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Sustained expression of GLP-1 receptor differentially modulates β -cell functions in diabetic and nondiabetic mice

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Abstract

Glucagon-like peptide 1 (GLP-1) has been shown to play important roles in maintaining β -cell functions, such as insulin secretion and proliferation. While expression levels of GLP-1 receptor (*Glp1r*) are compromised in the islets of diabetic rodents, it remains unclear when and to what degree *Glp1r* mRNA levels are decreased during the progression of diabetes. In this study, we performed real-time PCR with the islets of *db/db* diabetic mice at different ages, and found that the expression levels of *Glp1r* were comparable to those of the islets of nondiabetic *db/misty* controls at the age of four weeks, and were significantly decreased at the age of eight and 12 weeks. To investigate whether restored expression of *Glp1r* affects the diabetic phenotypes, we generated the transgenic mouse model *Pdx1^{PB}-CreERTM; CAG-CAT-Glp1r (β *Glp1r*)* that allows for induction of *Glp1r* expression specifically in β cells. Whereas the expression of exogenous *Glp1r* had no measurable effect on glucose tolerance in nondiabetic *β Glp1r;db/misty* mice, *β Glp1r;db/db* mice exhibited higher glucose and lower insulin levels in blood on glucose challenge test than control *db/db* littermates. In contrast, four weeks of treatment with exendin-4 improved the glucose profiles and increased serum insulin levels in *β Glp1r;db/db* mice, to significantly higher levels than those in control *db/db* mice. These differential effects of exogenous *Glp1r* in nondiabetic and diabetic mice suggest that downregulation of *Glp1r* might be required to slow the progression of β -cell failure under diabetic conditions.

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Conflicts of interest

The authors declare that they have no conflict of interest.

Keywords

GLP-1 receptor; GLP-1; Diabetes; β Cell failure; Exendin-4

1. Introduction

Diabetes mellitus is a progressive and chronic metabolic disorder, in which the sustained elevation of blood glucose levels leads to serious complications, such as blindness, kidney failure, etc. As all types of diabetes result from the absolute or relative deficiency of insulin secretion from pancreatic β cells [1], it is a potential target of therapies to protect or improve the damaged β cells [2].

Glucagon-like peptide-1 (GLP-1), a peptide secreted from enteroendocrine L cells in the gut, is known to exert its insulinotropic actions through GLP-1 receptors expressed in pancreatic β cells. *In vivo* and *in vitro* studies with GLP-1 and its analogs have revealed that GLP-1 not only potentiates glucose-stimulated insulin secretion (GSIS), but also increases insulin synthesis, stimulates β cell proliferation/neogenesis, and inhibits β cell apoptosis [3]. Although clinical studies demonstrated that GLP-1 receptor agonists improved glycemic controls in diabetic patients accompanied by increased insulin secretion [4], this class of medications is not always effective for diabetic patients and residual β -cell function may be associated with the efficacy of this treatment [5] [6] [7] [8].

Recently it has been reported that the expression levels of GLP-1 receptor (*Glp1r*) were reduced not only in the islets of rodent diabetic models, such as pancreatectomized rats and diabetic *db/db* mice [9] [10], but also in the islets of human diabetic patients [11] [12]. Therefore, we hypothesized that the decreased expression of *Glp1r* is associated with the pathogenesis or progression of β -cell failure, and that sustained expression of *Glp1r* may improve the metabolic profiles of diabetic mice. In order to test our hypothesis, we generated a novel transgenic mouse model in which the expression of *Glp1r* is maintained specifically in β cells only after Cre-mediated recombination following tamoxifen treatment. Whereas exogenous *Glp1r* had no effect on metabolic profiles in nondiabetic mice, persistent expression of *Glp1r* under diabetic conditions in *db/db* mice resulted in further impaired β -cell function, but a significantly greater response to GLP-1 receptor agonist, suggesting that the persistent activation of GLP-1 signaling does not necessarily exert beneficial effects on β -cell function, and even worse can lead to the progression of β -cell failure under diabetic conditions.

2. Materials and methods

2.1. Mice

Obese diabetic C57BL/KsJ-db/db (*db/db*) male mice and nondiabetic C57BL/KsJ-db/misty (*db/m*) male mice were purchased from Japan SLC. *Pdx1^{PB}-CreERTM* and *CAG-CAT-Glp1r-IRES-eGFP* (β *Glp1r*) transgenic mice were generated as described previously [13] [14]. The β *Glp1r* mice were backcrossed onto the C57BL/KsJ background for more than 8 generations by crossing β *Glp1r* mice with C57B/KsJ-db/misty mice.

Tamoxifen (Sigma–Aldrich, St Louis, MO, USA) was prepared at 20 mg/mL in corn oil and injected subcutaneously into *βGlp1r* mice and their littermates at 1.5 mg/10 g body weight twice every other day at the age of four weeks. To assess the effects of GLP-1 receptor agonist exendin-4, the mice were infused with exendin-4 at 24 nmol/kg body weight per day, or with volume-matched saline as controls, using a mini-osmotic pump (ALZET, model 1004, Cupertino, CA, USA), which delivers the solution at a constant rate for four weeks, from the age of six to 10 weeks.

Mice were housed on a 12-h light–dark cycle in a controlled climate. All animal procedures were approved by the Ethics Review Committee for Animal Experimentation of Osaka University Graduate School of Medicine.

2.2. RNA extraction and real-time PCR

To isolate the islets of *db/m* and *db/db* mice, collagenase (Liberase TL, Roche, Mannheim, Germany) was injected into the pancreatic ducts. Isolated pancreata were digested in a 37 °C incubator for 20 min, shaken gently, and washed with 0.25 mol/L sucrose. Islets were handpicked under a dissection microscope.

Total RNA was extracted from isolated islets using RNeasy Plus Micro Kit (Qiagen, Valencia, CA, USA), and cDNA was synthesized using WT-Ovation RNA Amplification System (Nugen, San Carlos, CA, USA) according to the manufacturer's protocols. Real-time PCR was performed using TaqMan Custom Arrays (Applied Biosystems, Foster City, CA, USA). The relative expression level for each gene was presented relative to the control mRNA level of *β-glucuronidase (Gusb)*.

2.3. Measurement of metabolic parameters

Oral glucose tolerance tests (OGTT) were performed after 6-h fasting by oral administration of glucose (1.0 g/kg mouse body weight). Blood glucose levels and plasma insulin levels were measured using a portable glucose meter (Sanwa Kagaku Co., Ltd., Nagoya, Japan) and insulin ELISA kit (Morinaga Institute of Biological Science, Yokohama, Japan), respectively. HbA1c levels were measured using a DCA Vantage analyzer (Siemens, Berlin, Germany).

2.4. Histology

Immunohistochemistry was performed for the observation of GFP-expressing cells, as described previously [14]. The primary antibodies used in this study were the following: guinea pig anti-insulin (1:1000; Dako, Carpinteria, CA, USA), chicken anti-insulin (1:1000; Abcam, Cambridge, MA, USA), rabbit anti-GFP (1:200; MBL, Nagoya, Japan), and anti-Ki67 (1:100; BD Pharmingen, Franklin Lakes, NJ, USA). Insulin-positive area and total pancreas area were measured using a BZ-X analyzer (Keyence, Osaka, Japan), and β -cell mass was calculated as relative β -cell area multiplied by the pancreatic weight. For the detection of Ki67, mounted sections were microwaved at 95 °C for 20 min in citrate buffer (pH 6.0) for antigen retrieval, before being incubated with blocking serum. Slides were observed using an Olympus FV1000D confocal laser-scanning microscope. In order to

detect apoptotic cells, TUNEL assay was performed using an apoptotic detection system (Merck Millipore, Darmstadt, Germany) according to the manufacturer's protocol.

2.5. Statistical analysis

Results were expressed as means $\pm$ SE. Data were analyzed by one-way ANOVA or unpaired *t* test (two tailed). *P* value < 0.05 was considered to be statistically significant.

3. Results

3.1. Age-dependent decrease in incretin receptor expression levels in db/db islets

While the expression level of GLP-1 receptor (Glp1r) was reported to be reduced in the islets of diabetic *db/db* mice at the age of 16 weeks, it has not yet been examined yet at earlier stages in the course of development of diabetes. To investigate age-dependent alterations in gene expression profiles for Glp1r and other pancreas-specific genes in *db/db* mice, TaqMan real-time PCR array was performed for 46 pancreas-specific genes in the islets of *db/db* diabetic and *db/m* nondiabetic mice, at four, eight, and 12 weeks of age. The expression levels of insulin 1 (*Ins1*) and insulin 2 (*Ins2*) in *db/db* mice were comparable to those in control (*db/m*) mice at the age of 4 and 8 weeks, and significantly decreased at 12 weeks of age (Supplementary Fig. 1), when fasting blood glucose levels rise above 250 mg/dL. There was no significant difference in glucagon (*Gcg*) and somatostatin (*Sst*) mRNA levels between *db/m* and *db/db* mice at least 12 weeks of age.

In contrast to the mRNA levels of endocrine hormones, those of *Glp1r* and GIP receptor (*Gipr*) were significantly decreased in *db/db* mice at the age of 8 weeks as well as 12 weeks (Fig. 1). Glp1r mRNA levels in *db/db* mice were less than one third of those in *db/m* mice at both ages. On the other hand, expression levels of insulin receptor (*Insr*) and glucagon receptor (Gcgr), which are highly expressed in adult β cells in mice, were comparable between *db/db* and *db/m* mice at all ages that were examined.

Among the pancreas-specific transcription factors that are known to be expressed in adult endocrine cells, Mafa was substantially decreased in *db/db* islets, showing 90% and 87% reductions compared with *db/m* islets at the age of eight and 12 weeks, respectively (Supplementary Fig. 2). On the other hand, levels of Neurog3 mRNAs showed a 6-fold increase in the islets of *db/db* mice.

3.2. Generation of β -cell-specific Glp1r-overexpressing mice

The robust reduction of *Glp1r* mRNAs during the progression of hyperglycemia led to the question as to whether the continuous expression of *Glp1r* in β cells could positively affect the metabolic profiles in this diabetic mouse model. To address this issue, we generated a novel transgenic mouse model, β *Glp1r*, in which the expression of Glp1r can be induced specifically in β cells. *CAG-CAT-Glp1r-IRES-eGFP* mice [14], which conditionally and specifically express *Glp1r* and *eGFP* by Cre-mediated recombination, were interbred with *Pdx1^{PB}-CreERTM* transgenic mice that express tamoxifen-activated Cre recombinase under the control of the β -cell-specific *Pdx1^{Area I/II}* enhancer [13]. The β *Glp1r* mice were crossed with C57BL/KsJ-db/misty (*db/m*) mice to generate both β *Glp1r,db/m* and β *Glp1r,db/db*.

Because the *eGFP* reporter gene was placed downstream of the *Glp1r* cDNA via an IRES element (Fig. 2A), the exogenous *Glp1r*-expressing β cells in β *Glp1r* mice are lineage labeled as green-fluorescent cells.

When β *Glp1r;db/db* mice were treated with tamoxifen at the age of four weeks and sacrificed one week later, immunostaining against eGFP and insulin revealed that all eGFP-expressing cells were positive for insulin and $72.5\% \pm 8.4\%$ of insulin-expressing cells were positive for eGFP (Fig. 2B). Consistent with the immunostaining results for eGFP, *Glp1r* mRNA levels in the isolated islets of 12-week-old β *Glp1r;db/db* mice were significantly higher than those of control *db/db* littermates and were restored to similar levels to those in *db/m* mice at the same age (Fig. 2C).

3.3. Exogenous expression of *Glp1r* did not affect metabolic profiles in nondiabetic mice but worsened hyperglycemia in *db/db* mice

To induce the exogenous expression of *Glp1r* before the onset of overt hyperglycemia, β *Glp1r;db/db* mice and their nondiabetic *db/m* littermates were injected with tamoxifen at the age of four weeks. There was no significant difference in body weight between β *Glp1r;db/m* and control *db/m* littermates or between β *Glp1r;db/db* and control *db/db* littermates, although the body weights of β *Glp1r;db/db* mice were slightly lower than those of control *db/db* mice (Fig. 3A). In addition, oral glucose tolerance test (OGTT) in β *Glp1r;db/m* demonstrated no significant difference in blood glucose and plasma insulin levels between β *Glp1r;db/m* and control *db/m* littermates (Fig. 3B, C), showing that exogenous *Glp1r* did not affect glucose profiles in nondiabetic *db/m* mice.

On the other hand, restored expression of *Glp1r* in diabetic β *Glp1r;db/db* mice significantly increased fasting blood glucose levels after 10 weeks of age (Fig. 4A), and there were no significant effects on HbA1c levels compared with those of control *db/db* mice (Fig. 4B). When OGTT was performed on β *Glp1r;db/db* mice without GLP-1 receptor agonist treatment, blood glucose levels at 2 h after glucose administration were significantly higher in β *Glp1r;db/db* mice (Fig. 4C), with a significant decrease in plasma insulin levels (Fig. 4D), compared with control *db/db* littermates. These findings suggest that exogenous *Glp1r* expression in diabetic *db/db* mice causes a further impairment in glucose tolerance due to insufficient insulin secretion in response to elevated glucose levels.

3.4. A GLP-1 receptor agonist improves glucose profiles and insulin secretion in *Glp1r*-overexpressing *db/db* mice

To investigate whether activation of GLP-1 receptor signaling would improve metabolic profiles in diabetic mice, we treated β *Glp1r;db/db* mice with the GLP1R agonist exendin-4 at 0.1 mg/kg body weight/day for four weeks using an osmotic pump. Administration of exendin-4 significantly ameliorated both fasting blood glucose and HbA1c levels in β *Glp1r;db/db* mice and control *db/db* littermates (Fig. 4A, B). In addition, blood glucose and plasma insulin levels during OGTT were significantly improved in exendin-4-treated β *Glp1r;db/db* mice and control *db/db* littermates, compared with the mice treated with saline (Fig. 4C, D). In particular, plasma insulin levels in β *Glp1r;db/db* mice treated with

exendin-4 were significantly higher than those in control *db/db* littermates, suggesting that restored expression of *Glp1r* efficiently enhanced insulin secretion in response to exendin-4.

3.5. Decreased proliferation and increased apoptosis in β cells of β *Glp1r*; *db/db* mice

To investigate the underlying mechanisms of β -cell failure in β *Glp1r*; *db/db* mice, histological analyses were performed on their paraffin-embedded tissues of the transgenic mice. When sections immunostained for insulin were assessed for measurement of β -cell area, there was no statistical difference in β -cell mass between β *Glp1r*; *db/db* mice and control *db/db* littermates, although there was a trend toward a smaller β -cell mass in β *Glp1r*; *db/db* mice (Fig. 4E). Ki67 staining demonstrated a significant decrease in β -cell proliferation in β *Glp1r*; *db/db* mice treated with exendin-4 (Fig. 4F), and the TUNEL assay showed a significant increase in apoptotic β cells in the same mutant mice (Fig. 4G). These findings suggest that restored expression of *Glp1r* and activation of its signaling in diabetic *db/db* mice lead to impaired β -cell turnover and apoptosis, although β -cell responsiveness to exendin-4 was enhanced in the transgenic mice.

4. Discussion

Incretin-based therapies, such as GLP-1 receptor agonists and DPP-4 inhibitors, have been widely used for diabetic patients [15]. Although GLP-1 and its analogs have potent effects on stimulating insulin secretion, there is some evidence for the impairment of incretin effect; (1) β -cell responsiveness to GLP-1 was impaired in diabetic patients compared with nondiabetic subjects [16] and (2) GLP-1 analogs are effective in only some diabetic patients, which might be associated with residual β -cell function [6] [7]. In addition, as the mRNA levels of *GLP1R/Glp1r* were reduced in both human diabetic patients and rodent diabetic models [9] [10] [11] [12], we hypothesized that the decreased expression of *Glp1r* may affect metabolic profiles by changing the response to endogenous GLP-1 and/or exogenous GLP-1 analogs.

To approach this issue, we used *db/db* diabetic mice and investigated when and how much the incretin receptors are downregulated during the progression of hyperglycemia. Gene expression analysis at three different ages revealed that the expression levels of *Glp1r* started to decrease at eight weeks of age, the same time point as a decline in Mafa mRNAs (Fig. 1). As the mRNA levels of *Glp1r* were comparable at four weeks of age between *db/db* and *db/m* mice, the decrease in *Glp1r* mRNA levels of *db/db* mice did not precede the development of hyperglycemia. Together with our recent finding that alleviation of glucotoxicity markedly increases *Glp1r* expression in *db/db* mice [17], this age-dependent decline of *Glp1r* mRNAs appears to be a consequence, rather than a cause of hyperglycemia.

We next questioned whether sustained expression of *Glp1r* might improve the metabolic profiles in *db/db* mice, and generated a transgenic mouse in which the expression of *Glp1r* can be induced specifically in β cells only after Cre-mediated recombination following tamoxifen treatment. Contrary to our expectation that sustained expression of *Glp1r* would have protective effects on β -cell failure in *db/db* mice, β *Glp1r*; *db/db* mice exhibited more profound glucose intolerance compared with control *db/db* littermates, accompanied by impaired insulin secretion (Fig. 4A–D). As *Glp1r* mRNA levels in the islets of β *Glp1r*; *db/db*

mice are similar to those in the islets of nondiabetic *db/m* mice (Fig. 2C), exogenous *Glp1r* mRNA was not overexpressed to toxic levels. In other words, sustained expression of *Glp1r* in β cells under diabetic conditions, even at the physiological levels of *Glp1r* mRNAs in nondiabetic mice, can lead to β -cell dysfunction. This indicates that the downregulation of *Glp1r* observed at the age of eight and 12 weeks might be partially protective and required for preventing rapid progression of β -cell failure under diabetic conditions.

It is noted that exogenous *Glp1r* had no effect on glucose profiles in nondiabetic *db/m* mice (Fig. 3B, C), which is consistent with a previous report showing that transgenic mice expressing human GLP1R in β cells exhibited normal insulin secretion [18]. Thus, there is a substantial difference in the effect of exogenous *Glp1r* on diabetic *db/db* mice and nondiabetic *db/m* mice, suggesting that the adverse effects of exogenous *Glp1r* result from β -cell dysfunction, under chronic hyperglycemia and/or leptin signaling deficiency, while insulin secretion in response to a GLP-1 receptor agonist was improved. It would be of interest to treat *β Glp1r;db/db* mice with antidiabetic agents so that we could improve such adverse effects of transgenic *Glp1r* and see any beneficial effects of the sustained expression of *Glp1r*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.bbrc.2016.01.177>.

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Fig. 1.

Age-dependent decrease in *Glp1r* expression levels in db/db mouse islets. Expression levels of incretin receptors and two other receptors. Islets were isolated from db/m and db/db mice at the age of four, eight, and 12 weeks, and TaqMan real-time PCR was performed. All expression levels were normalized to β -glucuronidase. *, $p < 0.05$.

Fig. 2.

Generation of transgenic mice that overexpress Glp1r in β cells. (A) A schematic representation of *Pdx1^{PB}-CreERTM; CAG-CAT-Glp1r-IRES-eGFP* and its recombination by Cre recombinase. Before recombination, the transcription of exogenous *Glp1r* is blocked by the floxed STOP cassette. When the mice are mated with Cre-expressing *Pdx1^{PB}-CreERTM* mice, the floxed sequence is removed by Cre recombinase, and the CAG promoter can activate the expression of *Glp1r*. (B) β Glp1r; *db/db* mice were treated with tamoxifen at the age of four weeks, and sacrificed at the age of 10 weeks. Immunostaining with antibodies against insulin (red), eGFP (green), and DAPI (blue) demonstrated that all eGFP-expressing cells were positive for insulin. Scale bars, 50 μ m. (C) β Glp1r;*db/db* mice and control littermates were treated with tamoxifen at the age of four weeks, and the islets were isolated

at 12 weeks of age. The expression levels of *Glp1r* were quantified by TaqMan PCR. *, $p < 0.05$; $n = 4$.

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Fig. 3.

Metabolic profiles in β Glp1r; *db/m*, β Glp1r; *db/db*, and their control littermates. (A) Whole body weights were measured every week as indicated. (B, C) Blood glucose levels and insulin levels during OGTT were measured in β Glp1r; *db/m* mice and control littermates (n = 6 for each group).

Fig. 4. Metabolic profiles and β -cell function of β Glp1r; db/db mice treated with exendin-4 (Ex4). (A, B) Fasting blood glucose and glycated hemoglobin (HbA_{1c}) levels were measured weekly or biweekly in saline-treated β Glp1r; db/db mice (open diamonds, n = 13), saline-treated control db/db mice (open circles, n = 13), Ex4-treated β Glp1r; db/db mice (black diamonds, n = 14), and Ex4-treated control db/db mice (gray circles, n = 13). (C, D) Blood glucose levels and insulin levels during OGTT were measured in β Glp1r; db/db and control littermates (n = 6e8). #, $p < 0.05$, saline-treated control db/db mice vs. saline-treated β Glp1r; db/db mice; §, $p < 0.05$, Ex4-treated control db/db mice vs. Ex4-treated β Glp1r; db/db mice; †, $p < 0.05$, saline-treated control db/db mice vs. Ex4-treated control db/db mice; *, $p < 0.05$, saline-treated β Glp1r; db/db mice vs. Ex4-treated β Glp1r; db/db mice. (E, F, G) Immunostaining with antibodies against insulin, eGFP, and Ki67 were

performed and β -cell mass (E), Ki67-positive cells (F), and TUNEL-positive cells (G) were calculated (*, $p < 0.05$; $n = 4$).

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