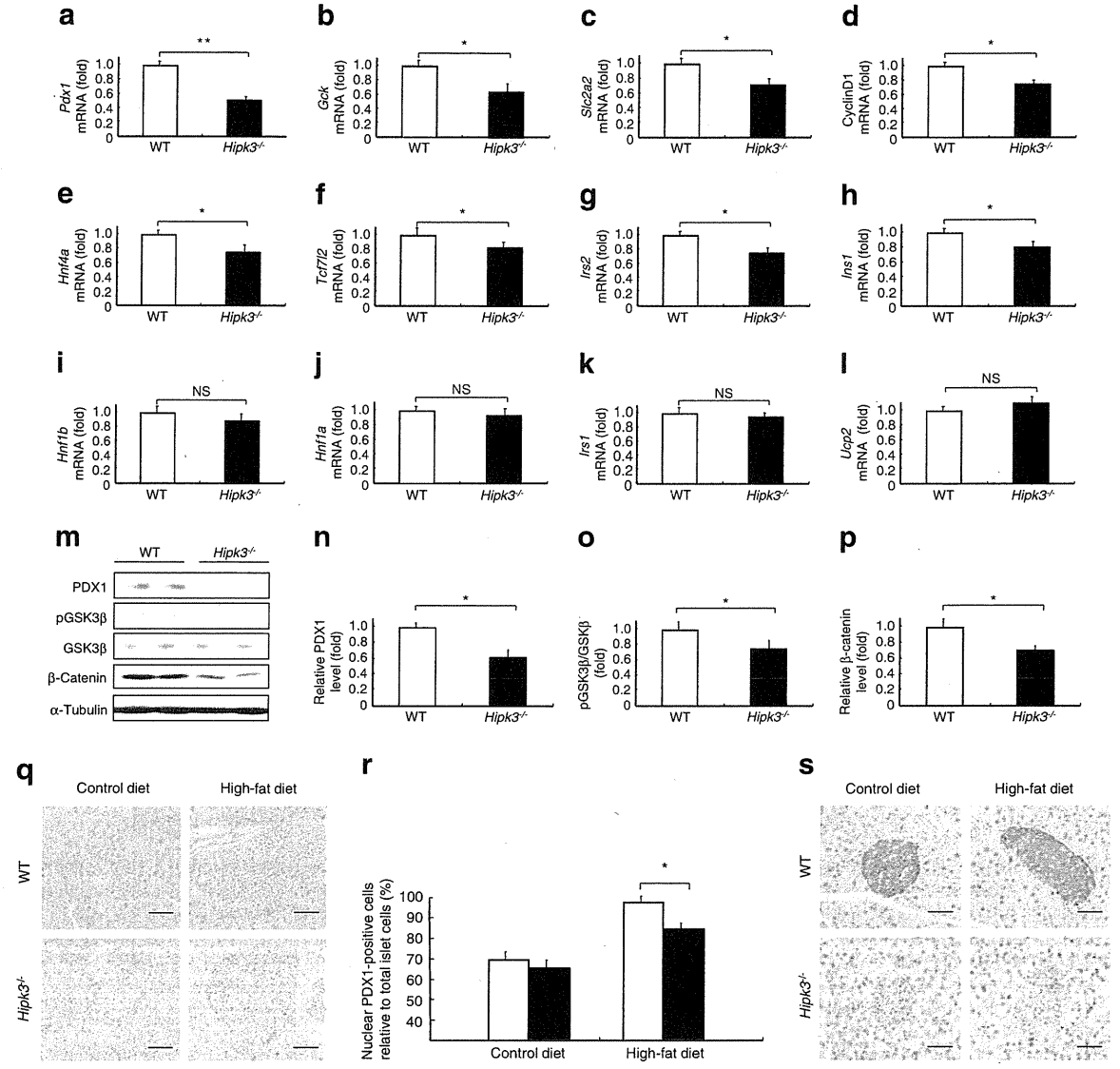

Fig. 6 (**a**) Quantitative RT-PCR analysis of islet-enriched transcription factors and glucose-sensing proteins *Pdx1*, (**b**) *Gck*, (**c**) *Slc2a2*, (**d**) Cyclin D1, (**e**) *Hnf4a*, (**f**) *Tcf7l2*, (**g**) *Irs2*, (**h**) *Ins1*, (**i**) *Hnf1b*, (**j**) *Hnf1a*, (**k**) *Irs1* and (**l**) *Ucp2*. Analysis was in islets isolated from wild-type (WT) and *Hipk3*$^{-/-}$ mice (n=4) on a high-fat diet for 12 weeks. Samples were normalised to *Gapdh*. **m** Protein samples from lysates of isolated islets from *Hipk3*$^{-/-}$ mice were separated by SDS-PAGE and western blot analyses performed using the antibodies as labelled (n=4). (**n**) Quantification of relative PDX1, (**o**) pGSK3β and (**p**) β-catenin levels. (**q**) Pancreatic sections were stained with anti-PDX1 antibody. PDX1 immune staining analysis was based on the counting of 1,845 and 1,683 cells, respectively, per islet sample from wild-type (n=4) and *Hipk3*$^{-/-}$ (n=4) mice fed a high-fat diet for 12 weeks, and (control diet) on the counting of 1,545 and 1,609 cells, respectively, per islet sample from wild-type (n=4) and *Hipk3*$^{-/-}$ (n=4) mice. (**r**) Ratio of the number of nuclear PDX1-positive cells to the total number of islet cells. (**s**) Histological analysis of pancreatic islet phosphorylated GSK3β in wild-type and *Hipk3*$^{-/-}$ mice. Pancreatic sections were stained with anti-phspho-GSK3β(Ser9) antibodies. Analysis was based on the counting of 1,705 and 1,692 cells, respectively, per islet sample from wild-type (n=4) and *Hipk3*$^{-/-}$ (n=4) mice fed a high-fat diet for 12 weeks, and (control diet) on the counting of 1,875 and 1,586 cells, respectively, per islet sample from wild-type (n=4) and *Hipk3*$^{-/-}$ (n=4) mice. Scale bars (**q**, **s**), 100 μm. Data represent means±SEM: *p<0.05 and **p<0.01 compared with wild-type control mice

utero, and exhibit exencephaly and severe defects in haematopoiesis, vasculogenesis and angiogenesis. In our experiments, expression of Hipk genes was detected in adult islets, and the HIPK family additively regulated insulin secretion. Our data also indicate that high-fat diet leads most significantly to *Hipk3* upregulation. The other two Hipk genes correlate with glucose tolerance and may play roles in glucose homeostasis. Given the inability of the intact *Hipk3* gene to support early development in *Hipk1*$^{-/-}$; *Hipk2*$^{-/-}$ mice and the contribution of HIPK3 to impaired

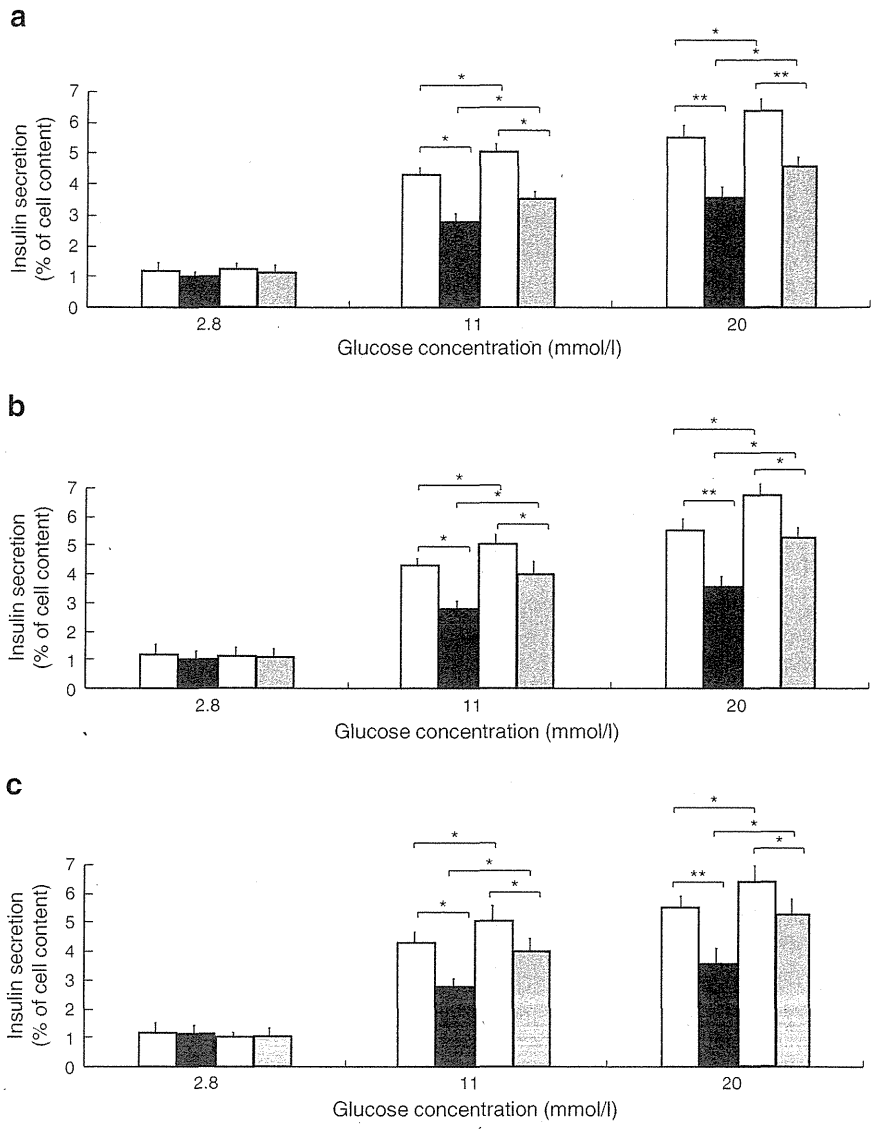

Fig. 7 Wnt3a regulates HIPK3-induced insulin secretion. (**a**) Insulin secretion was decreased by Wnt3a (n=4). Insulin secretion by islets from wild-type (white bars; light grey bars, wild-type+Wnt3a) and *Hipk3*$^{-/-}$ (black bars; dark grey bars, *Hipk3*$^{-/-}$+Wnt3a) mice was measured in KRB with a glucose concentration of 2.8, 11 or 20 mmol/l. Static incubation was performed for 1 h on ten islets per tube at 37°C, after preincubation with the basal glucose concentration for 20 min. Insulin levels were determined with a mouse insulin ELISA kit. (**b**) The insulin secretion of isolated islets was decreased by *Hipk3* deficiency and increased by the GSK3 inhibitor LiCl (n=4). Key, as above (**a**), except: light grey bars, wild-type+LiCl; dark grey bars, *Hipk3*$^{-/-}$+LiCl. (**c**) Insulin secretion of isolated islets from *Hipk3*$^{-/-}$ mice was increased by Wnt signalling activation by the GSK3 inhibitor, 1-azakenpaullone (n=4). Key, as above (**a**), except: light grey bars, wild-type+1-azakenpaullone; dark grey bars, *Hipk3*$^{-/-}$+1-azakenpaullone. Data represent means±SEM; *p<0.05 and **p<0.01

insulin secretion, we anticipate that HIPK3 may serve unique physiological roles especially in diabetes-prone circumstances such as a high-fat diet.

We also analysed *Hipk3*$^{-/-}$ mice. Major changes in gene phenotype were found with very modest increases in glucose levels, such as impaired glucose tolerance. Changes in islet gene expression can be explained by *Hipk3* deficiency and glucose toxicity.

HIPK2 positively influences PDX1 levels and transcriptional activity, and directly phosphorylates the C-terminal portion of PDX1 [28]. HIPK2 is a potential kinase for PDX1 Ser-269, with high glucose concentrations decreasing the degree of phosphorylation on PDX1 Ser-269 in beta cells [42]. We showed that the number of PDX1-positive cells was reduced by 20% in *Hipk3*-knockout mice on a 12 week high-fat diet (Fig. 6r). The insulin content was reduced by 18% in the same conditions (Fig. 4f). Thus, we speculate that decreased PDX1 positivity is related to beta cell mass reduction. Considering the role of PDX1 in beta cells and non-beta cells, HIPK may affect beta cells and non-beta cells, and reduce beta cell mass. Further studies are needed to clarify the mechanisms of beta cell reduction that decreased PDX1 positivity. HIPK3 has been shown to augment transcription of androgen receptor [32], SF-1 [35] and runt-related transcription factor 2 [36]. In this study, we identified HIPK3 as a novel modulator of PDX1 abundance. Several transcription factors converge on regulatory areas within the *Pdx1* promoter [43–45], including NKx2.2, a member of the NK-2 family of homeobox genes [44, 45]. HIPKs possess a homeodomain-interacting domain that is important for enhancing the repressor activities of NK homeodomain transcription factors [22], allowing the

possibility that HIPK3 may affect PDX1 levels via interacting factors that promote PDX1 transcription.

We showed that insulin transcript levels are correlated with the results of luciferase assays. We used rat insulin promoter instead of mouse insulin promoter, and this difference in species may have affected our results. Concerning the effect of HIPK on insulin expression, HIPK may interact with the insulin promoter or act by modulating PDX1 levels. Further studies are needed to elucidate the mechanisms by which HIPK affects insulin expression.

This study also revealed the involvement of the Wnt–GSK3β–β-catenin cascade in the action of HIPK3. Wnt signalling influences endocrine pancreas development and modulates mature beta cell functions, including insulin secretion, beta cell survival and beta cell proliferation [6–9]. Treatment with Wnt ligands increases glucose-induced insulin secretion in wild-type islets, but not in islets deficient in the Wnt co-receptor, lipoprotein receptor-related protein 5, suggesting a deficit in glucose sensing in islets when canonical Wnt signalling is impaired [46]. β-Catenin is sufficient to induce proliferation markers and to increase beta cell mass in transgenic mice [4]. Adipocyte-derived Wnt signalling molecules induce cyclin D1 transcription, and the proliferation of beta cell lines and primary murine beta cells [5]. Beta cell-specific *Gsk3b* knockout prevents diabetes in *Irs2*-knockout [13] and high-fat diet-fed mice [14] through expanded beta cell mass with increased beta cell proliferation. In line with these reports, we have shown that beta cell proliferation is decreased in *Hipk3*$^{-/-}$ mouse islets upon feeding a high-fat diet. In contrast to our findings, pancreas-specific depletion of β-catenin has been shown to lead to acinar cell hypoplasia, but not to change the mass and function of islets [47]. This discrepancy might be explained by β-catenin-independent signal transduction downstream of HIPK3.

Previous reports indicate that β-catenin and transcription factor 7 like 2, T cell specific, HMG box regulate proglucagon gene expression and GLP-1 synthesis in enteroendocrine cells [48, 49]. In this study, we obtained evidence that HIPK3 deficiency lowered GLP-1 concentrations and impaired glucose tolerance. It can thus be speculated that HIPK3 activates the Wnt signalling pathway for GLP-1 synthesis and secretion. Furthermore, HIPK3 may alter the response of intestinal K cells to known secretagogues, including nutrients, fatty acids and related molecules. These hypotheses deserve further examination.

In conclusion, HIPK3 has a role in insulin secretion, affecting the levels of pancreatic PDX1 and GSK3β phosphorylation. Thus, targeting HIPK3 with novel agonists could lead to new therapeutic strategies for preserving beta cell function. The present results provide new insight into the pathogenesis of type 2 diabetes.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement NS, KH and TK planned and designed the study. NS, KH, KU and TK conceived and designed the experiments, and wrote the manuscript. HF and MH contributed to the conception and design, the analysis and interpretation of the data, and drafting the article. NT carried out the two-photon excitation imaging and contributed to drafting the article. IT, MO, HA, MN, NK and TY provided advice, and contributed to the conception and interpretation of the data and drafting the article. All authors approved the final version.

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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.

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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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

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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 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

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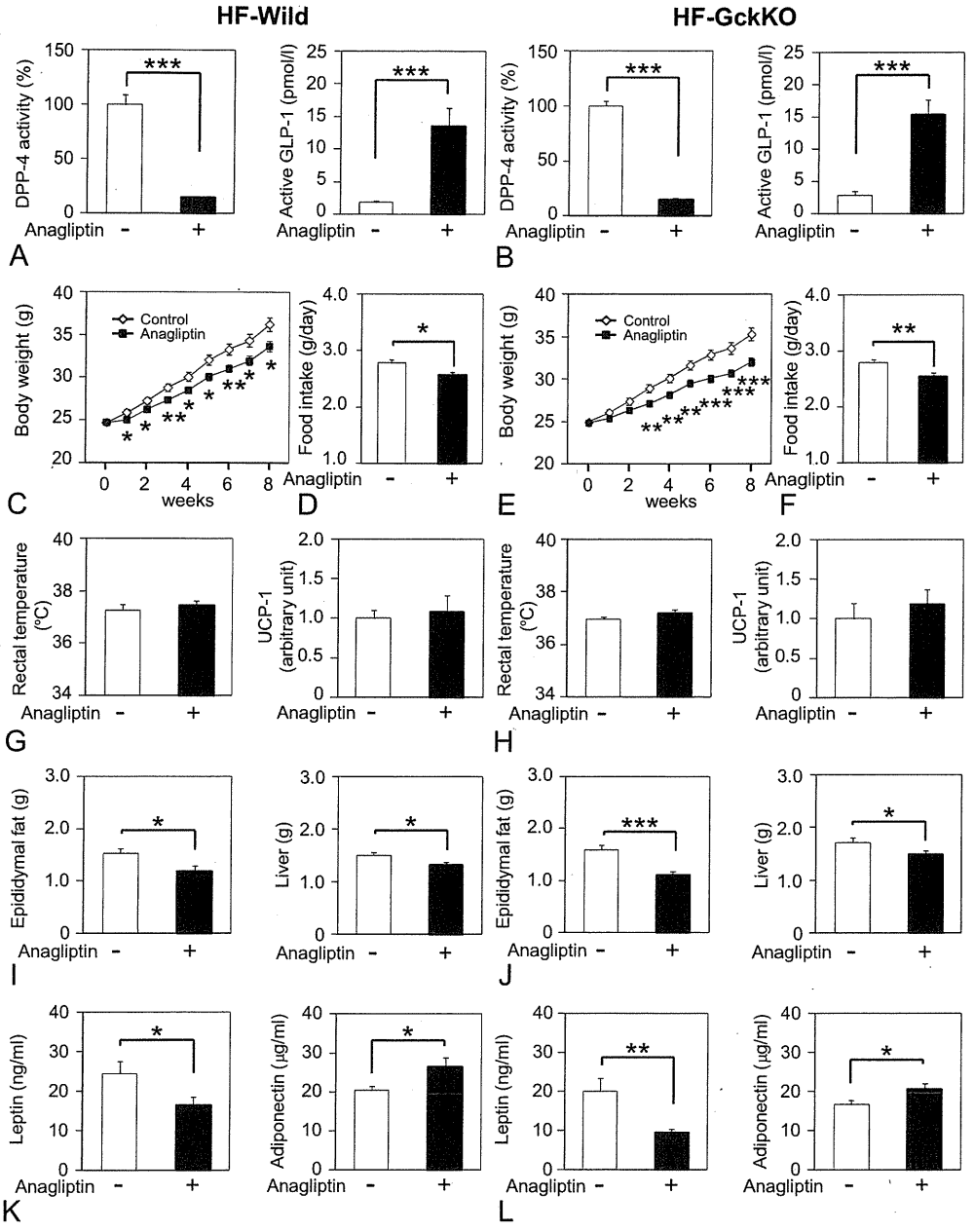

Fig. 1 – Treatment with 0.3% anagliptin decreased body weight gain and food intake. A and B, DPP-4 activities (left panels) and active GLP-1 levels (right panels) in wild-type (A) and GckKO (B) mice not treated (open bars) or treated (closed bars) with anagliptin (n=11–12). C and D, body weights (left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

pronounced in the 0.3% anagliptin-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

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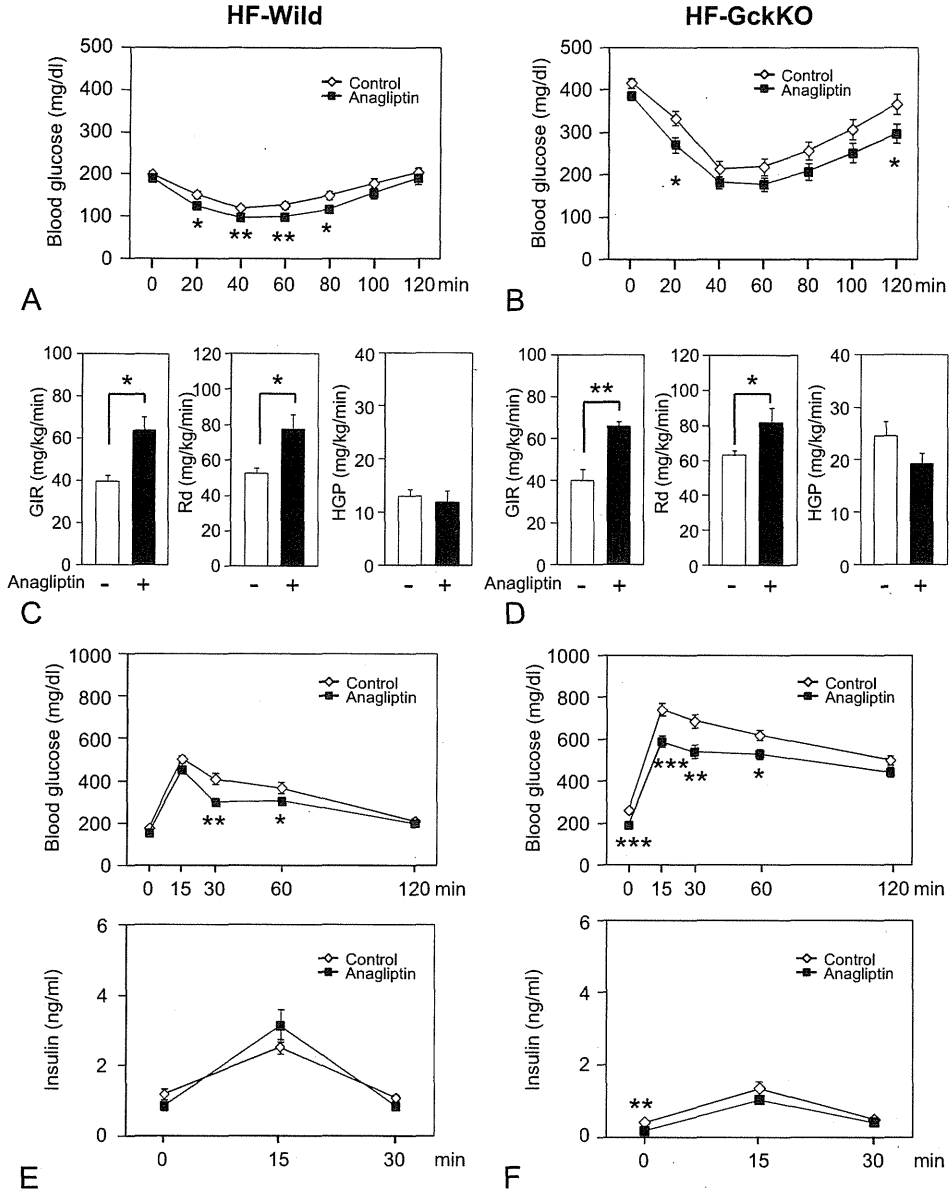

Fig. 2 – Treatment with 0.3% anagliptin improved insulin resistance and glucose tolerance in the wild-type and GckKO mice. A and B, blood glucose levels during the ITT in wild-type (A) and GckKO (B) mice not treated (open diamonds) or treated (closed squares) with anagliptin (n=30). C and D, GIR (left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

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.

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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 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.

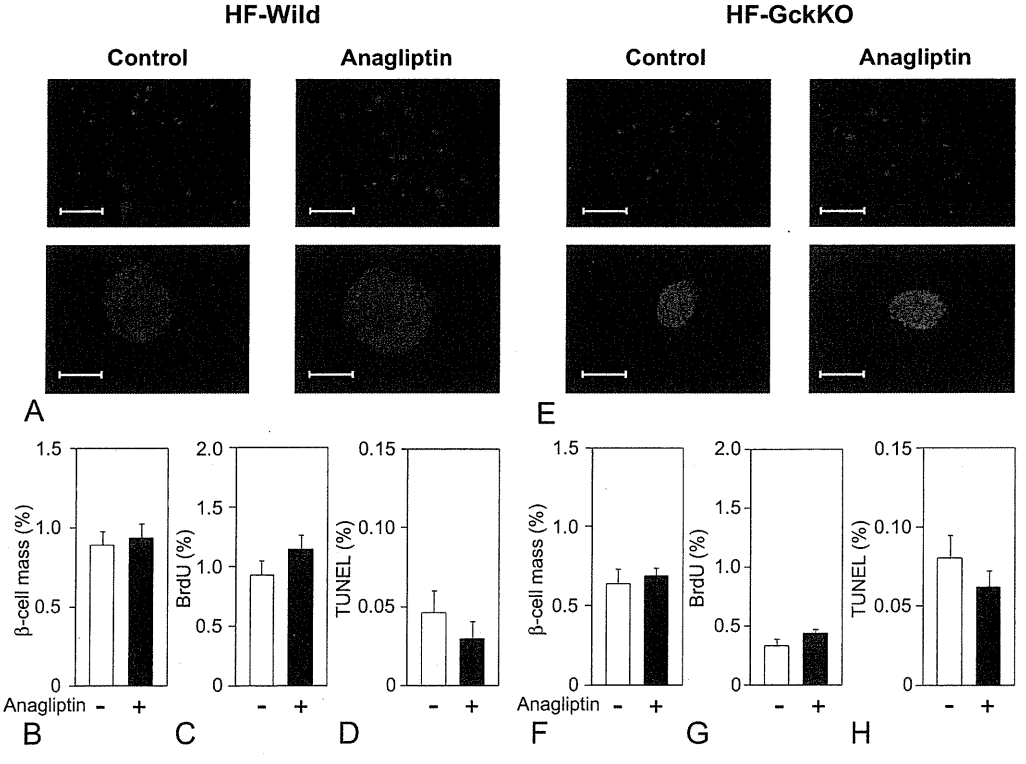

Fig. 3 – Effect of 0.3% anagliptin on the β-cell mass. Histological analysis of pancreatic β-cells (A and E) (upper panels; scale bar = 1000 μm, lower panels; scale bar = 100 μm), quantitation of β-cell mass (B and F), BrdU incorporation (C and G) and TUNEL staining (D and H) in wild-type (A–D) and GckKO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

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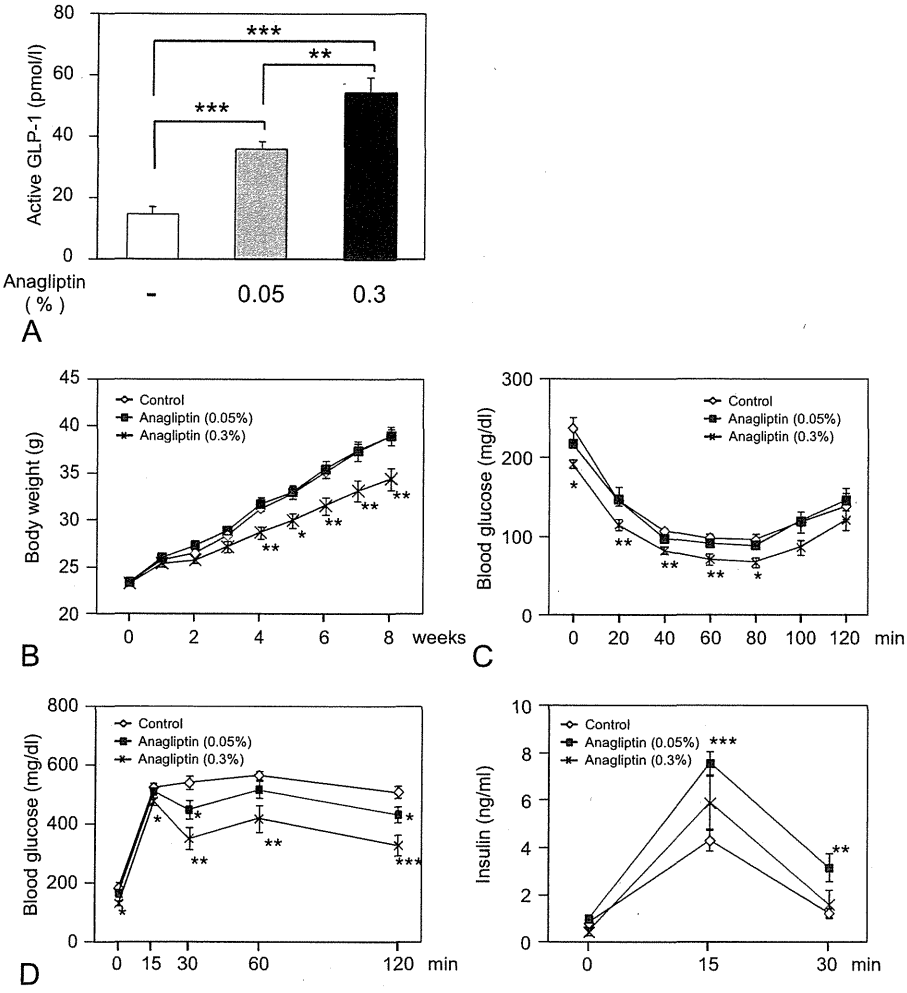

Fig. 4 – Treatment with 0.05% anagliptin had no effect on the body weight or insulin sensitivity, but improved glucose tolerance by increasing insulin secretion in the wild-type mice. A, plasma active GLP-1 levels in wild-type mice not treated (open bars) or treated (0.05%: gray bars, 0.3%: closed bars)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

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.

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

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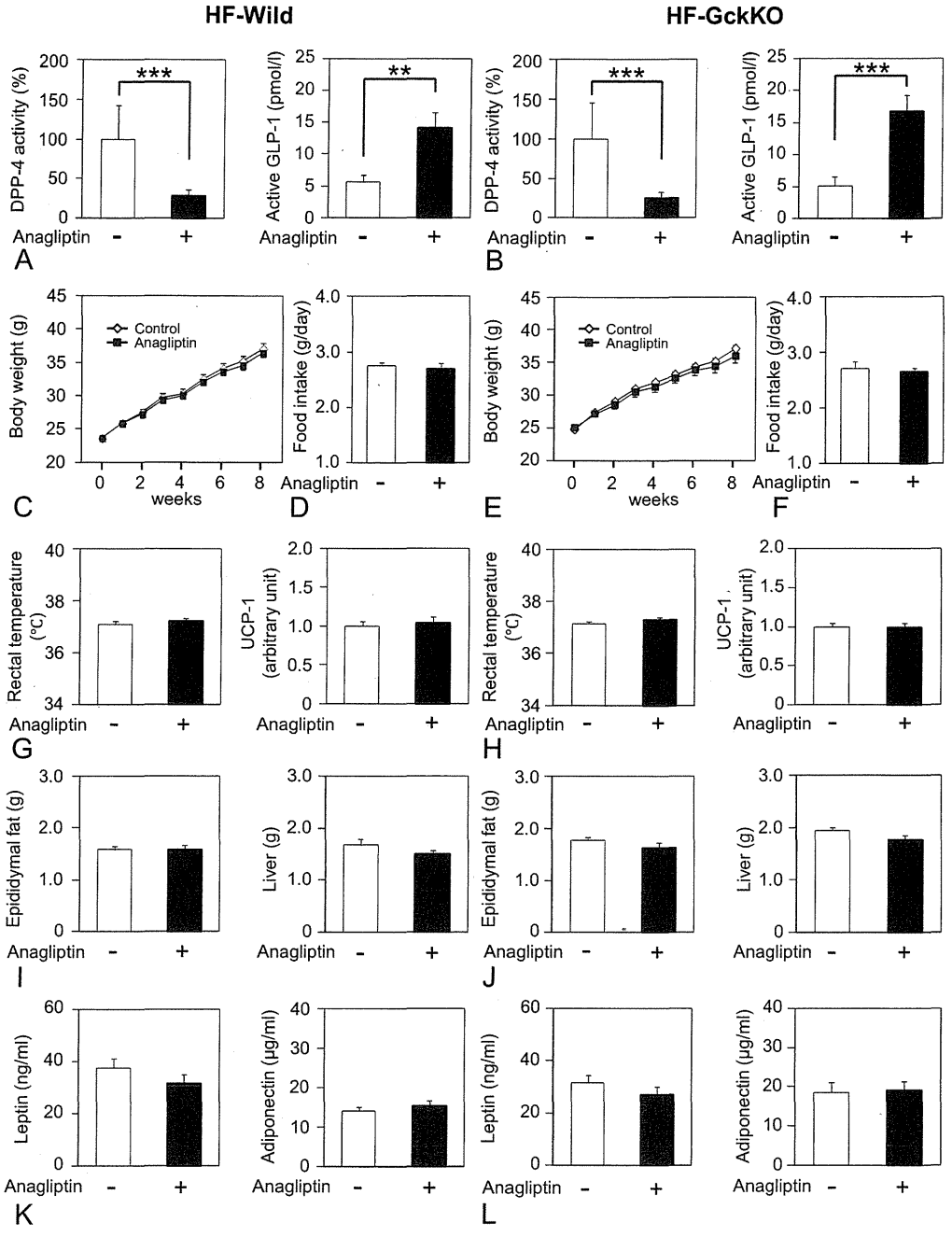

Fig. 5 – Treatment with 0.05% anagliptin had no effect on the body weight or food intake in either the wild-type or GckKO mice. A and B, DPP-4 activities (left panels) and active GLP-1 levels (right panels) in wild-type (A, n=15–16) and GckKO (B, n=11–12) mice not treated (open bars) or treated (closed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

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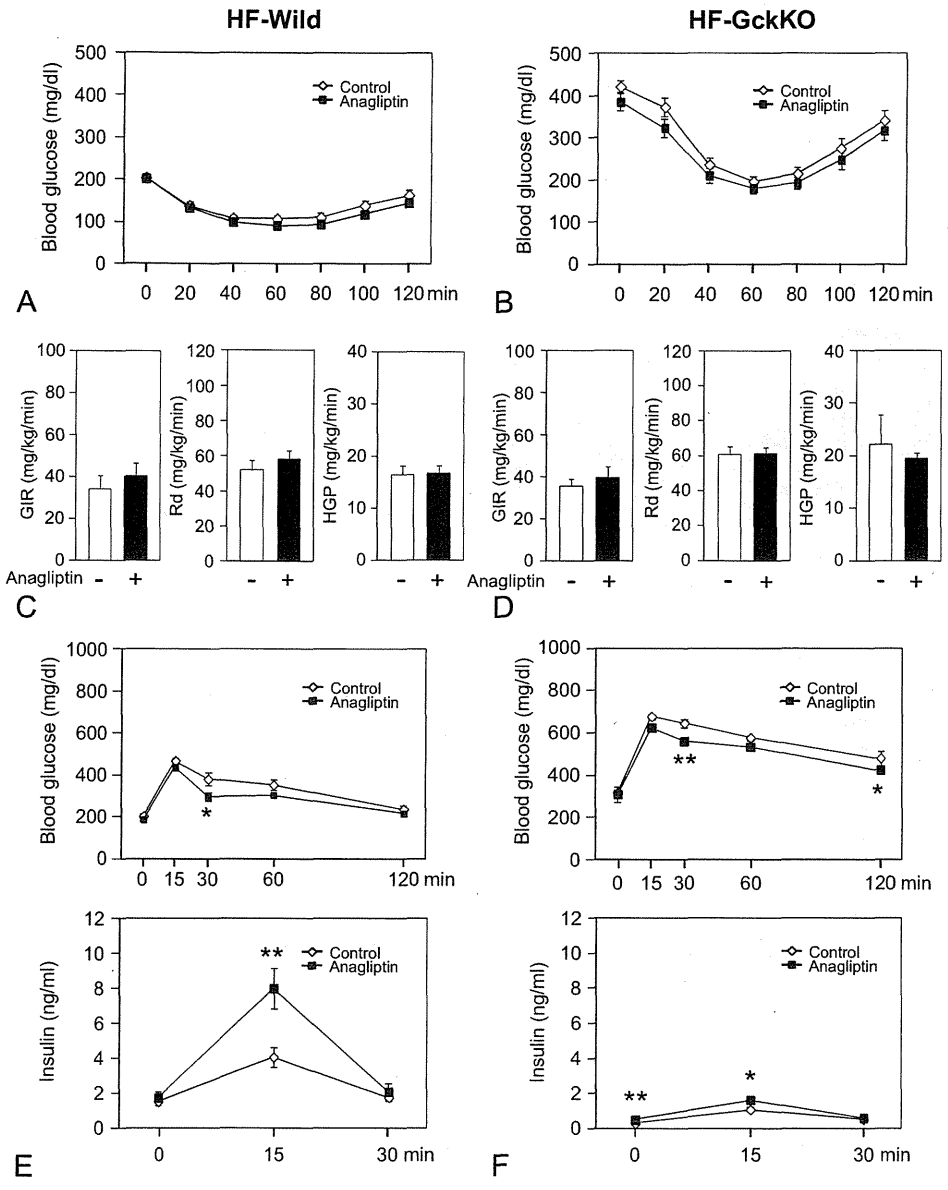

Fig. 6 – Treatment with 0.05% anagliptin improved glucose tolerance by increasing the insulin secretion in both wild-type and GckKO mice. A and B, blood glucose levels during the ITT in wild-type (A, n=15–16) and GckKO (B, n=18–21) mice not treated (open diamonds) or treated (closed squares) with anagliptin. 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. *, $p<0.05$. **, $p<0.01$.

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.

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

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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

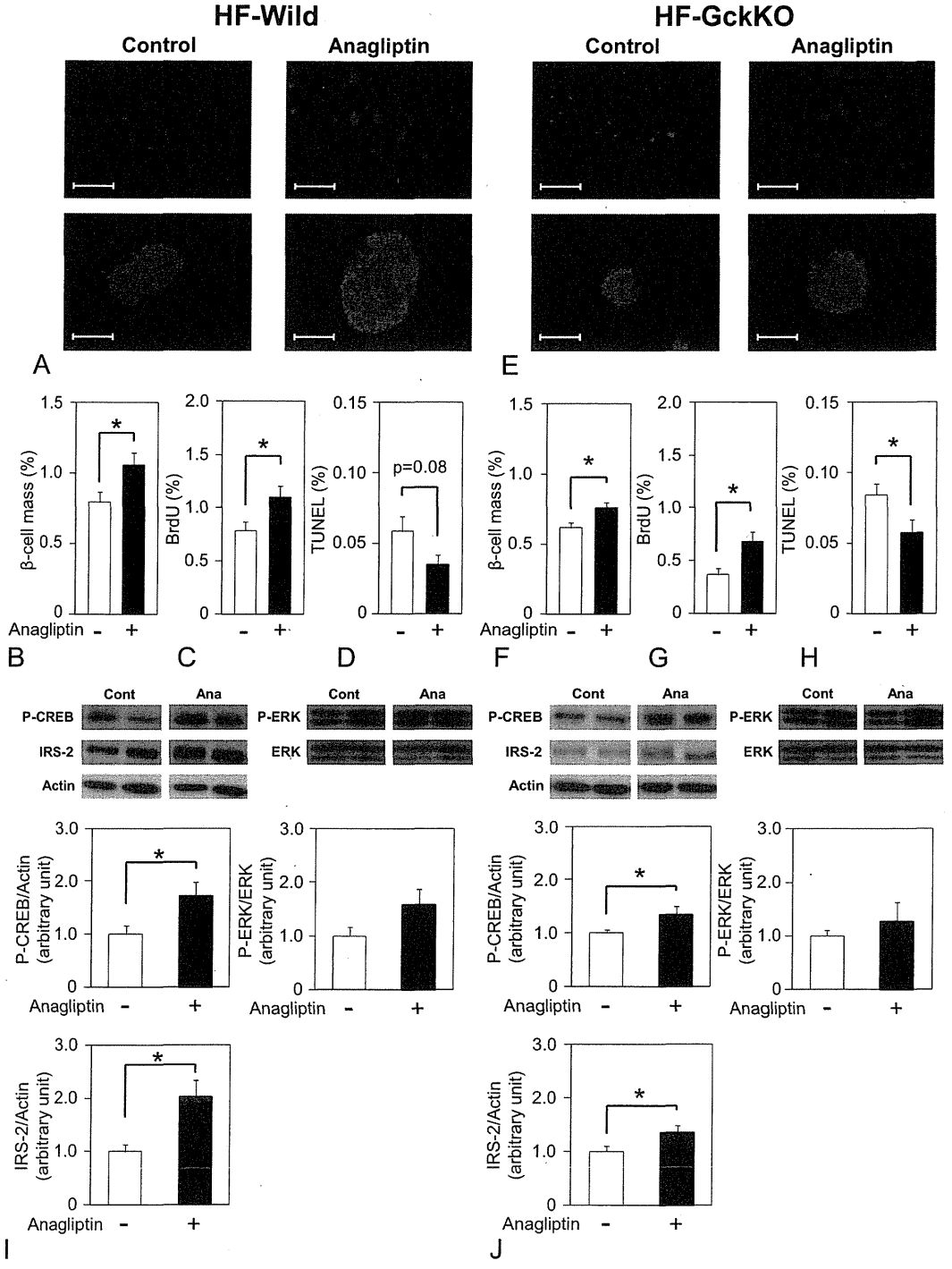

Fig. 7 – Treatment with 0.05% anagliptin increased the β-cell mass. Histological analysis of pancreatic β-cells (A and E) (upper panels; scale bar=1000 μm, lower panels; scale bar=100 μm), quantitation of β-cell mass (B and F), BrdU incorporation (C and G) and TUNEL staining (D and H) in wild-type (A–D)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

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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

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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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