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

Satoshi Kawashima [a], Taka-aki Matsuoka [a,*], Hideaki Kaneto [a], Yoshihiro Tochino [a], Ken Kato [a], Kaoru Yamamoto [a], Tsunehiko Yamamoto [a], Munehide Matsuhisa [b], Iichiro Shimomura [a]

[a] Department of Metabolic Medicine, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita City, Osaka 565-0871, Japan
[b] Diabetes Therapeutics and Research Center, Tokushima University, 3-18-15 Kuramoto, Tokushima City, Tokushima 770-8503, Japan

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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*.

* Corresponding author. Fax: +81 6 6879 3739.
E-mail address: matsuoka@endmet.med.osaka-u.ac.jp (T.-a. Matsuoka).

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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