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Effect of alogliptin, pioglitazone and glargine on pancreatic β-cells in diabetic db/db mice

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ABSTRACT

Objective: Progressive β-cell dysfunction and loss of β-cell mass are fundamental pathogenic features of type 2 diabetes. To examine if anti-diabetic reagents, such as insulin, pioglitazone (pio), and alogliptin (alo), have protective effects on β-cell mass and function *in vivo*, we treated obese diabetic db/db mice with these reagents. *Methods:* Male db/db mice were treated with a chow including pio, alo, or both of them from 8 to 16 weeks of age. Insulin glargine (gla) was daily injected subcutaneously during the same period. *Results:* At 16 weeks of age, untreated db/db mice revealed marked increase of HbA1c level, whereas those treated with pio, pio + alo, or insulin revealed the almost same HbA1c levels as non-diabetic db/m mice. Islet mass evaluated by direct counting in the whole pancreas and insulin content in isolated islets were preserved in pio, pio + alo and gla groups compared with untreated or alo groups, and there was no difference among pio, pio + alo and gla groups. To precisely evaluate islet β-cell functions, islet perifusion analysis was performed. In pio, pio + alo and gla groups, biphasic insulin secretion was preserved compared with untreated or alo groups. In particular, pio + alo as well as gla therapy preserved almost normal insulin secretion, although pio therapy improved partially. To examine the mechanism how these reagents exerted beneficial effects on β-cells, we evaluated expression levels of various factors which are potentially important for β-cell functions by real-time RT-PCR and immunohistochemistry. The results showed that expression levels of MafA and GLP-1 receptor were markedly decreased in untreated and alo groups, but not in pio, pio + alo and gla groups. *Conclusion:* Combination therapy with pio and alo almost completely normalized β-cell functions *in vivo*, which was comparable with gla treatment.

1. Introduction

Type 2 diabetes is a progressive disease characterized by insulin resistance and β-cell dysfunction. Under diabetic conditions, chronic hyperglycemia and subsequent augmentation of reactive oxygen species (ROS) deteriorates β-cell function and increases insulin resistance which leads to the aggravation of type 2 diabetes [1–3]. Among a variety of anti-diabetic reagents, a couple of medicines such as insulin, thiazolidinediones (TZD), and dipeptidyl peptidase 4 (DPP-4) inhibitor, are recognized to have protective effects on β-cell function [4–6].

TZD is an agonist of PPAR-γ which stimulates insulin signals in both liver and periphery. Some reports suggest that TZD has protective effects against β-cell dysfunction and loss of β-cell mass under diabetic conditions, mainly via reduction of gluco- and lipo-toxicity [5,7]. PPAR-γ is also expressed in β-cells, and there are some reports showing that PPAR-γ agonists directly activate several genes related to glucose-sensing in β-cells [8–10]. But the fundamental role of TZD in β-cells is not fully understood. Insulin therapy also improves glycemic and lipid control, which leads to protection of β-cell function. Although several reports indicated that activating insulin receptor is important for β-cell function and growth [11,12], it is still unclear whether long-term insulin therapy itself actually has protective effects against β-cell dysfunction found in type 2 diabetes. DPP-4 inhibitor is a new class of anti-diabetic reagent. Inhibition of DPP-4 extends the half-life of native glucagon-like peptide (GLP)-1, and thereby prolongs the effects of GLP-1 [6,13]. GLP-1 is characterized to have short term effect on insulin secretion, and chronic effect on insulin biosynthesis, β-cell proliferation, and neogenesis [14,15]. Although the effects of TZD and DPP-4 inhibitor on glycemic control is quite clear, it remains to be elucidated whether there is some difference between these reagents concerning the effects on preservation of functional β-cells *in vivo*.

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MafA is a critical transcription factor, which promotes the expression of β-cell factors involved in insulin biosynthesis and secretion [16–18]. MafA also plays key roles for β-cell maturation in the embryonic pancreas [19,20]. Importantly, expression level of MafA is reduced under diabetic conditions, and their reduction contributes to deterioration of β-cell function [21–25]. These findings suggest that sustained expression of MafA is necessary for preserving β-cell function. Interestingly, there are some reports showing that expression level of GLP-1 receptor is also reduced under diabetic conditions [26,27], and that the expression level of GLP-1 receptor is regulated by MafA [28]. From these findings, MafA appeared to be closely related with GLP-1 signaling.

In this study, we examined in detail if anti-diabetic reagents, such as insulin, TZD, and DPP-4 inhibitor, have protective effects on β-cell function *in vivo* using diabetic db/db mice.

2. Materials and methods

2.1. Treatment of mice

Obese diabetic C57B/KsJ-db/db (db/db) mice and non-diabetic C57B/KsJ-db/misty (db/m) mice were obtained from CLEA Japan. All mice were housed in individual metal cages in a room with controlled temperature (23 °C), humidity (45%) and lighting (lights on from 8:00 am to 8:00 pm) and were maintained on a laboratory chow diet (MF, Oriental yeast Co., Tokyo). After an acclimation period of 7 days, 8-week-old db/db mice were divided into five groups, and each group was fed a diet containing no medicine, 0.03% alogliptin (equivalent to 76.4 mg kg^{-1} day^{-1}) alone, 0.02% pioglitazone (equivalent to 50.9 mg kg^{-1} day^{-1}) alone, or both 0.03% alogliptin (equivalent to 76.4 mg kg^{-1} day^{-1}) and 0.02% pioglitazone (equivalent to 50.9 mg kg^{-1} day^{-1}) from 8 to 16 weeks of age. Insulin glargine was injected subcutaneously daily during the same period.

2.2. Assays for metabolic components

Blood glucose levels were measured with a portable glucose meter. HbA1c was measured by DCA Vantage™ analyzer (SIEMENS, Berlin, Germany). Plasma triglyceride levels were measured by SRL (Tokyo, Japan). Insulin levels were determined by enzyme-linked immunosorbent assay kit (Morinaga Institute of Biological Science, Inc., Yokohama, Japan).

2.3. Insulin tolerance test

At the end of treatment period (16 weeks of age), insulin tolerance test was performed. Regular insulin (2 U/kg BW) was intraperitoneally injected after the overnight fasting. Blood glucose levels were measured with a portable glucose meter. Mice in glargine group were excluded because of the prolonged effect of glargine for a week.

2.4. Histological analysis

Pancreata were dissected and fixed overnight in 4% paraformaldehyde. Fixed tissues were processed routinely for paraffin embedding and sectioned 4 μm in thickness. After treatment with 1% blocking goat serum, sections were immunostained for MafA and insulin with the following antibodies and dilutions: rabbit antibody against MafA [18]; guinea pig antibody against insulin (DAKO, Glostrup, Denmark) 1:2000; mouse antibody against 8-OHdG (Nikken Seil, Shizuoka, Japan) 1:100. For the double staining of insulin with MafA, we used Alexa Fluor 555 donkey anti-rabbit IgG (for MafA) and Alexa Fluor 488 goat anti-guinea pig IgG (for insulin). Fluorescent images were captured using a BIO-RAD Radience 2100 confocal microscope.

2.5. Measurements of islet mass

Measurements of islet mass were performed as reported previously [29]. Whole pancreas was squashed flat by a pair of glass plates to the thickness of 200 μm (lager than the size of islets) after dithizone staining. The area of pancreatic islets was calculated by WinRoof® (Mitani Corporation, Japan).

2.6. Isolation of mouse pancreatic islets

To isolate mouse islets, 0.4 mg/ml of collagenase (Liberase TL, Roche), was injected into pancreatic duct. Isolated pancreas was digested in a 37 °C incubator for 20 min. After washing and precipitating with 0.25 mol/L sucrose, islets were hand picked.

2.7. Measurements of insulin content

Isolated islets were homogenized with Poly Tron in acid–ethanol and incubated for overnight at 4 °C. Insulin content of the islet extraction was determined with the insulin ELISA Kit (Morinaga Biochemicals, Yokohama, Japan) after neutralization of acid and evaporation of ethanol, and was corrected by total cellular protein of each sample.

2.8. Islet perifusion analysis

Islets isolated from each group were cultured in medium containing 5 mM glucose. After the overnight incubation, 20 islets were placed in chamber, and perifused for 1 h with 40 mg/dl glucose, followed by 18 min with 400 mg/dl glucose. The effluent was collected every 30 s intervals for 5 min, every 1 min intervals for 5 min, and every 2 min intervals for 8 min. Insulin concentration was corrected by concentration of whole cell protein of each sample.

2.9. Real-time PCR analysis

Real-time PCR analysis was performed as described previously [25]. Primer sets for mouse MafA (forward: TTCAGCAAGGAG GAGGTCAT, reverse: CCGCCAACTTCTCGTATTTC), mouse GLP-1 receptor (forward: GGGTCTCTGGCTACATAAGGACAAC, reverse: AAGGATGGCTGAAGCGATGAC) and mouse β-actin (forward: GCTCT TTTGCAGCCTTCG, reverse: GCTCTTTTGCAGCCTTCG) were utilized to detect specific bands for each factor. Levels of *MafA* and *GLP-1R* mRNA expression were normalized with β-actin.

2.10. Statistical analysis

Data are expressed as means ± SE. Statistical analysis was performed using one-way ANOVA. A value of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effect of each treatment on body weight, blood glucose level, HbA1c level, and triglyceride level

Body weight of mice in pio, pio + alo and gla groups was higher compared with untreated db/db mice (Supplementary Fig. 1A and Table 1). Non-fasting blood glucose level and HbA1c levels were measured at 8, 11, 14, and 16 weeks of age. Non-fasting blood glucose level and HbA1c levels of the mice in pio, pio + alo and gla groups were significantly decreased compared with untreated db/db mice (Fig. 1A, Supplementary Fig. 1B and Table 1). Triglyceride level was significantly decreased in all treatment groups

Table 1
Metabolic features after the treatment period.

	Untreated	Alogliptin	Pioglitazone	Pio + alo	Glargine
Body weight (g)	49.2 ± 2.3	52.0 ± 1.5	69.0 ± 1.2**	71.0 ± 1.3**	56.3 ± 1.8
Non-fasting blood glucose (mg/dl)	507.7 ± 26.0	523.6 ± 25.9	113.0 ± 17.2**	109.1 ± 6.9**	118.8 ± 39.6**
HbA1c (%)	9.8 ± 0.8	9.9 ± 0.4	4.1 ± 0.1**	4.1 ± 0.1**	3.8 ± 0.1**
Triglyceride (mg/dl)	219.3 ± 46.6	74.8 ± 11.3*	37.5 ± 10.3**	31.0 ± 3.7**	73.0 ± 11.0*

Body weight (n = 7–8), non-fasting blood glucose (n = 5–7), HbA1c (n = 7–8) and triglyceride (n = 7–8) of each treatment group after the treatment period (16 weeks of age). Data are presented as means ± SE.
* $p < 0.05$ vs. untreated.
** $p < 0.01$ vs. untreated.

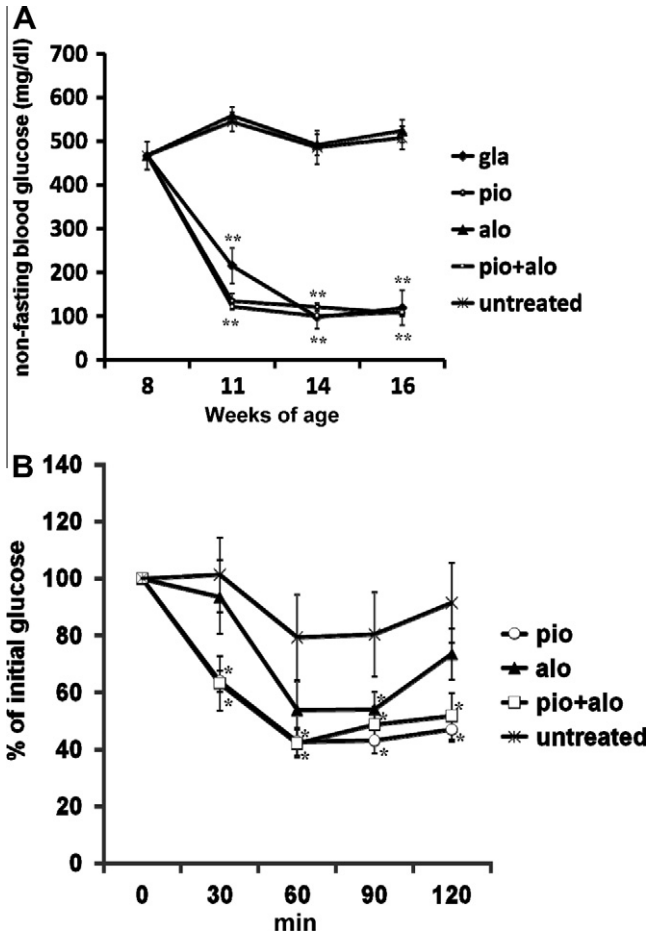

Fig. 1. Time-related change of (A) non-fasting blood glucose (n = 5–7), (B) decrease of insulin resistance by pioglitazone. Regular insulin (2 U/kg BW) was injected intraperitoneally after overnight fasting (n = 7–8). Data are presented as means ± SE. $^{**}p < 0.01$; $^{*}p < 0.05$ vs. untreated.

(Supplementary Fig. 1C and Table 1). These findings clearly demonstrated that pio and gla treatment effectively improved glucose and lipid metabolism in db/db mice, although those treatment induced obesity in the condition of free fed.

3.2. Improvement of insulin sensitivity by pioglitazone

Insulin tolerance test at 16 weeks of age showed that the reduction of blood glucose levels after insulin load was significantly larger in pio and pio + alo groups compared to untreated group (Fig. 1B). These results indicate that reducing insulin resistance in db/db mice leads to almost normal glucose and lipid metabolism, although we cannot exclude the possibility that pio has direct effects on β-cell function.

3.3. Preservation of islet mass by the treatment with glargine or pioglitazone

Pancreatic islets were atrophic in untreated and also groups at 16 weeks of age (Supplementary Fig. 2A), although temporal enlargements of islets were observed even in those groups at the younger age (data not shown). But, in pio, pio + alo, and gla groups, islet mass appeared to be preserved and hypertrophic (Supplementary Fig. 2A). To accurately measure the islet mass, we made preparation of the whole pancreas between the glasses after staining pancreatic islets with dithizone (Supplementary Fig. 2B), and calculated the area of islets stained in red. Islet mass was decreased in untreated group compared with that of non-diabetic db/m mice as expected. On the other hand, islet mass in pio, pio + alo and gla groups were significantly larger than untreated group, and there was no difference among these three groups (Fig. 2A). Since the

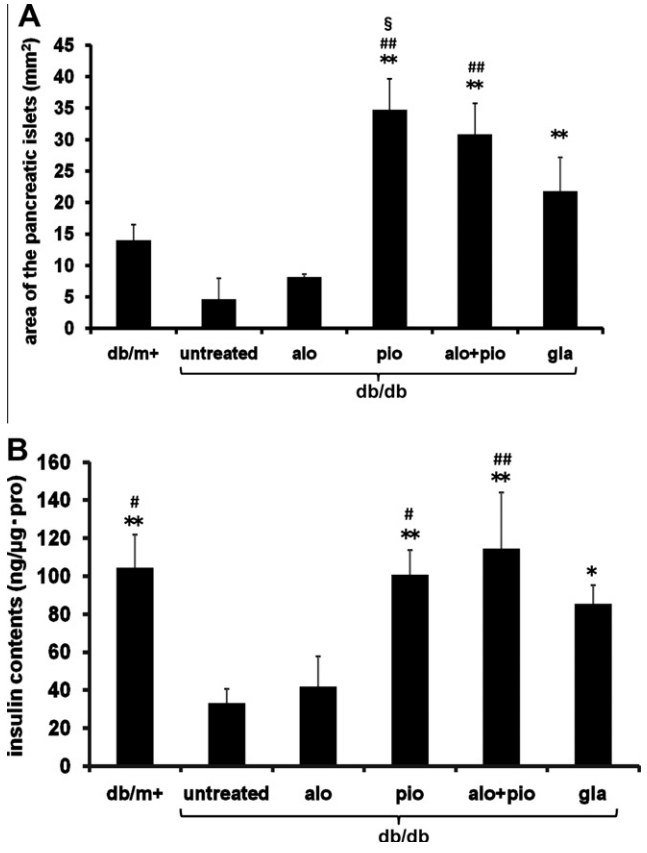

Fig. 2. (A) Islet area of the whole pancreas. Data are presented as means ± SE. $^{**}p < 0.01$; $^{*}p < 0.05$ vs. untreated. $^{\#\#}p < 0.01$; $^{\#}p < 0.05$ vs. alo. $^{\S}p < 0.05$ vs. db/m (n = 7–8). (B) Preservation of insulin contents by the treatment with pioglitazone and glargine. Insulin concentration was corrected by concentration of total protein of each sample. Data are presented as means ± SE. $^{**}p < 0.01$; $^{*}p < 0.05$ vs. untreated. $^{\#\#}p < 0.01$; $^{\#}p < 0.05$ vs. alo (n = 2–4).

islet mass of these three groups tended to be larger than that of db/m, it seemed that pio and gla preserved the compensative enlargement of islets against upregulated insulin resistance in db/db mice.

3.4. Preservation of insulin content by the treatment with pioglitazone or glargine

Insulin content in isolated islets of untreated group was significantly lower compared with that of db/m (32.8 ± 7.7 ng/μg pro vs. 104.2 ± 17.6 ng/μg pro, $p < 0.01$), while that of pio, pio + alo and gla groups were significantly higher compared with that of untreated group (pio; 100.6 ± 13.1 ng/μg pro, $p < 0.01$, pio + alo; 114.4 ± 29.8 ng/μg pro, $p < 0.01$, gla; 85.2 ± 1.0 ng/μg pro, $p < 0.05$). There was no difference between untreated and alo groups, and among pio, pio + alo and gla goups (Fig. 2B).

3.5. Normalization of glucose-stimulated insulin secretion by the treatment of alogliptin + pioglitazone or glargine

To precisely examine the ability of glucose-stimulated insulin secretion in each treatment group, we performed perifusion analysis using isolated islets (Fig. 3). In this method, we observed distinct biphasic insulin secretion pattern in non-diabetic db/m islets. However, insulin secretion response to glucose stimulation was markedly impaired and biphasic secretion pattern was unclear in untreated and alo groups. In pio, pio + alo and gla groups, the amount of insulin secretion was higher than untreated group and distinct biphasic insulin secretion pattern was clearly observed. Importantly, combination therapy of pio with alo led to significantly higher insulin secretion than pio alone. The combination therapy normalized glucose-induced insulin secretion to normal range as well as gla treatment in the experiment of islet perifusion analysis.

3.6. Preservation of MafA expression level by the treatment with pioglitazone and glargine

Expression level of MafA mRNA in isolated islets was lower in untreated and alo groups compared with non-diabetic db/m mice, and preserved in pio, pio + alo and gla groups (Fig. 4A). Similar results were obtained in protein level by immunohistochemistry (Fig. 4B). MafA amount in islets appeared to be parallel with insulin content, which is reasonable as MafA regulates the expression of insulin.

3.7. Preservation of GLP-1 receptor expression level by pioglitazone and glargine

To examine the mechanism why alo + pio treatment preserved insulin secretion compared with pio alone treatment, expression level of GLP-1 receptor in isolated islets was evaluated by real-time PCR analysis. Expression level of GLP-1 receptor was significantly lower in untreated group compared with non-diabetic db/m mice, and was preserved as almost same amount as non-diabetic db/m mice in pio, pio + alo and gla groups (Fig. 4C). These findings imply that enough amount of GLP-1 receptor expression preserved by pio is critical so that alo effectively functions for islet β-cells.

3.8. Decreased expression level of 8-OHdG by pioglitazone and glargine

To examine the influence of ROS on islet β-cells, expression level of 8-OHdG was evaluated by immunohistochemistry. Expression level of 8-OHdG was markedly decreased in pio, pio + alo and gla groups compared with that of untreated group (Supplementary Fig. 3). These findings suggest that removal of ROS by the treatment of pio or gla is critical for the preservation of MafA expression and normalization of β-cell functions.

4. Discussion

In the present study, we showed that the treatment with glargine or pioglitazone improved glycemic and lipid control in obese diabetic db/db mice. Both reagents exerted beneficial effects on β-cells; they increased islet mass, preserved insulin contents, and improved insulin secretion. Concerning about pioglitazone, our results were consistent with previous reports which indicated its protective effects on β-cell function and mass under diabetic conditions. So far, there was no report showing that insulin therapy could preserve β-cell mass and function in db/db mice, probably due to its extremely increased insulin resistance. In this study,

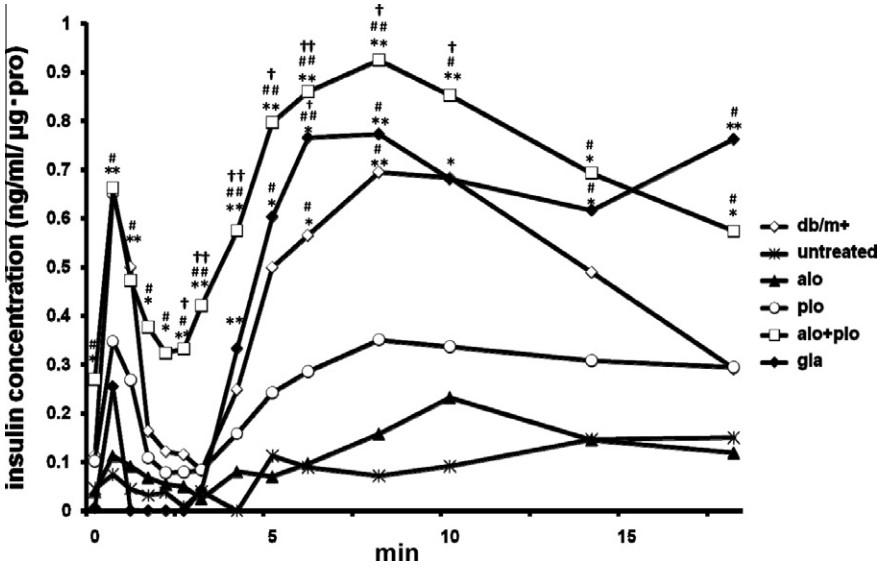

Fig. 3. Improvement of glucose-stimulated insulin secretion in isolated islets by the treatment of pioglitazone, alo + pioglitazone, glargine. The perifusate contained 40 mg/dl glucose for 1 h and 400 mg/dl glucose for 18 min. The effluent was collected in 30 s intervals for 5 min, 1 min intervals for 5 min and 2 min intervals for 8 min. Insulin concentration was corrected by concentration of whole cell protein of each sample. $^{**}p < 0.01$; $^{*}p < 0.05$ vs. untreated. $^{\#\#}p < 0.01$; $^{\#}p < 0.05$ vs. alo. $^{\dagger\dagger}p < 0.01$; $^{\dagger}p < 0.05$ vs. pio ($n = 5–7$).

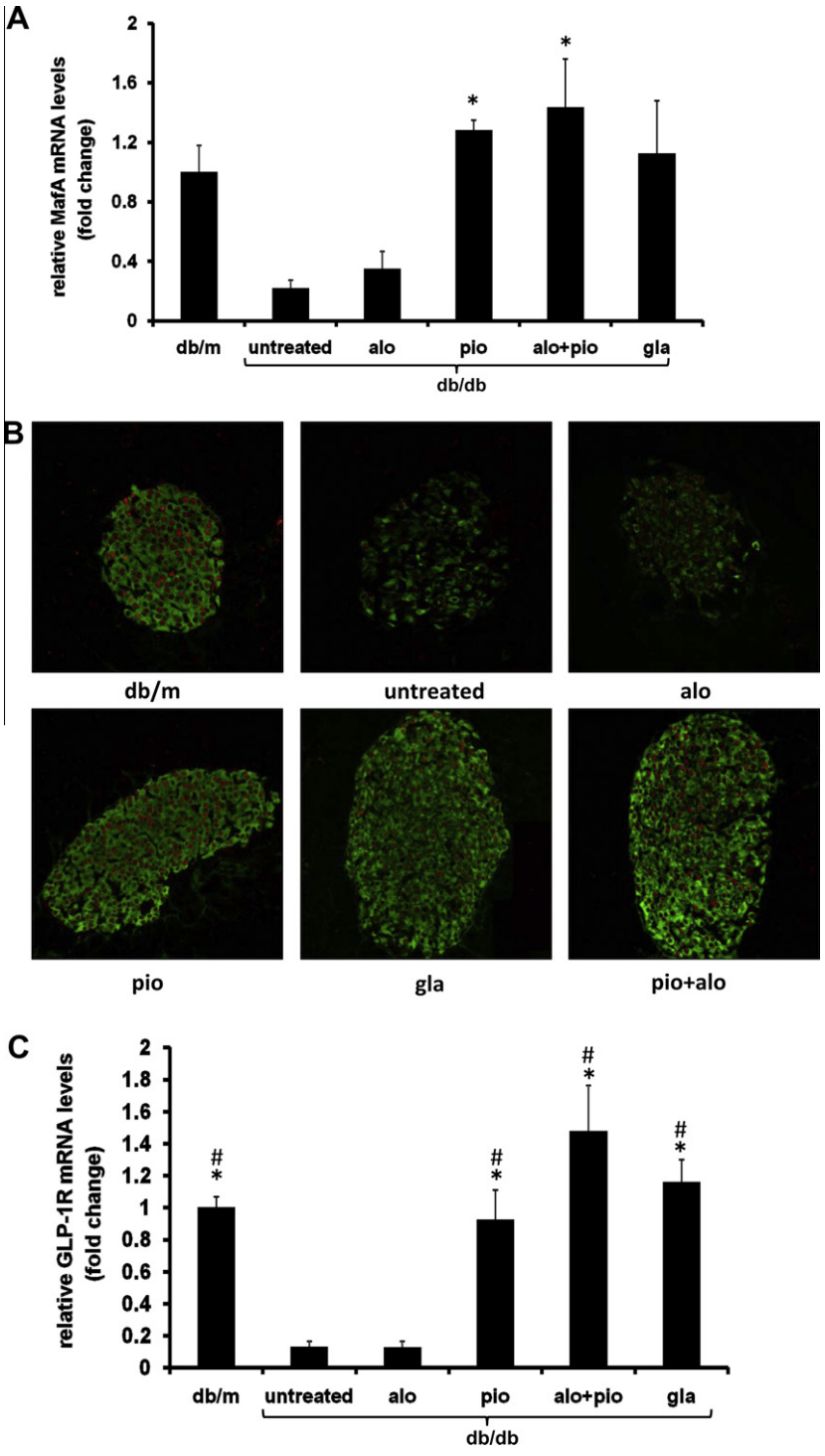

Fig. 4. Effect of alogliptin, pioglitazone, alogliptin + pioglitazone, and glargine treatment on expression levels of MafA, GLP-1R. (A) Levels of *MafA* mRNA expression were normalized with β-actin and shown as a change from non-diabetic db/m mice. Results are means ± SE. $^{*}p < 0.05$ to untreated ($n = 2–5$). (B) MafA protein expression assay in pancreatic sections of each group. Double staining for MafA in red and for insulin in green. (C) Levels of *GLP-1R* mRNA expression were normalized with β-actin and shown as a change from non-diabetic db/m mice. Results are means ± SE. $^{*}p < 0.05$ to untreated. $^{\#}p < 0.05$ to alo ($n = 4–6$).

we succeeded in achieving normal glycemic and lipid control in db/db mice by using enough amount of insulin glargine which is long acting insulin analog. Although overdose insulin therapy should be avoided on clinical case because of its potential influence on promoting atherosclerosis [30,31], it was protective at least on β-cell functions and compensative islet growth in db/db mice with severe insulin resistance and hyperinsulinemia.

Although the mechanism for the improvement of islet function by glargine or pioglitazone is still unclear, removal of glucose- and lipo-toxicity followed by reduction of ROS from islet β-cells are supposed to be a major reason, as we showed with the staining of 8-OHdG. As a result, expression of a critical islet β-cell factor MafA was preserved. As reported before, it is certain that preserved MafA expression is critical for maintaining the β-cell functions

[25,32]. In fact, potential targets of MafA, such as GLUT2 [28,33], pyruvate carboxylase [28] and ZnT8 [34], which are involved in glucose-stimulated insulin secretion, were also preserved by the therapy with glargine or pioglitazone (data not shown).

In spite of the same glycemic and lipid control, the glargine therapy improved insulin secretion up to normal level in islet perifusion analysis while the therapy of pioglitazone alone was less effective. One possible explanation for the difference between glargine and pioglitazone in the effects on insulin secretion is that glargine directly activates insulin signaling in β-cells. Indeed, it was previously reported that glucose-stimulated insulin secretion was decreased in β-cell specific insulin receptor knockout mice or isolated islets from IRS-1 knockout mice. These reports suggest that insulin signaling per se is important for glucose-stimulated insulin secretion [12,35].

More interestingly, combination therapy with alogliptin and pioglitazone improved glucose-induced insulin secretion up to the level of glargine-treated or normal mice islets in the islet perifusion analysis, although there was no difference in glycemic control between pioglitazone alone and the combination therapy. Since the db/db mice are extremely hyperphagic and keep eating, the improvement with the combination therapy detected by islet perifusion analysis might not be enough to reveal better glycemic control compared with pioglitazone alone. To examine the molecular mechanism how alogliptin could show additive effects to pioglitazone, we evaluated expression level of various factors which are potentially important for β-cell function by real-time RT-PCR. Among them, GLP-1R level was dramatically decreased in untreated db/db islets, and was restored by pioglitazone therapy. From these findings, we surmised GLP-1R level is critical for alogliptin to reveal maximum effects on islet β-cells. Since GLP-1R is one of the target genes of MafA [28], it is possible to think that pioglitazone restored GLP-1R, at least partially, by preserving MafA expression. Unfortunately, treatment with alogliptin alone did not show beneficial effects on islet β-cells in these model mice at the age around 8–16-week old. Its ineffectiveness might be due to the reduction of GLP-1R expression. These results suggest that the seasonable use of alogliptin is important for its proper effects on islet β-cell function.

In conclusion, the results in this study indicated that therapy with pioglitazone or glargine markedly improved β-cell functions, which was comparable with normal mice, under diabetic conditions *in vivo*, and suggested that preserved expression of GLP-1 receptor by pioglitazone was required to display the proper function of alogliptin.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.12.021.

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