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Beneficial effects of vildagliptin combined with miglitol on glucose tolerance and islet morphology in diet-controlled *db/db* mice

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ABSTRACT

Dipeptidyl peptidase-4 (DPP-4) inhibitors improve glycemic control in patients with type 2 diabetes primarily by increasing plasma active glucagon-like peptide-1 (GLP-1) levels. While various combination therapies based on DPP-4 inhibitors have been proposed for treatment of type 2 diabetes, the effects of combination therapy of DPP-4 inhibitors and alpha-glucosidase inhibitors on β-cell function are less characterized. We evaluated the effects of long-term treatment with vildagliptin, a DPP-4 inhibitor, on metabolic parameters and β-cell function, in combination with miglitol, an alpha-glucosidase inhibitor, in diet-controlled *db/db* mice. In this study, 6-week-old male *db/db* mice were provided with standard chow twice a day for 6 weeks. Meal tolerance tests and glucose tolerance tests showed that the combination therapy of vildagliptin with miglitol, but not each alone, suppressed postprandial glycemic excursion, enhanced postprandial active GLP-1 levels and prevented deterioration of glucose tolerance in the *db/db* mice. The combination treatment did not alter β-cell mass, but resulted in preserved expression of glucose transporter 2, Zinc transporter 8 and MafA and reduced the number of α cells. These results suggest that the combination of vildagliptin and miglitol prevents the development of overt diabetes in diet-controlled pre-diabetic *db/db* mice by normalizing postprandial glucose and incretin response, and by preserving β-cell structure and the expression of factors essential for β-cell function.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a heterogeneous and polygenic metabolic disease [1]. The pathology of this disease involves at least two mechanisms: dysfunction of pancreatic β-cells and insulin resistance in insulin sensitive organs [2]. Recent studies have suggested that the age-dependent deterioration of T2DM is associated with progressive loss of β-cell function, insulin secretion, and subsequent loss of glycemic control [3]. To date, various agents have been used to maintain β-cell function, including metformin, thiazolidinedione (TZD), and alpha-glucosidase inhibitors [4,5]. Among them, glucagon-like peptide-1(GLP-1) receptor agonists, a new class of anti-diabetic agents, exert their functions through multiple pathways; acting on the brain to induce satiety, inhibit gastric emptying and glucagon secretion, and potentiating glucose-stimulated insulin secretion and proliferation of β cells, all together leading to improved glucose homeostasis [6,7]. Thus, GLP-1 receptor agonists can potentially achieve long lasting good glycemic control by changing the natural history of T2DM.

DPP-4 inhibitors are a distinct class of oral glucose-lowering agents acting through enhancement of circulating levels of incretins, including GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) [8]. Vildagliptin is one of the prototype drugs of DPP-4 inhibitors [9]. Globally conducted clinical trials have shown that vildagliptin is well tolerated and efficacious in improving glycemic control in patients with T2DM [9]. Vildagliptin monotherapy trials have shown that significant HbA1c lowering is accompanied by body weight-neutral and lipid-neutral effects, low risk of edema, and low risk of hypoglycemia [10]. These characteristics make vildagliptin a favorable partner for combination therapy. While the usefulness of the combination therapy of

Abbreviations: DPP-4, dipeptidyl peptidase-4; GLP-1, glucagon-like peptide-1; IPGTT, intraperitoneal glucose tolerance test.

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vildagliptin and sulfonylurea or metformin has been assessed extensively in preclinical and clinical trials [11,12], no studies have been conducted to evaluate the efficacy of vildagliptin combined with alpha-glucosidase inhibitors. As a first step to elucidate the efficacy of the combination therapy of vildagliptin plus alpha-glucosidase inhibitors on glycemic control and maintenance of β cell function, *db/db* mice, a model of obesity-related T2DM, were treated for 6 weeks with the combination of vildagliptin and miglitol, an alpha-glucosidase inhibitor, and changes in various glycemic parameters and pancreatic islet morphology were investigated.

2. Materials and methods

2.1. Chemicals

The DPP-4 inhibitor, vildagliptin, was provided by Novartis Pharma. Vildagliptin was dissolved in sterile water and administered orally at a final concentration of 15 mg/kg/day. Miglitol, an alpha-glucosidase inhibitor, was provided by Sanwa Kagaku Kenkyusho (Mie, Japan). Miglitol was dissolved in sterile water and administrated orally at a final concentration of 5 mg/kg/day.

2.2. Animals and experimental design

The study was reviewed and approved by the Animal Care and Use Committee of Juntendo University and Nihon Bioresearch Inc (Gifu, Japan). Specific-pathogen-free male BKS.Cg- + *Lepr*^{db}/+*Lepr*^{db}/Jcl mice (*db/db* mice) were obtained from Clea Japan (Tokyo, Japan). Mice were housed in individual plastic cages in a clean room at controlled temperature (23 °C), humidity (55%) and lighting (lights on between 6:00 am and 6:00 pm) at Nihon Bioresearch Inc. After a 6-day acclimation period, 6-week-old *db/db* mice, weighing 22.1–24.8 g, were randomized into four groups (control, vildagliptin, miglitol, and vildagliptin plus miglitol) based on body weight and random blood glucose levels. The animals were provided with standard chow (22.6% protein, 53.8% carbohydrate, 5.6% fat, 6.6% mineral and vitamin mixture, and 3.3% fiber; total; 356 kcal/100 g, CRF-1, Charles River Japan, Yokohama, Japan) twice a day (from 9:00 to 11:00 am, and from 3:00 to 5:00 pm, total 4 h/day) and autoclaved tap water *ad libitum*. Thirty minutes prior to each meal, 7.5 mg/kg of vildagliptin or placebo vehicle mixed with 2.5 mg/kg of miglitol or placebo vehicle were administered through oral gavage (2.5 mL/kg in solution) for 6 weeks in each group.

2.3. Measurement of body weight and blood glucose

Body weight was measured on treatment days 1, 8, 15, 22, 29, 30, 34 and 42. Fasting blood glucose was measured by a portable glucose meter using G-checker (Sanko-junyaku, Japan) every week (from the day before treatment to day 29).

2.4. Intraperitoneal glucose tolerance test (IPGTT)

IPGTT was performed after 6-week treatment. Mice were injected intraperitoneally with glucose (0.5 g/kg body weight) after overnight fasting and blood samples were taken at various time points (0–120 min) from the tail vein. Blood glucose concentrations were measured using a standard method. Serum insulin concentrations were measured by insulin enzyme-linked immunosorbent assay (ELISA) kit (Morinaga,Yokohama).

2.5. Meal tolerance test

Meal tolerance test was performed on days 17 and 30. Standard chow was provided to mice after overnight fasting. Blood samples were taken from the tail vein at 0, 30 min, and 3 h, and blood glucose concentrations were measured. Plasma active GLP-1 levels (day 30 only) were determined by enzyme-linked immunosorbent assays (Shibayagi, Japan).

2.6. Intraperitoneal insulin tolerance test (ITT)

ITT was performed on day 34 with 2 units/kg body weight human insulin (Humalin; Eli Lilly) after overnight fasting. Blood samples were withdrawn from the tail vein at various time points (0–60 min), for measurement of blood glucose.

2.7. Immunohistochemistry

For immunoperoxidase, guinea pig anti-human insulin antibody (LINCO) was used at dilution of 1:2000. Biotinylated goat anti-guinea pig IgG secondary antibody (Chemicon), was used at 1:1000. The primary antibodies used for immunofluorescence were as follows: rabbit anti-rat GLUT2 (dilution: 1:500, Alpha Diagnostic International), rabbit anti-mouse zinc transporter 8 (dilution: 1:1000, [13]), rabbit anti-mouse MafA (dilution: 1:1000, Bethyl Laboratories), and rabbit anti-human glucagon (dilution: 1:500, DAKO).

2.8. Morphometric analysis

The β-cell area was quantified in at least 15 insulin-stained sections from three mice per group chosen at random (i.e., at least 15 sections per group were analyzed, with each section separated by at least 200 μm). Glucagon-positive cells were quantified in at least 80 islets from three mice per group chosen from the same mice used in the quantification of β-cell area.

2.9. Statistical analysis

All values were expressed as mean ± SEM. Differences in blood glucose concentration, body weight, food intake, and insulin levels during IPGTT and ITT among the different treatment groups were examined for statistical significance by one-way ANOVA, followed by post hoc analysis (Bonferroni's test). A p value less than 0.05 denoted the presence of a statistically significant difference.

3. Results

3.1. Vildagliptin plus miglitol combination therapy prevents age-related rise in fasting blood glucose

In this study mice had access to food only for 4 h/day to prevent massive obesity. Thus, food consumption of diet-controlled *db/db* mice [approximately 3.2 g/day for 12-week old] was around 60% of that of obese *db/db* mice [approximately 5.0 g/day for 12-week old] with free access to food (Table 1, data not shown). Accordingly, the mean body weight gain during the treatment period was modest in all groups: 23.2 ± 0.3 g (±SD) on average at 6 weeks of age and 29.8 ± 0.7 g at 12 weeks (Table 1). In comparison, the body weight of *db/db* mice with free access to food is 42.6 ± 2.9 g at 12 weeks of age. Body weight gain and food consumption were not significantly different among the treatment groups (Table 1). A gradual increase in fasting blood glucose levels was noted in the control, vildagliptin monotherapy and miglitol monotherapy groups. In contrast,the combination treatment of vildagliptin and miglitol resulted in statistically significant suppression of fasting blood glucose levels at treatment days 8, 22, and 29 (Fig. 1A). Thus, the age-associated increase in fasting blood glucose level was

Table 1
Effect of the different therapeutic regimens on body weight, food consumption.

	Control	Vildagliptin	Miglitol	Vildagliptin plus miglitol
n	6	7	6	6
Body weight (g)				
Day 1	23.2 ± 0.3	23.4 ± 0.5	23.2 ± 0.4	22.8 ± 0.2
Day 42	29.8 ± 0.7	30.6 ± 0.7	28.4 ± 1.2	28.2 ± 0.5
Food consumption (g)				
Day 5 – am	0.9 ± 0.1	1.1 ± 0.1	0.9 ± 0.1	1.0 ± 0.1
Day 5 – pm	1.4 ± 0.1	1.4 ± 0.1	1.3 ± 0.2	1.2 ± 0.1
Day 40 – am	1.6 ± 0.0	1.6 ± 0.0	1.3 ± 0.1	1.1 ± 0.0
Day 40 – pm	1.6 ± 0.1	1.6 ± 0.2	1.7 ± 0.1	1.5 ± 0.2

Data are mean ± SEM.

almost completely suppressed in *db/db* mice by vildagliptin plus miglitol.

3.2. Vildagliptin and miglitol combination therapy improves glucose tolerance

IPGTT was performed to determine the effect of chronic treatment with vildagliptin and/or miglitol on glucose tolerance. Blood glucose excursions at 30, 60, 90 min after glucose load in the vildagliptin plus miglitol group were significantly lower than the control, vildagliptin monotherapy, and miglitol monotherapy groups (Fig. 1B). Simultaneously measured plasma insulin concentrations were significantly lower in the combination group at 30 min after glucose challenge compared to the control group.(Fig. 1C).

3.3. Combination therapy suppresses postprandial blood glucose excursions

To examine the effect of chronic treatment with vildagliptin and/or miglitol on postprandial blood glucose excursions, blood glucose levels were measured in the morning of experimental day 30. Before meal, blood glucose levels tended to be lower in the combination therapy group compared to the other groups (Fig. 2A). Furthermore, blood glucose excursions at 30 min after feeding were markedly suppressed by the combination treatment of vildagliptin plus miglitol. Similar reductions were observed in the miglitol monotherapy group (Fig. 2A). Overall, postprandial blood glucose fluctuation was the smallest in the combination therapy group. The combination of vildagliptin and miglitol mark-

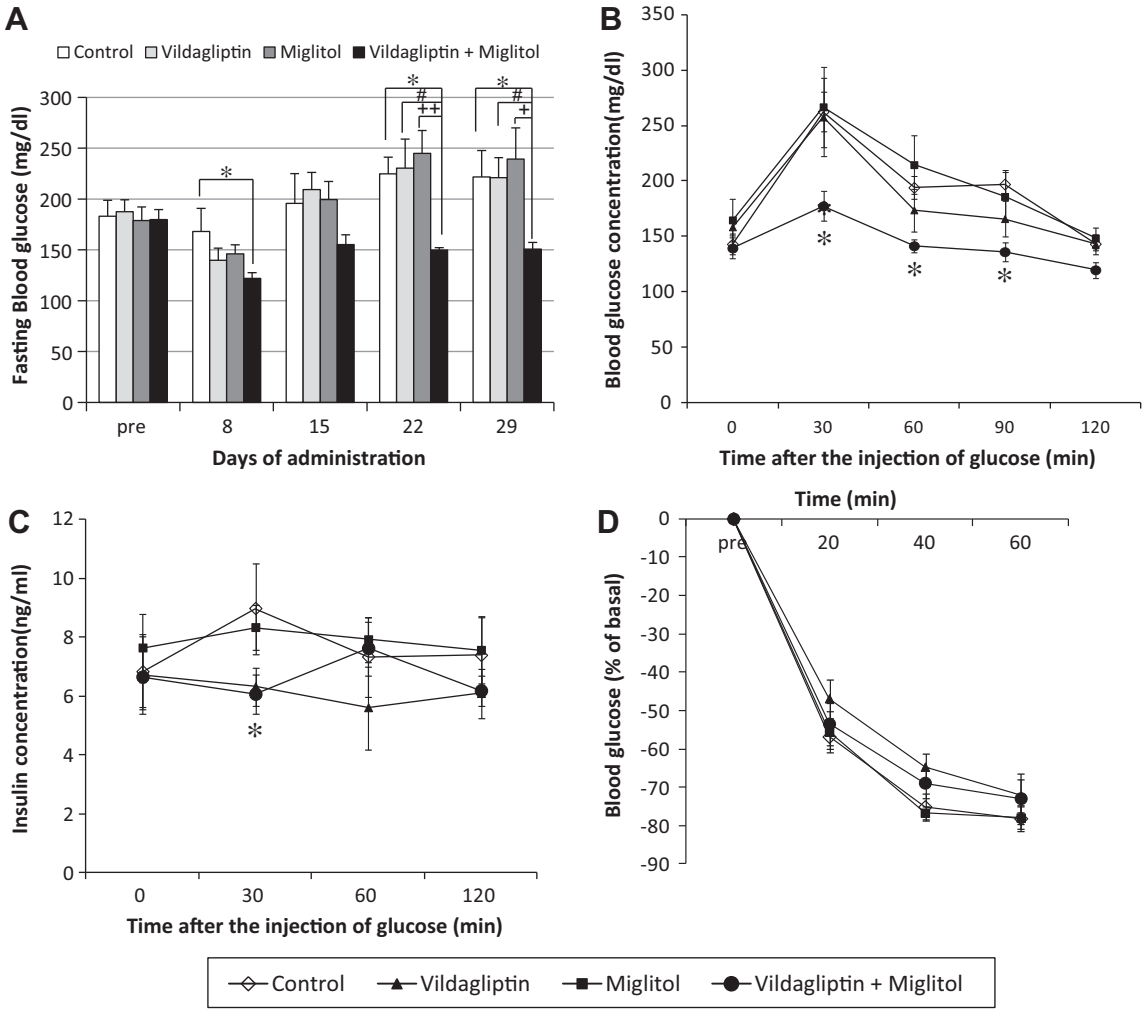

Fig. 1. Beneficial effects of the combination therapy of vildagliptin and miglitol on glucose homeostasis. (A) Effects of vildagliptin and/or miglitol on blood glucose levels. Six-week-old *db/db* mice were treated with vildagliptin (15 mg/kg/day) or placebo, combined with miglitol (5 mg/kg/day) or vehicle for 6 weeks. The blood glucose levels of each group of mice were monitored at 9 AM once a week until Day 29. Data are mean ± SEM (n = 6–7 mice/group). $^{*}P < 0.05$ control vs miglitol + vildagliptin, $^{\#}P < 0.05$ vildagliptin vs vildagliptin + miglitol, $^{++}P < 0.01$, $^{+}P < 0.05$ miglitol vs vildagliptin + miglitol. (B and C) Effect of vildagliptin and miglitol on glucose tolerance. Intraperitoneal glucose tolerance tests were performed on 12-week-old *db/db* mice. After an overnight fast, glucose was injected intraperitoneally at a dose of 0.5 g/kg, and blood glucose (B) and insulin (C) levels were measured.$^{*}P < 0.05$ control vs vildagliptin + miglitol. (D) Intraperitoneal insulin tolerance tests were performed in 11-week-old *db/db* mice of each group. After an overnight fast, insulin was injected intraperitoneally at a dose of 2 U/kg body weight, and blood glucose level was measured. Data are mean ± SEM (n = 6–7 mice/group).

edly increased active-GLP-1 levels after food feeding (Fig. 2B). A similar increase was observed in the vildagliptin monotherapy group, although it was statistically less than that observed after the combination therapy (Fig. 2B).

3.4. Combination therapy has no effect on IPITT

Intraperitoneal injection of insulin caused 70–80% reduction in glucose levels from the baseline and there was no significant difference in the reduction among the groups, including the vildagliptin plus miglitol group (Fig. 1D).

3.5. Combination therapy has little effect on β-cell mass

We also investigated the effects of treatment with vildagliptin and/or miglitol on islet morphology by immunostaining for insulin (Fig. 3A, B). Unlike previous studies [13,14], islets of untreated *db/db* mice did not show obvious disorganization or reduced β-cell staining, and there were no differences in pancreatic β-cell mass and pancreas weight among the four treatment groups [control: 278.5 ± 6.4 mg, vildagliptin: 264.1 ± 14.8 mg, miglitol: 287.8 ± 12.1 mg, vildagliptin plus miglitol: 309 ± 19.0 mg]. The difference in islet architecture between our study and the previous studies [13,14] could be due to differences in dietary protocols of free access to food used in previous studies and the restriction applied in the present study.

3.6. Combination therapy preserves mature function of pancreatic β-cells

Next, detailed immunohistochemical analysis was conducted by staining with zinc transporter-8 (ZnT8), GLUT2, glucagon and MafA (Fig. 3D), all of which are known to be implicated in the function of terminally differentiated β cells [15–17]. ZnT8 expression in islets was limited in mice of the vehicle-treated group and only weak expression of ZnT8 was observed in islets of mice treated with vildagliptin or miglitol alone. In the combination therapy group, ZnT8 expression was clearly noted throughout the islets. With regard to immunostaining for GLUT2, a key component of the glucose-stimulated insulin-secretion machinery [16], the staining was preferentially localized in β-cell membrane in islets of mice treated with the combination therapy (Fig. 3D), whereas it was undetectable in the majority of islets of the vehicle-treated animals. Glucagon-producing cells in the islets of mice of the vehicle-treated group were often intermixed with β cells, and not peripherally located as is seen in wild-type C57BL/6 J mice. The increase in glucagon-positive cells was normalized and peripheral localization of glucagon-producing cells in islets was restored in mice of the miglitol monotherapy group and the combination therapy group. While the expression of MafA was absent in mice of the vehicle-treated group, low level of MafA expression was maintained in the miglitol monotherapy group (Fig. 3D). Importantly, nuclear expression of MafA was clearly detected in islets of mice of the combination therapy group (Fig. 3D).

4. Discussion

The diabetic phenotype of *db/db* mice appears to be primarily caused by overeating due to defects in leptin signaling. The standard food consumption and body weight of male *db/db* mice at 12 weeks of age are around 5 g/day and 42.6 ± 2.9 g (±SEM), respectively, according to the data provided by the breeder (Clea Japan, Inc.). In our preliminary trials, the same combination therapy was tested in regular *db/db* mice fed normal chow *ad libitum*, but no beneficial effects were noted, possibly due to the pronounced metabolic disorders, including glucose toxicity and lipotoxicity, in regular *db/db* mice (data not shown). In the present study, mice had access to food only for 4 h/day (2 h × 2), and consequently, food consumption and average body weight were significantly smaller than usual: around 3.2 g/day and 29.8 ± 0.7 g (±SEM), respectively, in the control group. Consistent with a prediction from such smaller body weight, fasting blood glucose levels in this study were less than 300 mg/dL at 10 weeks of age in the control group, while standard plasma glucose levels of regular male *db/db* mice at 10 weeks of age are usually more than 500 mg/dL in our facility (data not shown). Based on these considerations, diet-controlled *db/db* mice established in this study represent an animal model of mild T2DM suitable for evaluation of the efficacy of various kinds of glucose-lowering agents.

In this study, we investigated whether and how vildagliptin treatment together with miglitol could improve several metabolic

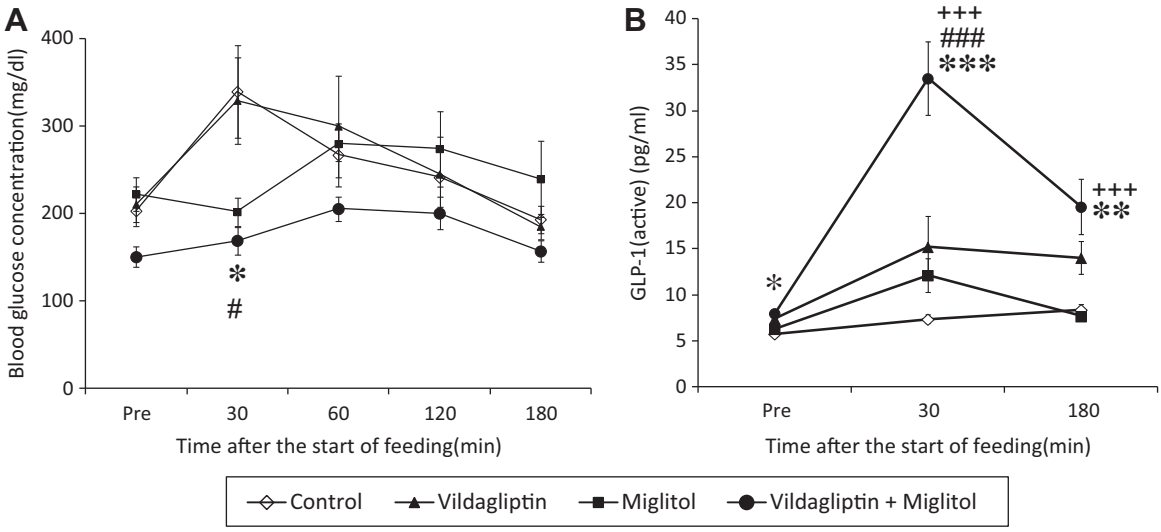

Fig. 2. Beneficial effects of the combination therapy of vildagliptin plus miglitol on postprandial glucose excursion and active GLP-1 levels. (A) Effects of treatment on postprandial glucose excursion. Meal tolerance tests were performed in *db/db* mice at experimental day 30. After an overnight fast, food (standard chow) was provided, and blood glucose was measured. Data are mean ± SEM (n = 6–7 mice/group). $^{*}P < 0.05$ control vs vildagliptin + miglitol, $^{\#}P < 0.05$ vildagliptin vs vildagliptin + miglitol. (B) After an overnight fast, at day 30, standard chow was provided, and active-GLP-1 concentration was measured. Data are mean ± SEM (n = 6–7 mice/group). $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ control vs vildagliptin + miglitol, $^{\#\#\#}P < 0.001$ vildagliptin vs vildagliptin + miglitol, $^{+++}P < 0.001$ miglitol vs vildagliptin + miglitol.

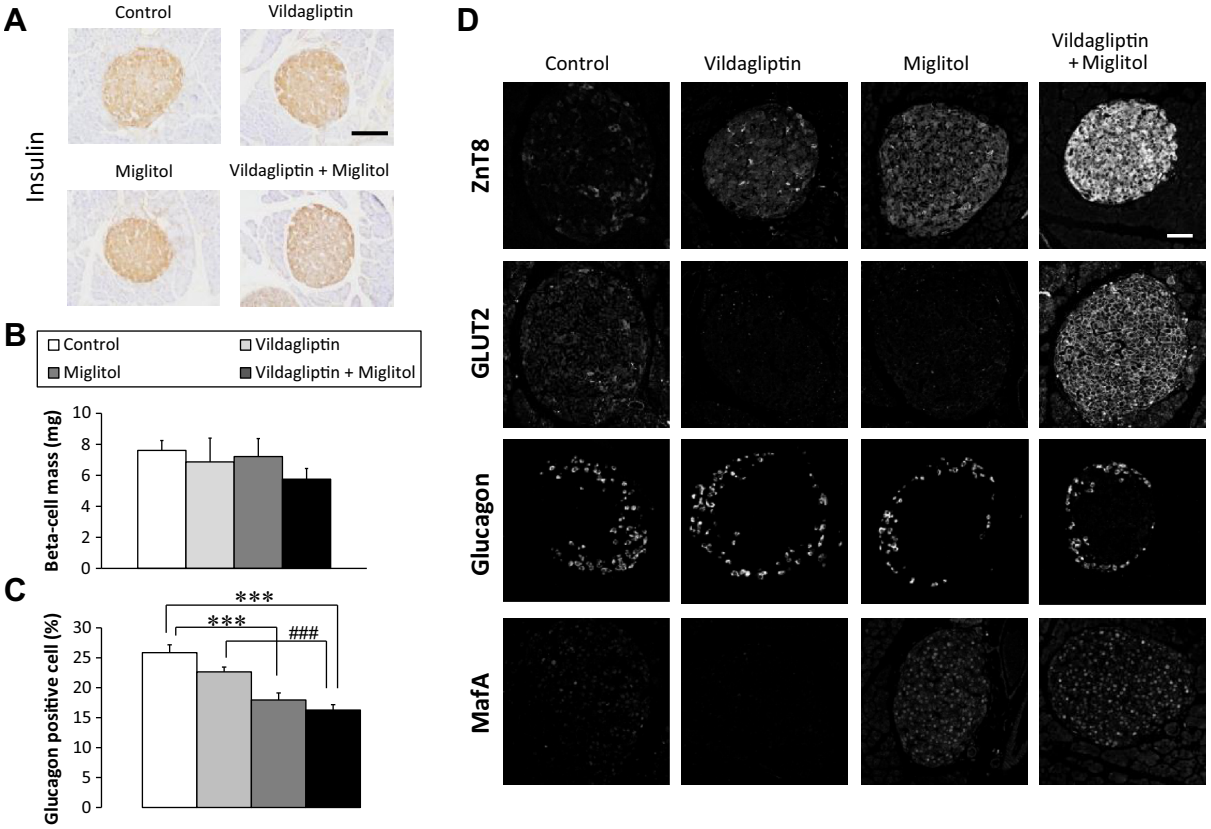

Fig. 3. Effects of vildagliptin and miglitol on β-cell mass and islet morphology. (A) Representative immunostaining for insulin in pancreatic tissue sections from vehicle-treated and vildagliptin and/or miglitol-treated mice. Scale bar = 100 μm. (B) β-Cell mass calculated by the formula: islet β-cell mass (mg) = (area stained by insulin antibody)/(area of the whole pancreas) × (pancreas weight). Data are mean ± SEM (n = 3 mice/group). (C) Proportion of glucagon-positive cells relative to the number of intra-islet cells. Data are mean ± SEM (n = 3 mice/group). ***P<0.001 control vs miglitol, control vs vildagliptin + miglitol, ###P<0.001 vildagliptin vs vildagliptin + miglitol. (D) Representative immunostaining for ZnT8, GLUT2, glucagon and MafA in islets of 12-week-old mice. Scale bar = 50 μm.

parameters in the diabetic state. Monotherapy with miglitol or vildagliptin did not significantly improve random blood glucose levels, IPGTT or meal test. In comparison, the combination treatment of vildagliptin and miglitol resulted in significant improvement of these metabolic parameters. In the combination therapy group, the glucose tolerance test showed no increase in glucose-stimulated insulin secretion, suggesting little involvement of insulin hypersecretion in the improved glucose tolerance. Although the exact mechanisms underlying the beneficial effects of the combination therapy on diet-controlled *db/db* mice are not clear, we speculate that significantly enhanced postprandial GLP-1 response by the combination therapy (Fig. 2B) [14], plays a major role in achieving improved glucose tolerance and islet morphology.

Insulin response during IPGTT was decreased in *db/db* mice that received the combination therapy, while glucose tolerance was significantly improved in these mice. We cannot completely rule out the possibility that modest increase in insulin sensitivity, which was not detected by ITT (Fig. 1D), was achieved by increased postprandial GLP-1 response, given that some previous studies reported that GLP-1 receptor agonists have also insulin sensitizing effects [18] .

Aberrant postprandial glucagon response is well described in type 2 diabetes patients and animal with experimental diabetes; glucagon release from α cells is not suppressed but rather paradoxically enhanced [19], leading to enhanced hepatic glucose output. Experiments in obese diabetic ZDF rat have demonstrated that GLP-1 receptor agonists preserve β-cell mass at the expense of α-cell mass [20]. Furthermore, GLP-1 can inhibit glucagon secretion from α-cells [21]. Thus, it is likely that the combination therapy normalized postprandial suppression of glucagon release from α cells by limiting α-cell number and function. Although α-cell function (reflected by change in plasma glucagon levels) was not directly assessed in the present study, the observation that the increase in glucagon-positive cells in *db/db* islets was ameliorated by the combination therapy (Fig. 3C), supports such possibility.

Postprandial glucose excursions observed in *db/db* mice were significantly ameliorated by the combination therapy (Fig. 2A). Repetitive postprandial glycemic excursions possibly induce glucose toxicity in pancreatic β-cells through the formation of reactive oxygen species (ROS), as demonstrated in vascular endothelial cells [22]. Transcription factors critically involved in the maintenance of mature function of pancreatic β-cells, including MafA and pdx1, appear to be sensitive to glucose toxicity [23]. It should be noted that ZnT8 and GLUT2, the molecular markers examined in this study, are reported to be controlled by both MafA and pdx1 [24–26]. Thus, the reduction in postprandial glycemic excursions by the combination therapy probably resulted in the preserved function of MafA and pdx1, leading to the maintenance of mature function of β cells, represented by the high expression of ZnT8, GLUT2 and MafA (Fig. 2A).

Recent clinical trials of an alpha-glucosidase inhibitor have demonstrated that these agents prevent the onset and progression of diabetes in individuals with impaired glucose tolerance [27,28]. Alpha-glucosidase inhibitors provide a unique approach in the management of diabetes. By competitive and reversible inhibition of intestinal alpha-glucosidases, alpha-glucosidase inhibitors delay carbohydrate digestion, prolongs the overall carbohydrate digestion time, and thus reduces the rate of glucose absorption. After

oral administration of alpha-glucosidase inhibitors, the postprandial rise in blood glucose is dose-dependently decreased, and insulin secretion is attenuated, saving insulin for when it is needed by the body [29]. The combination treatment of vildagliptin plus miglitol profoundly suppressed the rise in postprandial glucose and attenuated serum insulin levels (Fig. 2A and Fig. 1C). Thus, the combination treatment prevented the progression of diabetes in diet-controlled *db/db* mice presumably by reducing the burden of β cells.

In conclusion, the present study suggested that the combination treatment of vildagliptin and miglitol may be an effective pharmacological option, along with life style modification, in preventing the progression of diabetes by changing the natural history of T2DM.

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