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ARTICLE

Control of beta cell function and proliferation in mice stimulated by small-molecule glucokinase activator under various conditions

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Abstract
Aims/hypothesis We investigated changes in the expression of genes involved in beta cell function and proliferation in mouse islets stimulated with glucokinase activator (GKA) in order to elucidate the mechanisms by which GKA stimulates beta cell function and proliferation.
Methods Islets isolated from mice were used to investigate changes in the expression of genes related to beta cell function and proliferation stimulated by GKA. In addition, *Irs2* knockout (*Irs2*$^{-/-}$) mice on a high-fat diet or a high-fat diet containing GKA were used to investigate the effects of GKA on beta cell proliferation in vivo.
Results In wild-type mice, *Irs2* and *Pdx1* expression was increased by GKA. In *Irs2*$^{-/-}$ mice, GKA administration increased the glucose-stimulated secretion of insulin and *Pdx1* expression, but not beta cell proliferation. It was particularly noteworthy that oxidative stress inhibited the upregulation of the *Irs2* and *Pdx1* genes induced by GKA. Moreover, whereas neither GKA alone nor exendin-4 alone upregulated the expression of *Irs2* and *Pdx1* in the islets of *db*/*db* mice, prior administration of exendin-4 to the mice caused GKA to increase the expression of these genes.
Conclusions/interpretation GKA-stimulated IRS2 production affected beta cell proliferation but not beta cell function. Oxidative stress diminished the effects of GKA on the changes in expression of genes involved in beta cell function and proliferation. A combination of GKA and an incretin-related agent might therefore be effective in therapy.

Keywords Beta cell proliferation · Glucokinase activator · IRS2 · Oxidative stress

Abbreviations

CREB	cAMP-responsive element-binding protein
2-DG	2-Deoxyglucose
GKA	Glucokinase activator
HF	High-fat

Introduction

Glucokinase is the predominant enzyme involved in glucose phosphorylation in beta cells and hepatocytes, and it plays an important role as a glucose sensor in beta cells and as a regulator of glucose metabolism in the liver [1, 2]. In addition, glucokinase plays a pivotal role in regulating not only beta cell function, but also beta cell mass [3, 4].

Since the report by Grimsby et al in 2003 [5], several glucokinase activators (GKAs) have been developed, and these have been shown to lower blood glucose in several animal models of type 2 diabetes [5–12]. In a study of beta cell function, it was reported that a GKA stimulated insulin secretion in a Ca^{2+}-dependent manner in rodent islets and MIN6 cells [13], and we and others have reported that GKAs promoted beta cell proliferation and increased production of IRS2 [11, 14], which is critically required for beta

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cell growth and survival [3, 15–17]. However, the exact mechanisms by which GKAs stimulate beta cell function and proliferation are largely unknown.

Single- and multiple-dose placebo-controlled studies in human have recently reported that GKAs reduce the fasting and postprandial glucose levels of patients with type 2 diabetes and of healthy adults [18, 19]. Notably, however, another GKA, MK-0941, led to improvements in glycaemic control that were not sustained [20]. Therefore, a better understanding of the underlying mechanism is needed to determine whether glucokinase activation with GKAs is a feasible treatment goal for individuals with type 2 diabetes.

In the present study, we first investigated changes in the expression of genes involved in beta cell function and proliferation in mouse islets stimulated with GKA in order to elucidate the mechanisms by which GKA stimulated beta cell function and proliferation. We then explored therapeutic strategies by which GKA might work more effectively.

Methods

Chemicals A GKA (3-[(1*S*)-2-hydroxy-1-methylethoxy]-5-[4-(methylsulfonyl)phenoxy]-*N*-1,3-thiazol-2-yl benzamide) was prepared by Tsukuba Research Institute, Banyu Pharmaceutical, Tokyo, Japan, as previously described [21].

Animals *Irs2*$^{-/-}$ mice were generated as described elsewhere [16] and were then backcrossed with C57Bl/6J mice more than nine times. Both wild-type and *Irs2*$^{-/-}$ male mice were fed standard chow until 8 weeks of age, when they were given free access to either the standard chow, a high-fat (HF) diet, or an HF diet containing GKA. To evaluate the effect of GKA on glucose metabolism in vivo more thoroughly, wild-type and *Irs2*$^{-/-}$ mice were divided into four groups: wild-type mice fed the HF diet, wild-type mice fed the HF diet containing 0.04% GKA, *Irs2*$^{-/-}$ mice fed the HF diet, and *Irs2*$^{-/-}$ mice fed the HF diet containing 0.04% GKA. Five-week-old male *db/db* mice were purchased from Charles River Laboratories Japan (Yokohama, Japan). When they were 6 weeks of age, they were intraperitoneally injected with normal saline or exendin-4 (100 μg/kg; Sigma-Aldrich, Tokyo, Japan) once daily for 2 weeks. The mice were housed under a 12-h light/dark cycle. The animals were maintained in accordance with standard animal care procedures based on the institutional guidelines.

Diet protocol Standard chow (MF; Oriental Yeast, Tokyo, Japan) and an HF diet (High Fat Diet 32; Clea Japan, Tokyo, Japan) were used. GKA was administrated in the form of a 0.04% (wt/wt) admixture to the HF diet as previously described [11].

Glucose tolerance test Mice were fasted for 4 h before the study, and then orally loaded with a 1.5 mg/g body weight dose of glucose. Blood glucose was measured with a Glutest Neo portable glucose meter (Sanwa Chemical, Nagoya, Japan).

Immunohistochemical analysis to estimate beta cell mass Isolated pancreases were immersion-fixed in 10% formalin at 4°C overnight. Tissue was then routinely processed for paraffin embedding, and 5 μm sections mounted on glass slides were immunostained with rabbit anti-human insulin (diluted 1:1,000) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The area of the beta cells was calculated with WinROOF software (Mitani, Tokyo, Japan) as described elsewhere [3]. Approximately 100 islets per mouse were counted in each group.

Analysis of BrdU incorporation Wild-type and *Irs2*$^{-/-}$ mice on the HF diet for 10 weeks were divided into two groups: an HF diet group, and a group given 0.04% GKA mixed into the HF diet. After 3 days, the mice were intraperitoneally injected with BrdU (Nacalai Tesque, Kyoto, Japan), and the pancreases were removed 6 h later. Immunohistochemical detection of BrdU was performed with a commercial kit (BD Biosciences, Franklin Lakes, NJ, USA). Approximately 100 islets per mouse were counted in each group.

Islet isolation Islets were isolated by using liberase RI (Roche Diagnostics, Indianapolis, IN, USA) or collagenase XI (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's instructions, as described elsewhere [3, 11].

Analysis of insulin secretion Insulin secretion was measured after culturing islets overnight in RPMI 1640 medium containing 11 mmol/l glucose supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich, St Louis, MO, USA). Ten islets were incubated at 37°C for 1.5 h in Krebs–Ringer bicarbonate buffer containing 5.6 or 22 mmol/l glucose in the absence or presence of GKA. The insulin concentration of the assay buffer was measured with an insulin ELISA kit (Morinaga, Yokohama, Japan).

Real-time quantitative PCR Total RNA was isolated with the RNeasy Mini Kit (Qiagen, Hilden, Germany) and used as the starting material for complementary DNA (cDNA) preparation. cDNA was synthesised by using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA), and TaqMan quantitative PCR was performed with the ABI Prism 7500 PCR instrument (Applied Biosystems).

Western blot analysis The anti-phospho [Ser133] cAMP-responsive element-binding protein (CREB), total CREB,

and cyclin D2 antibody were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-IRS2 antibody was purchased from Upstate (Temecula, CA, USA). Protein was prepared from more than 100 islets pooled from several mice in the same group, and 20 μg of protein samples were applied to the gel. Protein bands were visualised with the ECL Plus Western Blotting Detection System (GE Healthcare, Amersham, UK).

Statistical analysis Results are expressed as mean ± SE (*n*). Differences between two groups were analysed for statistical significance by Student's *t* test. Individual comparisons between more than two groups were assessed by the post hoc Fisher's PLSD test. A p value <0.05 was considered statistically significant.

Results

Mechanism of the upregulation of Irs2 expression in response to GKA-induced glucokinase activation Because we had hypothesised that glucose metabolism via glucokinase would increase the phosphorylation of CREB and the production of IRS2 [3, 22], we investigated whether the GKA was able to stimulate the phosphorylation of CREB. Ser133 phosphorylation of CREB was significantly increased at 22 mmol/l glucose in comparison with 5.6 mmol/l glucose, and the GKA stimulated the Ser133 phosphorylation of CREB in the isolated islets of wild-type mice at 5.6 mmol/l glucose (Fig. 1a). The phosphorylation of CREB paralleled the upregulation of IRS2 production.

As it has been reported that glucose treatment of isolated islets potently increases *Irs2* expression via glucose metabolism and Ca^{2+} influx [23], we examined changes in *Irs2* mRNA levels in response to GKA administration or high-glucose stimulation under several conditions to identify the mechanism of *Irs2* upregulation. First, we evaluated *Irs2* mRNA expression in response to the GKA and sulfonylureas. Real-time quantitative PCR showed that *Irs2* mRNA was significantly increased with 22 mmol/l glucose in comparison with 5.6 mmol/l glucose (Fig. 1c). The GKA also significantly increased *Irs2* mRNA with 5.6 mmol/l glucose, but neither gliclazide nor glibenclamide upregulated the *Irs2* mRNA level (Fig. 1c).

Next, we used the non-metabolisable analogue of glucose, 2-deoxyglucose (2-DG), to investigate whether glucose metabolism was required for GKA-stimulated *Irs2* expression. When glucose was replaced by 2-DG, the *Irs2* mRNA levels did not increase in response to the GKA or a stimulatory concentration of 2-DG (22 mmol/l; Fig. 1d), indicating that glucose metabolism is necessary for GKA-induced *Irs2* expression to occur in islet beta cells.

We also conducted experiments using an L-type calcium channel blocker (nifedipine) and calcineurin inhibitor

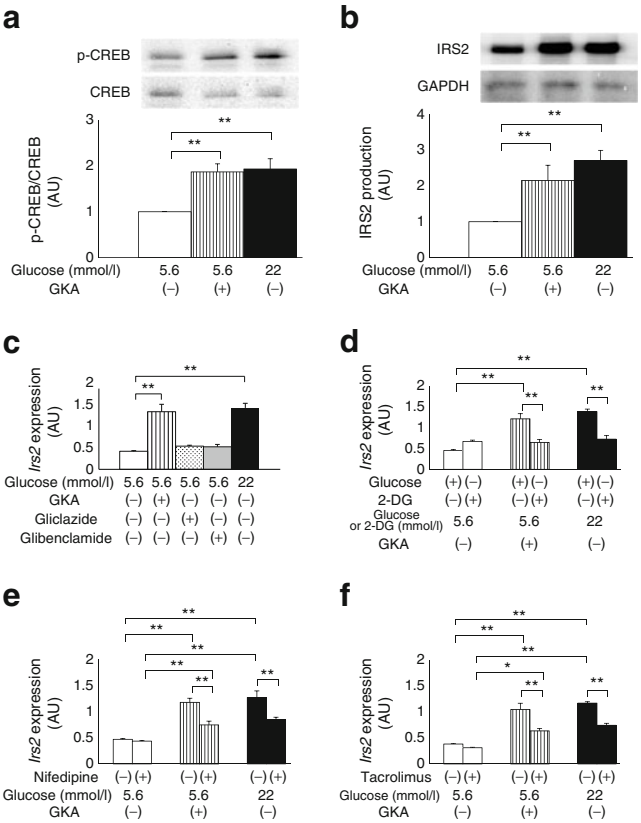

Fig. 1 Effect of GKA on upregulation of *Irs2* expression. **a** Western blot assay of phospho [Ser133] CREB (p-CREB) and total CREB levels in islets. **b** Production of IRS2 in islets. Islets from wild-type mice were stimulated with 5.6 mmol/l glucose in the absence or presence of 6 μmol/l GKA or with 22 mmol/l glucose alone. Equal amounts of lysates were blotted with the p-CREB, total CREB, IRS2 and GAPDH antibody. Expression levels were quantified. The intensity of p-CREB was normalised to total CREB expression, and the intensity of IRS2 was normalised to GAPDH expression (white bar, 5.6 mmol/l glucose; hatched bar, 5.6 mmol/l glucose plus GKA; black bar, 22 mmol/l glucose) (n=5). **c–f** Effects of (**c**) sulfonylureas (10 μmol/l gliclazide and 1 nmol/l glibenclamide), (**d**) 2-deoxyglucose (DG), (**e**) 50 μmol/l nifedipine, and (**f**) 10 μmol/l tacrolimus on *Irs2* expression. The *Irs2* and beta-actin (control) mRNA levels of isolated islets in wild-type mice were measured by real-time quantitative PCR. Data have been normalised to beta-actin expression (white bars, 5.6 mmol/l glucose; hatched bars, 5.6 mmol/l glucose plus GKA; dotted bars, 5.6 mmol/l glucose plus gliclazide; grey bar, 5.6 mmol/l glucose plus glibenclamide; black bars, 22 mmol/l glucose) (n=3 or 4). Values are mean ± SE. *p<0.05; **p<0.01

(tacrolimus). The results showed that both nifedipine (50 μM) and tacrolimus (10 μM) significantly inhibited the upregulation of *Irs2* mRNA levels induced by GKA administration or high-glucose stimulation, although the *Irs2* mRNA levels remained slightly but significantly increased (Fig. 1e, f). These results indicated that the GKA-induced *Irs2* upregulation in islets is at least partly Ca^{2+}-dependent and mediated by calcineurin.

Effect of the GKA on changes in gene expression in isolated islets Next, we investigated changes in the expression levels of genes involved in beta cell function and proliferation in the

islets of wild-type mice in the absence or presence of the GKA. *Pdx1* is the major regulator of glucose-stimulated insulin gene transcription, and its mRNA level was significantly increased at 22 mmol/l glucose in comparison with 5.6 mmol/l glucose; in addition, the GKA stimulated *Pdx1* mRNA production with 5.6 mmol/l glucose in the isolated islets of wild-type mice (Fig. 2a). The increased *Pdx1* levels paralleled the upregulation of *Glut2*, *Gck*, *Ins1* and *Ins2* expression (Fig. 2a). Gliclazide, however, failed to increase the expression levels of these (Fig. 2b). These results indicated that the GKA improved beta cell function at the transcriptional level.

As the results of a DNA microarray analysis we previously reported showed decreased expression of *Pdpk1* and *Ccnd2* in $Gck^{+/-}$ mice in comparison with wild-type mice on the HF diet [3], we investigated the expression levels of these and other cell-cycle-related genes in the present study. GKA and high-glucose stimulation significantly increased *Pdpk1*, *Ccnd1*, *Ccnd2* and *Ccnd3* mRNA expression, but the expression of *Cdk4* and p27 was unaltered (Fig. 2c). Cyclin D2 protein levels were also increased by the GKA and high-glucose stimulation (Fig. 2d). These results suggested an involvement of cell cycle signalling, such as by cyclin D2, in GKA-stimulated beta cell proliferation.

Effect of the GKA on glucose metabolism and beta cell mass in $Irs2^{-/-}$ mice We used $Irs2^{-/-}$ mice to determine whether IRS2 was required for the therapeutic effects of the GKA, and divided the animals into four groups: wild-type mice fed the HF diet (WT group), wild-type mice fed a diet containing 0.04 % GKA mixed into the HF diet (WT+GKA group), $Irs2^{-/-}$ mice fed the HF diet (IRS2 group), and $Irs2^{-/-}$ mice fed 0.04% GKA mixed into the HF diet (IRS2+GKA group).

There were no significant differences in body weight between mice receiving and mice not receiving GKA in both the wild-type and $Irs2^{-/-}$ groups (Fig. 3a). The blood glucose level decreased shortly after consuming the HF diet containing the GKA. The blood glucose level of the WT+GKA group was significantly lower than that of the WT group, and the blood glucose level of the IRS2+GKA group was significantly lower than that of the IRS2 group (Fig. 3b). We also performed an OGTT on these four groups of mice (Fig. 3c). The area under the curve (0–120 min) of the blood glucose levels during the OGTT was significantly decreased in both the wild-type and $Irs2^{-/-}$ mice that received the GKA compared with the mice that did not receive the GKA (Fig. 3d). Thus, GKA demonstrated glucose-lowering efficacy without affecting body weight in both the wild-type and $Irs2^{-/-}$ mice on the HF diet.

Next, to investigate the effect of GKA on beta cell mass, we measured the beta cell mass of the mice. Histological analysis revealed that the area of the beta cells was significantly increased in the wild-type mice in comparison with the $Irs2^{-/-}$ mice (Fig. 3e), consistent with our previous report [3], but no further increase was observed in response to administering GKA to either genotype of mouse (Fig. 3e).

As we previously reported [11], the absence of any effect of the GKA on the beta cell mass of the $Irs2^{-/-}$ mice may be attributable to a suppression of beta cell proliferation due to

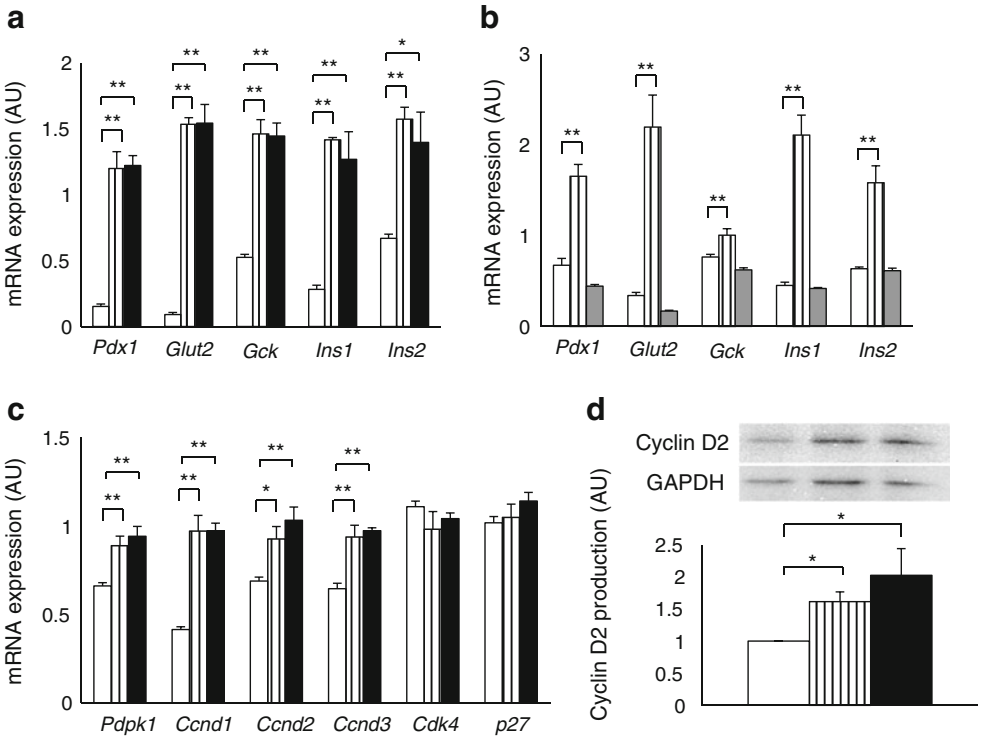

Fig. 2 Changes in gene expression levels in islets stimulated with GKA. **a–c** The mRNA levels of (**a, b**) *Pdx1*, *Glut2*, *Gck*, *Ins1* and *Ins2*, and of (**c**) *Pdpk1*, *Ccnd1*, *Ccnd2*, *Ccnd3*, *Cdk4* and *p27* in islets measured by real-time quantitative PCR. Data have been normalised to beta-actin expression. **d** Western blot assay of cyclin D2 levels in islets. Equal amounts of lysates were blotted with the cyclin D2 antibody and GAPDH antibody. Protein levels were quantified, and the cyclin D2 data have been normalised to GAPDH expression (white bars, 5.6 mmol/l glucose; hatched bars, 5.6 mmol/l glucose plus GKA; grey bars, 5.6 mmol/l glucose plus gliclazide; black bars, 22 mmol/l glucose) (n=3–5). Values are mean ± SE. *p<0.05; **p<0.01

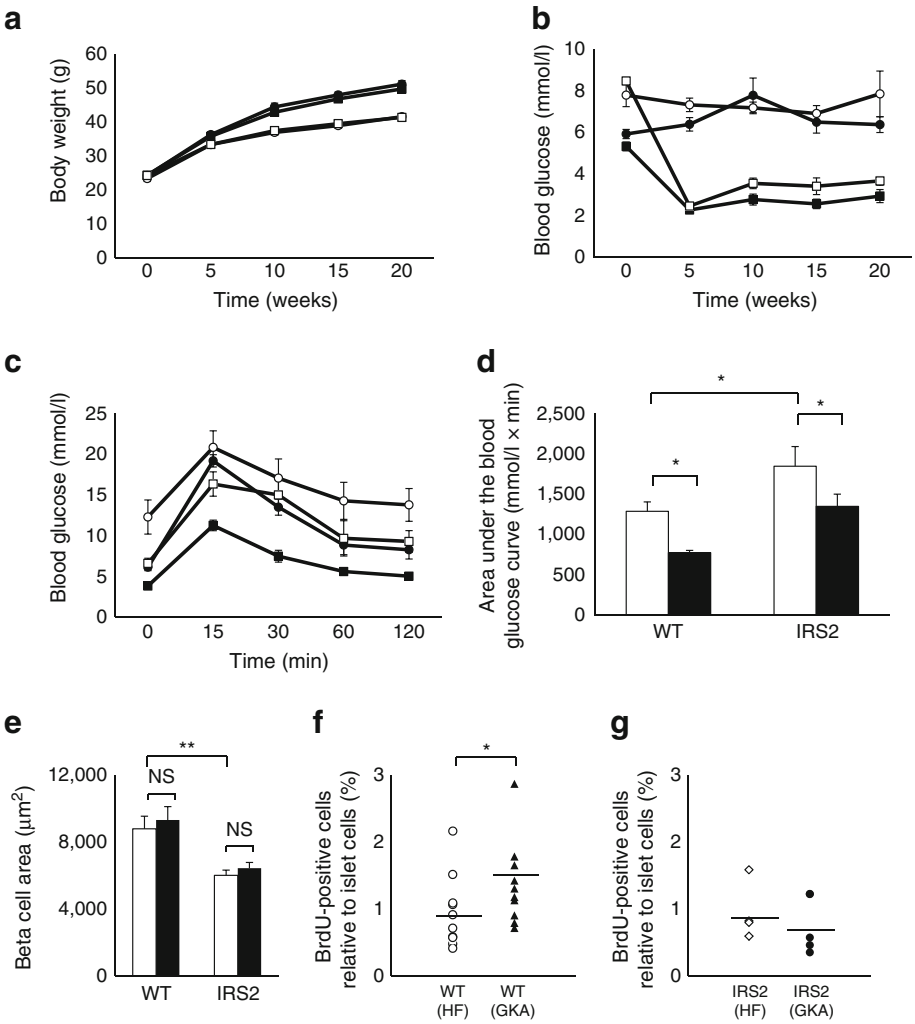

Fig. 3 Impact of GKA on glucose metabolism and on beta cell mass and proliferation in *Irs2*$^{-/-}$ mice. **a, b** Changes in (**a**) body weight and (**b**) fed blood glucose level. Wild-type mice on the HF diet (WT; black circles), wild-type mice fed the 0.04% GKA HF diet (WT+GKA; black squares), *Irs2*$^{-/-}$ mice fed the HF diet (IRS2; white circles) and *Irs2*$^{-/-}$ mice fed the 0.04% GKA HF diet (IRS2+GKA; white squares) (n=8). **c** Blood glucose levels during the OGTT in the WT group (black circles), WT+GKA group (black squares), IRS2 group (white circles) and IRS2+GKA group (white squares) (n=7 or 8). **d** Area under the curve of the glucose excursion during the OGTT in the mice on the HF diet (white bars) or on the 0.04% GKA HF diet (black bars) (n=7 or 8). **e** Quantification of beta cell mass. Histological analysis of pancreatic islets from the mice on the HF diet (white bars) and the 0.04% GKA HF diet (black bars) (n=4 or 5). **f, g** Replication rates, assessed by BrdU incorporation after administration of GKA for 3 days, were determined in (**f**) WT mice and (**g**) *Irs2*$^{-/-}$ mice after 10 weeks on the HF diet. Results are shown as vertical scatter plots for the wild-type mice without GKA (white circles) and with GKA (black triangles), and *Irs2*$^{-/-}$ mice without GKA (white diamonds) and with GKA (black circles) (n=4–11). Values are mean ± SE. *p<0.05; **p<0.01

the chronic reduction in ambient blood glucose levels induced by the GKA treatment rather than to the deficiency of IRS2. To investigate this possibility, we evaluated beta cell proliferation after administering the GKA on three consecutive days to mice fed the HF diet for 10 weeks; the results showed a significant decrease in the fed blood glucose level of both the wild-type and *Irs2*$^{-/-}$ mice shortly after administration of the GKA (data not shown). The BrdU incorporation ratio was significantly increased in the wild-type mice given the GKA for 3 days in comparison with the wild-type mice not given the GKA; this was not, however, seen in the *Irs2*$^{-/-}$ mice (Fig. 3f, g). These results support the concept that IRS2 could have an effect on beta cell proliferation stimulated by a GKA in vivo.

Effect of the GKA on the beta cell function of Irs2$^{-/-}$ *mice* To investigate the effect of the GKA on beta cell function ex vivo, we evaluated glucose-stimulated insulin secretion by the islets of *Irs2*$^{-/-}$ mice. Insulin secretion by *Irs2*$^{-/-}$ islets in response to 5.6 mmol/l glucose was significantly increased in the presence of GKA (Fig. 4a). The increase in insulin secretion in response to the application of 22 mmol/l glucose was less evident, suggesting that the secretion had peaked. These findings indicated that the GKA

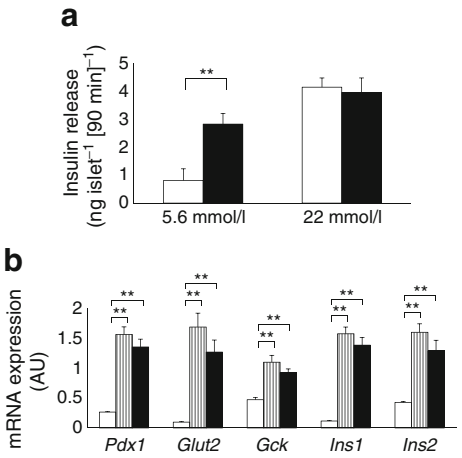

Fig. 4 Impact of GKA on glucose-stimulated insulin secretion ex vivo and changes in expression of genes involved in beta cell function in islets isolated from *Irs2*$^{-/-}$ mice. **a** Insulin secretion was measured with 5.6 mmol/l or 22 mmol/l glucose in the absence or presence of GKA. Results are shown as nanograms of insulin islet^{-1} 90 min^{-1} (n=7). Values are mean ± SE of data obtained from the analysis of islets isolated from *Irs2*$^{-/-}$ mice treated with vehicle (white bars) or 6 μmol/l GKA (black bars). **b** mRNA levels of *Pdx1*, *Glut2*, *Gck*, *Ins1* and *Ins2* in islets from *Irs2*$^{-/-}$ mice were measured by real-time quantitative PCR. Data have been normalised to beta-actin expression (white bars, 5.6 mmol/l glucose; hatched bars, 5.6 mmol/l glucose plus GKA; black bars, 22 mmol/l glucose) (n=3 or 4). Values are mean ± SE. **p<0.01

had stimulated glucose-stimulated insulin secretion by increasing glucose sensitivity without altering maximum insulin secretion by the *Irs2*$^{-/-}$ islets, which is consistent with our previous findings in wild-type and *Gck*$^{+/-}$ islet cells [11].

Next, we investigated whether the GKA affected the expression of genes involved in beta cell function in islets isolated from *Irs2*$^{-/-}$ mice. *Pdx1* mRNA significantly increased with 22 mmol/l glucose in comparison with 5.6 mmol/l glucose, and the GKA stimulated *Pdx1* mRNA expression at 5.6 mmol/l glucose in the isolated islets of *Irs2*$^{-/-}$ mice (Fig. 4b). In addition, there were no differences in *Pdx1* expression 5.6 mmol/l glucose in wild-type and *Irs2*$^{-/-}$ mice, and *Pdx1* expression was significantly increased with 22 mmol/l glucose or by the GKA in both genotypes of mouse to the same degree (data not shown). The increased *Pdx1* expression also paralleled the upregulation of *Glut2*, *Gck*, *Ins1* and *Ins2* levels (Fig. 4b). These results indicated that GKA enhanced beta cell function at the transcriptional level independently of IRS2.

Effect of oxidative stress on GKA-induced Irs2 and Pdx1 expression Exposure to exogenous H_2O_2 is known to reduce glucose-induced insulin secretion by impairing mitochondrial metabolism in beta cells [24]. This knowledge prompted us to evaluate the effect of oxidative stress on GKA-induced changes in the expression of genes related to beta cell function and proliferation. We investigated GKA-stimulated *Irs2* and *Pdx1* expression after H_2O_2 preconditioning. H_2O_2 significantly inhibited the upregulation of *Irs2* and *Pdx1* induced by GKA (Fig. 5a, b), and the inhibition was prevented by prior administration of alpha-tocopherol plus ascorbate (Fig. 5c, d). These results indicated that oxidative stress prevented GKA affecting the expression of genes related to beta cell function and proliferation.

Effect of GKA on Irs2 and Pdx1 expression in db/db mice Based on the above results, we next evaluated the effect of GKA on *Irs2* and *Pdx1* expression in *db*/*db* mice, a well-known model of type 2 diabetes accompanied by obesity and characterised by increased insulin resistance and severe damage to the pancreatic beta cells. The levels of gene expression for the reduced-form NADPH oxidase complex were coordinately elevated in islets isolated from *db*/*db* mice in comparison with islets isolated from wild-type mice (Fig. 6a–c), and thus the islets from the *db*/*db* mice could be exposed to oxidative stress. In contrast to the islets isolated from the wild-type mice, GKA failed to increase *Irs2* and *Pdx1* expression in islets isolated from the *db*/*db*

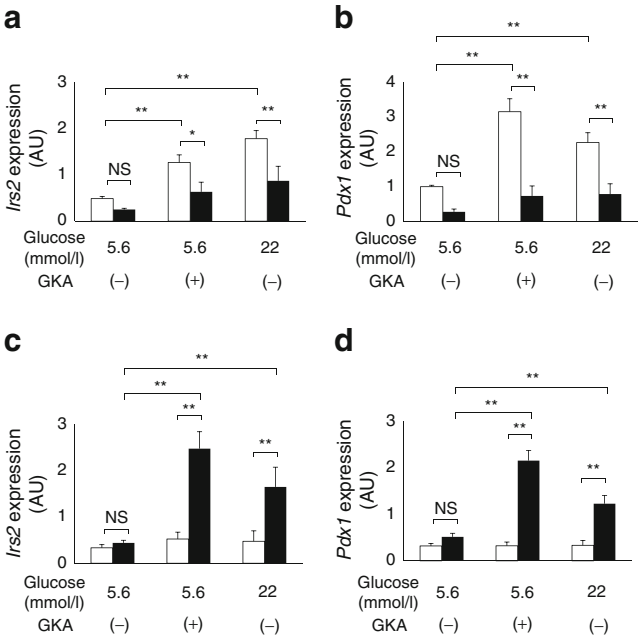

Fig. 5 Effect of GKA on changes in *Irs2* and *Pdx1* expression levels under oxidative stress. **a, b** mRNA levels of (**a**) *Irs2* and (**b**) *Pdx1* in islets from wild-type mice after H_2O_2 preconditioning were measured by real-time quantitative PCR. Isolated islets were incubated overnight with RPMI1640 medium containing 5.6 mmol/l glucose with or without 50 μmol/l H_2O_2 (white bars, absence of H_2O_2; black bars, presence of H_2O_2). **c, d** Effect of antioxidant treatment on GKA-stimulated *Irs2* (**c**) and *Pdx1* (**d**) expression. Isolated islets were incubated overnight with RPMI1640 medium containing 5.6 mmol/l glucose and 50 μmol/l H_2O_2 with or without alpha-tocopherol plus ascorbate (white bars, absence of alpha-tocopherol plus ascorbate; black bars, presence of alpha-tocopherol plus ascorbate). Data have been normalised to beta-actin expression (n=4). Values are mean ± SE. *p<0.05; **p<0.01

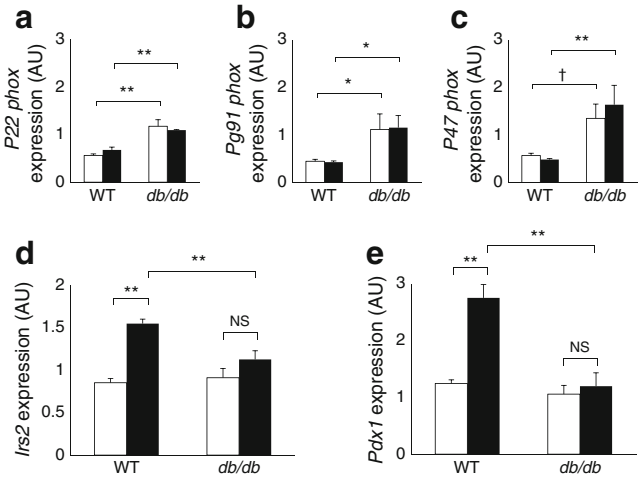

Fig. 6 Changes in expression of genes in isolated islets of *db/db* mice stimulated with GKA. mRNA levels of (**a**) *P22phox* (also known as *Cyba*), (**b**) *Pg91phox* (also known as *Cybb*), (**c**) *P47phox* (also known as *Ncf1*), (**d**) *Irs2* and (**e**) *Pdx1* in islets from 8-week-old wild-type and *db*/*db* mice were measured by real-time quantitative PCR. Data have been normalised to beta-actin expression (white bars, absence of GKA; black bars, presence of GKA) (n=4). Values are mean ± SE. *p<0.05; **p<0.01; †p=0.05

mice (Fig. 6d, e). These results showed that the effects of GKA on the expression of genes related to beta cell function and proliferation were diminished in the islets from the *db*/*db* mice.

Effect of exendin-4 on GKA-stimulated Irs2 and Pdx1 expression in db/db mice In order to increase *Irs2* and *Pdx1* expression stimulated by GKA in *db*/*db* mice, exendin-4 was intraperitoneally injected into *db*/*db* mice for 2 weeks before isolating the islets. Exendin-4 decreased blood glucose levels without affecting body weight (Fig. 7a, b). Remarkably, GKA was able to upregulate *Irs2* and *Pdx1* expression in the islets of *db*/*db* mice after prior administration of exendin-4 (Fig. 7c, d). It is noteworthy that exendin-4 alone was insufficient to upregulate these molecules. However, under our experimental conditions, the expression levels of genes for the reduced-form NADPH oxidase complex were unchanged (Fig. 7e–g).

Discussion

The results of the present study yielded three new findings. First, glucokinase activation by the GKA, a glucose-like activator of beta cell metabolism, increased IRS2 production; in addition, GKA-stimulated IRS2 production was able to affect beta cell proliferation, but not beta cell function. Second, the effects of the GKA on the expression of genes involved in beta cell function and proliferation were diminished in islets exposed to exogenous H_2O_2 and in those from *db*/*db* mice. Third, a combination of GKA and an incretin-related agent was effective in upregulating *Irs2* and *Pdx1* expression in these islets.

GKA increased the phosphorylation of CREB and IRS2 production (Fig. 1a, b), and 2-DG failed to increase *Irs2* expression by GKA (Fig. 1d). Both nifedipine and tacrolimus partly, but significantly, inhibited upregulation of *Irs2* expression by GKA (Fig. 1e, f). Taken together with the previous study [25], these results suggest that GKA increased CREB phosphorylation and *Irs2* expression in islets via glucose metabolism, Ca^{2+} influx and, in part, a Ca^{2+}–calcineurin pathway. In contrast, *Irs2* expression was not increased by either of the sulfonylureas. Sulfonylureas are known to close ATP-sensitive potassium channels regardless of glucose metabolism, and their closure results in membrane depolarisation, an influx of Ca^{2+} through voltage-dependent Ca^{2+} channels, and an increase in cytosolic free Ca^{2+} concentration, thereby triggering insulin secretion [26]. If that is true, why did the sulfonylureas fail to increase *Irs2* expression despite being able to increase the Ca^{2+} influx? One possible explanation is that the glucose flux per se may also be needed to increase *Irs2* expression and lead to beta cell proliferation [3, 22], since the sulfonylurea glibenclamide is unable to increase beta cell

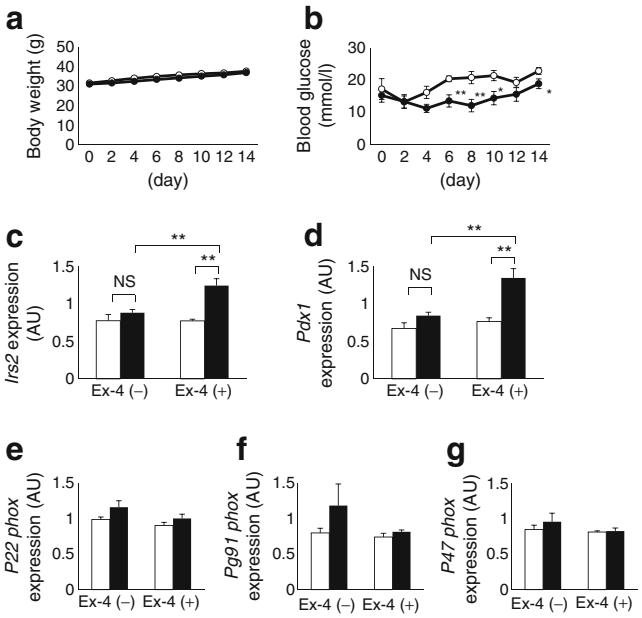

Fig. 7 Effect of a combination of exendin-4 and GKA on *Irs2* and *Pdx1* expression in *db/db* mice. **a, b** Changes in (**a**) body weight and (**b**) fed blood glucose levels of *db/db* mice injected or not injected with exendin-4 (Ex-4) for 2 weeks (white circles, not injected with Ex-4; black circles, injected with Ex-4) (n=8). The mRNA levels of (**c**) *Irs2*, (**d**) *Pdx1*, (**e**) *P22phox* (also known as *Cyba*), (**f**) *Pg91phox* (also known as *Cybb*) and (**g**) *P47phox* (also known as *Ncf1*) in islets from *db*/*db* mice were measured by real-time quantitative PCR. Data have been normalised to beta-actin expression (white bars, absence of GKA; black bars, presence of GKA) (n=5). Values are mean ± SE. *p<0.05; **p<0.01

proliferation in wild-type mice [27]. We therefore hypothesised that both glucose metabolism and Ca^{2+} influx are required to increase *Irs2* expression and lead to beta cell proliferation. This hypothesis is supported by a report that glibenclamide increases beta cell proliferation in the presence of an increased glucose flux [27].

The *Pdx1* expression level has been reported to be severely reduced in the beta cells of *Irs2*$^{-/-}$ mice with a certain genetic background [28], suggesting that IRS2 may directly regulate the expression and function of *Pdx1*, and thereby maintain beta cell growth and function. However, GKA upregulated the expression of *Pdx1* and several downstream genes, *Glut2*, *Gck*, *Ins1* and *Ins2*, in the isolated islets of *Irs2*$^{-/-}$ mice with a C57BL/6 background (Fig. 4b). In regard to this point, Suzuki et al reported finding that *Pdx1* expression in *Irs2*$^{-/-}$ mice is regulated in a strain-dependent manner [29]. *Pdx1* expression was not downregulated in our *Irs2*$^{-/-}$ murine beta cells that had a C57BL/6J background [29]. We therefore assume that GKA increased glucose-stimulated insulin gene transcription independently of IRS2. Since Kushner et al reported that transgenic overexpression of *Pdx1* restored beta cell mass in *Irs2*$^{-/-}$ mice [28], *Pdx1* may play a role in regulating beta cell mass. Nevertheless, because a haploinsufficiency of *Pdx1* led to impaired beta cell function, but not to decreased beta cell mass [30], and because beta cell mass was smaller in our *Irs2*$^{-/-}$ mice than in wild-type mice despite the *Pdx1* expression level being maintained [29], two different pathways are involved in the stimulation of beta cell function and proliferation by a GKA.

The results of our study have clinical implications. As stated above, MK-0941 lacked durability in glycaemic control [20]. In these patients, disease-related characteristics were a mean baseline HbA_{1c} of 9.0 % (75 mmol/mol), a mean duration of diabetes of 12 years, and a mean insulin glargine (A21Gly,B31Arg,B32Arg human insulin) dose of 45 U/day. These data suggest that the insulin secretion or the beta cells themselves were severely impaired. From these clinical trials, it is possible that a pancreatic effect of GKAs could not be expected when pancreatic beta cells have been impaired. It is well known that the level of 8-hydroxydeoxyguanosine, a marker of oxidative stress, is increased in diabetic patients and independently associated with mean HbA_{1c} [31]; in addition, increased oxidative stress influences beta cell function, and antioxidant treatment can exert beneficial effects in diabetes, with a preservation of beta cell function [32]. We therefore investigated the effect of oxidative stress on GKA-induced changes in the expression of genes involved in beta cell function and proliferation. As expected, the results showed that oxidative stress inhibited the upregulation of *Irs2* and *Pdx1* in response to GKA, although the inhibition was prevented by prior administration of an antioxidant (Fig. 5). Moreover, the levels of these molecules were not increased by GKA in islets isolated from *db*/*db* mice (Fig. 6).

Although our findings could support the observation that GKA is ineffective in patients with type 2 diabetes whose pancreatic beta cells have been impaired, there are some limitations to our study. First, with regard to the experiment with exogenous H_2O_2, we could not rule out the possibility that the absence of increasing *Irs2* and *Pdx1* expression after H_2O_2 preconditioning was caused by not only impaired mitochondrial function, but also an impaired biological response of these islets independently of GKA activity. It is also possible that *Gck* expression level might have an effect on the inhibition of upregulation of *Irs2* and *Pdx1* induced by 5.6 mmol/l glucose plus GKA. Second, with regard to the experiment with the *db*/*db* mice, GKA compounds act to reduce glucose levels in the *db*/*db* mouse model, as shown with MK-0941 and other agents [8, 33]. The background of the *db*/*db* mice or the difference in compounds could influence the effect of GKAs. Thus, greater study of the glucose-lowering effect of the GKA in *db*/*db* mice is needed.

In the present study, we also explored the therapeutic strategy by which GKA worked more effectively in these impaired beta cells. Based on the results of this study, we propose that GKA should be used before pancreatic beta cell failure. First, GKA should be used earlier, as diabetes is progressing, since beta cell failure has already progressed before the diagnosis of diabetes is made [34, 35]. The efficacy of long-term GKA therapy should be assessed in patients with mild type 2 diabetes or impaired glucose tolerance. Second, GKA should be used in combination with incretin therapy in case GKA monotherapy is ineffective. The results of our study indicated that GKA stimulated *Irs2* and *Pdx1* expression in the islets of *db*/*db* mice when exendin-4 had been administered in advance (Fig. 7). This finding suggested that combination therapy consisting of a GKA and an incretin may be useful for patients with type 2 diabetes. We previously speculated that the increase in *Irs2* expression in the islets of *db*/*db* mice in response to the combination of these drugs was due to the reduction of oxidative stress or the additive effect of Ca^{2+} influx via glucose signalling and cAMP signalling through the human glucagon-like peptide-1 receptors [22]. However, the levels of expression of the genes for the reduced-form NADPH oxidase complex were unchanged by prior administration of exendin-4 under our experimental conditions (Fig. 7e–g). Since *Irs2* expression was not increased by exendin-4 alone (Fig. 7c), we assume that exendin-4 amplifies GKA-stimulated calcium signalling rather than imposing it. Further study is needed to test the combination in vivo on glycaemic control and the resultant changes in pancreatic gene transcription.

In conclusion, GKA-stimulated IRS2 production affected beta cell proliferation, but not beta cell function. Oxidative

stress was able to prohibit the ability of GKA to change the expression of genes involved in beta cell function and proliferation. A combination of GKA and an incretin-related agent might be effective here. These findings suggest that the GKAs should have outstanding potential for the treatment of diabetes and related disorders.

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