Both sitagliptin analogue & pioglitazone preserve the β-cell proportion in the islets with different mechanism in non-obese and obese diabetic mice

Jin-A Yeom#, Eun Sook Kim#, Heon-Seok Park, Dong-Sik Ham, Chenglin Sun, Ji-Won Kim, Jae-Hyoung Cho & Kun-Ho Yoon*

Division of Endocrinology & Metabolism, Department of Internal Medicine, The Catholic University of Korea, Seoul 137-701, Korea

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INTRODUCTION

Type 2 diabetes (T2DM) is characterized by reduced insulin sensitivity and progressive deterioration of β-cells (1, 2). Several previous studies have shown that diabetic subjects already have significant deficits in β-cell mass at the time of diagnosis, and experience steady declines in their β-cell volumes (3), which can be devastating since no currently available treatments are capable of reversing this decline (4).

In particular, lean subjects with T2DM appear to be at a relatively high risk of β-cell failure since there is ample evidence to suggest that innate lean diabetic patients have smaller β-cell masses (5) and more defective insulin secretion than obese patients. Thus, the preservation of β-cell mass constitutes a key objective in efforts to impede the progression of diabetes, particularly in non-obese subjects with diabetes. Dipeptidyl peptidase-4 (DPP-4) inhibitors have evaluated as potential therapeutic drugs for the treatment of this pathophysiology, due to both their glucose-lowering effects in response to metabolic demands and to their expected effects in β-cell preservation (6, 7), both of which are caused by an elevation in the levels of biologically active incretins (8). Considering their relevant mechanisms and underlying pathophysiology, DPP-4 inhibitors may be more suitable for the treatment of non-obese subjects with diabetes, specifically in regard to the potentiation of insulin secretion and β-cell mass preservation (6-8). Some clinical studies in which DPP-4 inhibitors were examined in Asian diabetics with relatively low body mass index (BMI) have already demonstrated their greater efficacy in lowering levels of HbA1c (9, 10). Another drug proposed for the preservation of β-cells is thiazolidinedione (TZD), which is generally believed to reduce glucolipotoxicity by improving insulin sensitivity (11-13).

Consequently, this study was conducted to determine whether or not treatment with a sitagliptin analogue (a DPP-4 inhibitor) or pioglitazone (a TZD) exerted different effects on glucose homeostasis and β-cell preservation in lean and obese mouse models of type 2 diabetes.

RESULTS

Changes in body weight and non-fasting glucose levels and effects on glucose tolerance

The db/db mice gained weight rapidly during the experiment. The degree of weight gain was slightly lower in the SITA- and PIO-treated groups relative to the control group (SITA vs. control: 122.2±8.4% vs. 131.2±8.7%; PIO vs. control: 113.0±9.3% vs. 131.2±8.7%). In the Akita mice, the SITA- and PIO-treated groups also gained slightly less weight than the controls (SITA vs. control: −2.6±4.8% vs. 7.7±6.0%; PIO vs. control: −5.4±3.7% vs. 7.7±6.0%).

After SITA or PIO treatment, non-fasting blood glucose levels were significantly reduced at 3-5 days, and this reduction was maintained throughout the experiment for both the db/db and Akita mice (Fig. 1). In comparison with the controls, the area under the glucose curve for SITA- or PIO-treated db/db and Akita
mice was significantly reduced (AUCh; all P < 0.01).

To evaluate the effects of each drug on glycemic control, an intraperitoneal glucose tolerance test (IP-GTT) was conducted after 4-6 weeks of treatment (Fig. 1Ab-f, Bb-f). The SITA and PIO group had significantly reduced glycemic excursion and AUCh both in the Akita and db/db mice models (Fig. 1Abf, Bbf).

Effects of SITA and PIO treatments on islet mass and morphology

We noted no changes in β-cell mass in the SITA- or PIO-treated groups relative to the controls in both the db/db and Akita mice groups (Fig. 2Ac, d). However, increased β-cell-to-islet-area ratios were detected in the db/db and Akita mice after treatment with both drugs (both P < 0.01; Fig. 2Aa, b). As is shown in Fig. 2B, in addition to the islet proportion, islet architecture was maintained in the SITA and PIO groups in both the db/db and Akita groups, with an increased insulin-stained area observed in the center and a diminished glucagon-stained area within the periphery. In addition, both the db/db and Akita control mice had a diminished insulin-stained area and an expanded glucagon-stained area.

**Islet mass dynamics**

Ki-67 staining and analysis were performed in order to evaluate the effects of the drugs on the proliferation of islet cells. As illustrated in Fig. 3, the percentage of Ki-67-positive staining in the SITA-treated Akita mice was significantly increased, whereas no differences were noted in the db/db mice. For PIO treatment, the proliferation rate was not increased in the db/db mice and was significantly reduced in the Akita mice. We also evaluated SITA-dependent inhibition of cell apoptosis via a TUNEL assay (Fig. 4). Both the SITA- and PIO-treated db/db mice exhibited reduced apoptosis relative to the controls, whereas no differences were observed for the Akita mice.

**DISCUSSION**

In this study, SITA treatment was shown to exert a more prom-
Fig. 2. β-Cell composition, mass, and morphology. The SITA and PIO groups had higher β-cell ratios in islets than in the controls (Aa, b). The SITA and PIO treatment restored the islet structures with increased levels of central β-cells and peripheral α-cells relative to the controls (B); n = 3-8 mice per condition. **P < 0.01, vs. control.

Fig. 3. Comparison of proliferation represented by Ki-67 staining. The SITA-treated group exhibited an increased rate of proliferation in the Akita mice, whereas the PIO-treated group exhibited no increases in proliferation in either model and a slight reduction in the Akita mice (A). Ki67 staining is shown in (B). The arrows indicate Ki-67-positive cells (×400 magnification); n = 3-8 mice per condition. *P < 0.05, vs. control and **P < 0.01, vs. control.

Fig. 4. Comparison of the apoptotic ratio, shown by TUNEL staining. The percentage of TUNEL-positive cells was significantly lower with SITA or PIO treatment relative to the controls in the db/db mice group, whereas no difference was observed in the Akita mice after SITA or PIO treatment (A). The TUNEL staining is shown in (B) (×200 magnification). The arrows indicate 2 TUNEL-positive cells; n = 3-8 mice per condition. *P < 0.05, vs. control and **P < 0.01, vs. control.

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diabetes, via the potentiation of glucose-dependent insulin secretion (16) and prevention of β-cell mass loss in diabetic animal models (17, 18), which has never previously been achieved by any treatments. Theoretically, SITA, which is a DPP-4 inhibitor, should be more potent in Akita mice due to an improvement in the principal defect, i.e., impaired insulin secretion, but not in db/db mice, which exhibit basal hyperinsulinemia that results from insulin resistance. Conversely, PIO, which is an anti-diabetic agent that reduces insulin resistance via PPARγ activation (19), exerts similar glucose-lowering effects in both lean and obese mice, by overcoming relative or absolute insulin deficiencies via its potentiating activity.

The beneficial results of SITA and PIO on islet structure, such as its morphogenic characteristics and β-cell ratio, indicate that both drugs improve islet cell dynamics, resulting in the future preservation of the β-cell mass. PIO treatment preserved β-cells via an induced reduction in apoptosis, which is the principal defect responsible for the loss of β-cell mass (20). Related mechanisms include either the indirect effects of the glucose-lowering action or the direct effects of TZD on islets by lowering the intracellular triglyceride content and attenuating oxidative stress (21). No reductions in apoptosis were noted in the Akita mice after PIO treatment.

Moreover, the results of other studies (7, 18, 31). We suggest that the 4-6-week treatment period noted in the Akita mice after PIO treatment. This was consistent with the results reported by Izumi et al. (22), who detected apoptosis in fewer than one cell per islet in Akita mice, which is in contrast to the results reported in a previous study (23). We do not yet know the mechanisms underlying this phenomenon, but it may be the consequence of the rapid clearance of apoptotic material, rather than the infrequent occurrence of apoptosis as suggested by O’Brien et al. (24).

Meanwhile, SITA exerted beneficial effects on the islets via both reduced apoptosis and increased proliferation. These results indicate that SITA itself exerts additional effects on islet preservation aside from lowering glucotoxicity, thereby resulting in an anti-apoptosis effect. According to the findings of previous studies, exposure to SITA may result in the preservation of β-cells due to an induced increase in proliferation or neogenesis via the maintenance of high GLP-1 (25, 26) or GIP (27, 28) levels through DPP-4 inhibition, which activates PI-3 kinase and causes PKCδ or PKB, PDX-1 elevation, and cyclin D1/D2 activation (29, 30). There is currently no obvious explanation for the lack of an increased proliferation rate in the db/db mice as compared with the Akita mice. This may be a consequence of unresolved hyperglycemic excursion or a characteristic difference specifically inherent to the db/db mouse itself.

In this study, islet mass did not increase after administration of either SITA or PIO in either model, which is inconsistent with the results of other studies (7, 18, 31). We suggest that the 4-6-week treatment period used in this study was not sufficiently long for the β-cell mass to be altered significantly in either animal model. Moreover, the β-cell mass at any specific time should not be viewed as an accurate reflection of the actual β-cell reservoir, since islet cells retain their plasticity via a dynamic mechanism involving regeneration, proliferation, and apoptosis (32-34). Unexpectedly, fasting glucose levels during GTT were a bit higher than nonfasting levels in db/db mice treated with SITA (Fig. 1), which was possibly caused by acute stressful conditions before IP-GTT. Nevertheless, the effects of the drugs on glycemic dynamics were evident when compared with the control mouse.

In conclusion, the results of this study showed that both SITA and PIO may exert some beneficial effects on islet structure in addition to glycemic control, induced via the different effects of these compounds on apoptosis and proliferation. In regards to β-cell dynamics, only SITA increased the proliferation of β-cells in Akita mice, indicating a possible additional benefit on β-cell mass in the lean mouse model. The results of this study demonstrate that SITA and PIO exert comparable effects in regards to slowing the progression of T2DM by preserving β-cell mass. Further studies will be necessary to corroborate this, and to ascertain whether or not SITA exerts different effects in lean and obese subjects.

MATERIALS AND METHODS

Reagents

The DPP-4 inhibitor utilized in this study was a des-fluoro analogue of sitagliptin—7-{[3R]-3-amino-1-oxo-4-(2,5-difluorophenyl)butyl]-5,6,7,8-tetrahydro-3-(trifluoromethyl)-1,2,4-triazolo[4,3-a]pyrazine L-tartaric acid salt and the compound is referred to hereafter as des-fluoro-sitagliptin. Des-fluoro-sitagliptin was prepared by Process Research, Merck Research Labs ( Rahway, NJ). Pioglitazone was provided by Lianyungang Guiyuan Chempharm Co., LTD (China).

Mice

5 week-old male non-obese heterozygous Ins2+/- Akita mice and obese C57BL/KsJ-db/db mice were purchased from Jackson Laboratories (Bar Harbor, ME). The mice were housed in a specific pathogen-free facility with a 12-h light, 12-h dark cycle. The mice had free access to standard rodent chow and water, except when fasting. The mice were permitted to acclimate to the animal facility for 1 week prior to treatment.

Treatment

Des-fluoro-sitagliptin and pioglitazone were premixed with the normal chow diet and administered orally. The target doses were 11 g/kg body weight (des-fluoro-sitagliptin) and 100 mg/kg body weight (pioglitazone) based on previous studies (35, 36). In our pilot experiment, we found that even a dose of 500 mg/kg/day sitagliptin inhibited only about 60% of DPP-4 enzyme activity, which was too weak to produce a blood glucose lowering effect. As demonstrated by Lamont and Drucker (36), inhibition of DPP-4 activity up to 90% was achieved by treating mice with a concentration of 11 g/kg of sitagliptin. Therefore, this dose was selected for this study. The mice were randomly assigned to independent treatment groups: Akita mice [control (n = 5), SITA (n = 8), PIO (n = 7)]; db/db mice [control (n = 5), SITA (n = 10), PIO (n = 10)]. Non-fasting blood glucose levels were measured from tail bleeds using a glucometer (ARKRAY Inc., Kyoto, Japan) and body weights were monitored three times per week.
Glucose tolerance
Glucose tolerance was evaluated via an intraperitoneal glucose tolerance test (IPGTT) 4-6 weeks after treatment (Akita mice at 4 weeks, db/db mice at 6 weeks after intervention). The mice were bled after overnight fasting in order to measure the basal metabolic parameters, after which they were intraperitoneally injected with a volume of 25% glucose solution calculated to deliver glucose at a dosage of 2 g/kg body weight. Blood samples for glucose determinations were drawn from the tail vein at 30, 60, 90 and 120 min after glucose loading. Blood glucose levels were measured using a glucometer, and the area under the glucose curve (AUCg) was calculated.

Immunohistochemistry
After 4-6 weeks of treatment (Akita mice at 4 weeks, db/db mice at 6 weeks after intervention), the mice were sacrificed and their pancreata were secured for histologic analysis. The pancreatic tissues were fixed in 10% buffered formalin and embedded in paraffin. Immunohistochemical staining was conducted on 7-μm-thick paraffin sections using streptavidin-biotin-peroxidase complex techniques (Vectastain ABC kit, Vector Lab Inc., Burlingame, USA). Color development was evaluated using 3′-diaminobenzidine tetrabromochloride (DAB, Sigma Chemical Co., MO, USA). The β-cell mass was measured using a point-counting method after hematoxylin and insulin staining with a guinea-pig anti-insulin antibody (1:100; ZYMED, San Francisco, CA). Beta-cell proliferation rates were evaluated via Ki67 staining using the rabbit anti-Ki67 antibody (1:200; abcam, Cambridge, UK) on an average of 24 islets and 2,400 β-cell nuclei per mouse (n = 3-8 mice per group) and apoptosis was assessed via terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL; Chemicon International, Millipore, Billerica, MA) on an average of 13 islets and 2000 β-cell nuclei per mouse (n = 3-8 mice per group). The β-cell ratio was calculated in the islets, which were stained with insulin (1:100; ZYMED, San Francisco, CA) and glucagon (1:100; ZYMED, San Francisco, CA) using primary antibodies and FITC and Texas Red immunofluorescent secondary antibodies.

To inhibit endogenous peroxidase, sections of pancreatic tissue were quenched with hydrogen peroxide solution (3% H2O2 in PBS) for 15 minutes. For antigen retrieval, the sections were microwaved in citrate buffer at neutral pH. Nonspecific binding was blocked via incubation with PBS containing 5% bovine albumin from the species in which the secondary antibody was raised for 1 hour at room temperature.

Analytical techniques
The relative volume of β-cells in the pancreas was counted via the point-counting method (18, 19) using a BH-2 microscope (Olympus Corp., Tokyo, Japan) connected to a video camera (Samsung Aerospace Ind., Seoul, Korea) that was equipped with a color monitor using a 100-point transparent overlay. In brief, immunostained slide pancreas sections were visualized under 100× magnification and positioned under a regular lattice overlay on a color monitor. The β-cells were counted simultaneously in the insulin-immunostained slides. The absolute β-cell mass for each animal was calculated by multiplying the relative volume of β-cells in the pancreas and the weight of the pancreas prior to fixation.

The ratio of α/β-cells in the islets was calculated after counting both types of cells in the islets after immunofluorescence staining. After double immunostaining for insulin and glucagon, images were captured using Image J software with a Zeiss Axiovert 200 fluorescence microscope and the 20× objective. Images of total islets were captured from each section (-2,500 cells per section), and the cell numbers in the islets were counted. The frequency of TUNEL and Ki-67 positive cells were presented as the percentage of total β-cells per section. Immunolabeled sections were prepared for all groups. The experimental protocols employed in this study were approved by the animal ethics review committee at our institution.

Statistical analysis
The results are expressed as the means ± SE of at least three independent experiments. Analysis of variance tests were used for between-group comparisons. Statistical significance was evaluated via Student’s t test, and a P value of <0.05 was considered significant.

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