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A B S T R A C T
The aim was to compare the protective effects of pioglitazone (PIO) and/or liraglutide (LIRA) on β-cells with the progression of diabetes. Male db/db mice were treated with PIO and/or LIRA for 2 weeks in an early and advanced stage. In an early stage insulin biosynthesis and secretion were markedly increased by PIO and LIRA which was not observed in an advanced stage. In concomitant with such phenomena, expression levels of various β-cell-related factors were up-regulated by PIO and LIRA only in an early stage. Furthermore, β-cell mass was also increased by the treatment only in an early stage. Although there was no difference in apoptosis ratio between the two stages, β-cell proliferation was augmented by the treatment only in an early stage. In conclusion, protective effects of pioglitazone and/or liraglutide on β-cells were more powerful in an early stage of diabetes compared to an advanced stage.

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1. Introduction
It is well known that type 2 diabetes mellitus is a chronic and progressive disease and its underlying pathology comprises pancreatic β-cell dysfunction and insulin resistance in peripheral tissues (Taylor, 1999). Pancreatic β-cell function such as insulin biosynthesis and secretion is broadly decreased by the time when an individual is diagnosed with type 2 diabetes (Butler et al., 2003; Taylor, 1999). To prevent diabetic complications, maintenance of strict glycemic control is necessary (Ohkubo et al., 1995; The Diabetes Control and Complications Trial Research Group, 1993; UK Prospective Diabetes Study (UKPDS) Group, 1998), but this requires the preservation of β-cell function. It is known that chronic hyperglycemia is a cause of impairment of insulin biosynthesis and secretion. Once hyperglycemia becomes apparent, β-cell function gradually deteriorates. This process is called “β-cell glucose toxicity” which is often observed under diabetic conditions (Kaneto et al., 2005; Robertson, 2004; Robertson et al., 2003; Weir et al., 2001). In the diabetic state, chronic hyperglycemia decreases insulin biosynthesis and secretion and finally brings about apoptosis. It was previously shown that the loss of insulin biosynthesis is accompanied by decreased expression of various insulin gene transcription factors (Kaneto et al., 2001; Matsuoka et al., 1997). For example, Pdx-1 and MafA are very important insulin gene transcription factors in β-cells (Jonsson et al., 1994; Leonard et al., 1993; Matsuoka et al., 2003; Miller et al., 1994; Ohlsson et al., 1993; Olbrot et al., 2002) but expression levels of such factors are reduced in diabetes status.

It is known that long-term glycemic control protects β-cells against glucose toxicity. Indeed, some anti-diabetic drugs, thiazolidine (Kanda et al., 2010; Kawasaki et al., 2005), DPP4 inhibitors (Hamamoto et al., 2013) and glucagon like peptide-1 receptor agonist (Shimoda et al., 2011), possess a protective effect upon β-cells. In the condition of normal glucose tolerance, β-cell proliferation capacity to various stimulations is gradually attenuated with age (Rankin and Kushner, 2009). On the other hand, in obese type 2 diabetes model db/db mice exhibiting significant hyperglycemia, compensatory hypertrophy of the islets can be seen in the early stage of diabetes but the capacity of such compensation is
markedly attenuated in the advanced stage (Dalboge et al., 2013). It has been reported that one of the causes of decrease in β-cell mass, apoptosis proceeds to the same extent irrespective of age, but cell proliferation is significantly reduced with the progress of diabetic pathology (Dalboge et al., 2013). The aim of this study is to compare the protective effects of pioglitazone and/or liraglutide on pancreatic β-cells between in an early and advanced stage of diabetes.

2. Materials and methods

2.1. Animals

As an early stage model of diabetes, we used 7-week-old male BKS.Cg−Lepr(db/db) mice which showed the mild rise of fasting blood glucose levels but high concentration of casual blood glucose levels. As an advanced stage model of diabetes, we used 16-week-old male db/db mice which showed high concentrations of both fasting and casual blood glucose levels. They were purchased from Clea, Tokyo, Japan. They were housed two to three animals per cage in all experiments under controlled ambient conditions and a 14:10 h light/dark cycle with lights on at 07:00 hours. Animals were given free access to drinking water and conventional food and were maintained at 25 °C. These were divided into 4 groups, and which were received PIO (25 mg/kg, oral), LIRA (0.2 mg/kg, s.c., twice daily), PIO and LIRA combination, or vehicle (CTRL, control) (0.05% carboxymethylcellulose, PO and PBS s.c., twice daily) for 2 weeks (n = 5 for each group). This study was approved by the Animal Use Committee of Kawasaki Medical School (no. 11–063) and was conducted in compliance with the Animal Use Guidelines of the Kawasaki Medical School. Doses of the drugs used in the study were based on reviews of the literature (Kanda et al., 2010; Shimoda et al., 2011). Body weight and food intake were monitored weekly from 6 weeks or 15 weeks of age.

2.2. Measurement of biochemical markers

Blood samples were collected from the tail vein. Blood glucose levels were measured immediately using a commercially available enzyme electrode method. The concentration of plasma insulin (Insulin ELISA kit; Morinaga Institute of Biological Science, Yokohama, Japan), glucagon (Glucagon ELISA kit; Yanaihara Institute Inc, Shizuoka, Japan) were determined by ELISA. Plasma TG and plasma NEFA concentration were measured enzymatically using the Tri-glyceride E-Test Wako and NEFA C-Test Wako (Wako Pure Chemical Industries, Osaka, Japan).

2.3. Intraperitoneal insulin tolerance test (IPITT)

After a 4-h fast, insulin (1–2 units/kg body weight) was injected intraperitoneally. Blood samples were collected from the tail vein before and 15, 30, 60, 90 and 120 min after insulin administration, and blood glucose levels were measured.

2.4. Measurement of triglyceride content and insulin content in pancreatic islets

At the end of the study, pancreatic islets were isolated by collagenase digestion, as previously reported (Kitamura et al., 2001). The islets were incubated with RPMI medium (Sigma) containing 10% (vol/vol) fetal calf serum overnight at 37 °C in 5% CO2. On the following day, size-matched pancreatic islets were collected. The TG content and insulin content in the pancreatic islets were measured as previously reported (Hamamoto et al., 2013).

2.5. Glucose-stimulated insulin secretion from isolated pancreatic islets

Size-matched pancreatic islets were prepared (5 pancreatic islets/tube) and preincubated in KRB–HEPES buffer. The supernatant was replaced with either a 3 or 16.7 mmol/l glucose solution, and the mixture was incubated for an additional 60 min. The supernatant was recovered and stored at −80 °C until use.

2.6. Immunofluorescence

According to the previously established method (Hamamoto et al., 2013), the sections were incubated with a mixture of primary antibodies (mouse anti-glucagon antibody and rabbit anti-insulin antibody; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Pancreatic islet proliferation was identified by staining sections with rabbit anti-Ki-67 polyclonal antibody (Abcam, Cambridge, MA, USA). And to investigate cell apoptosis in pancreatic islets, a TUNEL assay was performed using a DeadEnd Fluorometric TUNEL System (DeadEnd; Promega, Madison, WI, USA).

2.7. Morphometric analysis

The image analysis software NIH Image (version 1.61: http://rsbweb.nih.gov/ij/) was used to calculate the pancreas area and islet area. Using a total of 15 sections (3 sections from three different areas of the pancreas) for each group of mice, β-cell mass was estimated via the following formula: cell mass (mg) = average of islet area per section/average of pancreas area per section × weight of pancreas × β-cell ratio (average of β-cell number/cell number in islet). Observations were made using a minimum of 50 islets and, when quantified, were expressed as a percentage of the total number of islet cells.

2.8. Laser capture microdissection (LCM)

According to the previously established method (Kanda et al., 2010), frozen 8-μm-thick slices were immediately stained with hematoxylin. After tissue staining, the islets were irradiated with a laser using a PixCell system (Arcturus, Mountain View, CA, USA) using 2 s duration, 7.5 μm laser beam at 95 mV power. The peripheral area was first removed, and then the β cell rich core area was collected.

2.9. Real-time PCR

RNA samples from the islet core tissue obtained by LCM were extracted using a PicoPure RNA Isolation Kit (PN 12206-01, Arcturus). The primers were designed using Primer Express, based on mRNA sequences downloaded from the GenBank nucleotide database.

A reaction mixture was prepared by combining 0.5 μl of sample, 1 μl of 50 nM primers, 5 μl of Sybr Green PCR Master Mix (Applied Biosystems) solution, and 3.5 μl of diluent solution. Dissociation curve analysis was performed in all experiments to determine the dissociation temperature, and the size of the PCR products was confirmed using agarose gel electrophoresis. To quantify gene expression, the 2−ΔCT was calculated using 18S rRNA as an internal control, and it was compared with the mRNA value for each control group of mice.

2.10. Statistical analysis

All data are shown as mean ± S.E. The comparison between 4 groups was performed using ANOVA and the multiple comparisons were performed using the Tukey–Kramer method. And p < 0.05
was regarded as significant. A Mann–Whitney U test was used to test for differences early and advanced model of CTL with \( p < 0.05 \) regarded as significant. Statistical analysis was performed using JMP9 (SAS, Cary, NC, USA).

3. Results

3.1. Metabolic variables after treatment of pioglitazone (PIO) and/or liraglutide (LIRA) in an early and advanced stage of diabetes

At the baseline (7 and 16 weeks of age), there was no difference in body weight, food intake, fasting blood glucose levels (FBG) among 4 groups (control (CTL), LIRA, PIO and combination (Comb) groups) in both early (Fig. 1a–c) and advanced stages of diabetes (Fig. 1d–f). Between an early and advanced stage of CTL group before the intervention, there was a significant difference in body weight and FBG. In an early stage, after 1 week of treatment, LIRA and Comb treatment significantly decreased food intake (Fig. 1a). These effects were sustained until 2 weeks (Fig. 1a). Body weight gain was suppressed in LIRA group but it was significantly accelerated by PIO group compared with CTL group (Fig. 1b). Fasting blood glucose levels were significantly lower in LIRA and PIO group compared with CTL group, which was further enhanced by combination treatment (Fig. 1c). In an advanced stage, after 2 weeks of treatment, food intake was lower in the LIRA and Comb groups as observed in an early stage (Fig. 1d). The change of body weight in drug-treated groups showed the same tendency as in an early stage (Fig. 1e). A significant reduction of fasting blood glucose levels was observed only in Comb group (Fig. 1f).

Similarly, at the baseline (7 and 16 weeks of age), there was no difference in fasting plasma insulin, glucagon, NEFA and TG levels among the 4 groups (CTL, LIRA, PIO and Comb groups) in both an early (Fig. 1g–j) and advanced stages of diabetes (Fig. 1k–n). Between an early and advanced stage of CTL group before the intervention, there was a significant difference in TG levels. In an early stage of diabetes, plasma insulin level in PIO group was lower compared to the other groups after 2 weeks of intervention (Fig. 1g). Glucagon levels in LIRA and Comb tended to be lower compared to the other groups, although it did not reach a statistical significance (Fig. 1h). Fasting plasma TG levels in PIO and Comb groups were lower than that of CTL (Fig. 1i), although there was no difference in plasma NEFA levels among the 4 groups after 2 weeks of intervention (Fig. 1j). Plasma insulin levels in an advanced stage was lower than those in early stage (Fig. 1k). Furthermore, LIRA and/or PIO treatment did not affect plasma insulin levels. LIRA and Comb treatments significantly reduced plasma glucagon concentrations compared with other groups (Fig. 1l). Significant reductions of fasting TG and NEFA levels were observed only in Comb group (Fig. 1m, 1n).

3.2. Glucose-stimulated insulin secretion from isolated pancreatic islets

There was a significant difference in insulin secretion between an early and advanced stage of CTL group in the presence of 3 mmol/l and 16.7 mmol/l glucose. In an early stage model, after 2 weeks of treatment, insulin secretion was increased in response to 16.7 mmol/l glucose in PIO and LIRA groups compared with CTL, although it did not reach a statistical significance. There was a statistically

![Fig. 1. (a–f) Metabolic variables in an early (7–9 weeks of age) and advanced stage of diabetes (16–18 weeks of age) treated for 2 weeks with vehicle (circles), pioglitazone (triangles), liraglutide (diamonds), or combination (crosses). (a, d) Food intake, (b, e) body weight, (c, f) fasting blood glucose level. (g–n) Metabolic variables in an early and advanced stage of diabetes treated for 2 weeks. (g, k) Fasting plasma insulin level, (h, l) fasting plasma glucagon level, (i, m) fasting plasma triglyceride level, (j, n) fasting plasma NEFA level. The multiple comparison was performed using the Tukey–Kramer method. Data are presented as mean ± S.E. n = 10. *: \( p < 0.05 \) vs CTL, §: \( p < 0.05 \) vs PIO, Ω: \( p < 0.05 \) vs Lira.
significant difference between CTL and Comb groups (Fig. 2a). On the other hand, in the advanced stage model, although insulin secretion was enhanced by combination treatment, it was not so marked compared to that of the early model (Fig. 2b).

3.3. Insulin contents and triglyceride (TG) contents in pancreatic islets

There was a significant difference in insulin content between an early and advanced stage of CTL group, although no difference was observed in TG content. In the early stage model, after 2 weeks of intervention, islet insulin contents in treated mice were higher than that in CTL mice in an early stage (Fig. 2c). Insulin content was increased by LIRA treatment which was further enhanced by combination treatment. TG contents in the pancreatic islets in treated mice were lower than that of CTL mice (Fig. 2d). In contrast, in the advanced stage model, there was no difference in both insulin and TG content among the 4 groups after 2 weeks of intervention (Fig. 2e, 2f).

3.4. Profiles of various gene expressions related with insulin and lipid biosynthesis

To examine how insulin content was recovered by such treatment in an early stage, but not in an advanced stage, we evaluated insulin gene expression levels. *Insulin* mRNA expression was up-regulated by LIRA and Comb treatments in an early stage, but not in an advanced stage, although it did not reach a statistical significance (Fig. 3a, e). Next, we evaluated expression levels of various insulin gene transcription factors such as *MafA*, *PDX-1* and *NeuroD* all of which are very important to maintain mature β-cell function. There was a significant difference in *PDX-1* and *NeuroD* levels between an early and advanced stage of CTL group. As shown in Fig. 3b, in an early stage, *MafA* expression was up-regulated by PIO or LIRA treatment which was further enhanced by Comb treatment. In contrast, in an advanced stage, *MafA* expression was not up-regulated even after the Comb treatment (Fig. 3f). *Pdx-1* expression was up-regulated after Comb treatment in an early stage (Fig. 3c), which was not observed in an advanced stage (Fig. 3g). Similar results were obtained with *NeuroD*; its expression was up-regulated after Comb treatment in an early stage (Fig. 3d), which was not observed in an advanced stage (Fig. 3h). Glut2 expression level in an advanced stage was markedly lower compared to that in an early stage (p < 0.05). Glut2 and Glucokinase expressions were up-regulated by PIO and LIRA in an early stage (Fig. 3i, j), but not in the advanced stage (Fig. 3l, m). GLP-1 receptor expression levels in an advanced stage were markedly reduced compared to those in an early stage (p < 0.05), although GLP-1 receptor expression levels were not up-regulated after 2 weeks of treatment in both stages (Fig. 3k, n). In addition, we evaluated gene expression related with lipid synthesis. Fas expression was down-regulated after Comb treatment in an early stage (Fig. 3o), which was not observed in an advanced stage (Fig. 3q), although there was no such phenomena in SREBP-1c expression (Fig. 3p, r). To examine the effects of anti-diabetic treatment on ER stress, we evaluated several markers of ER stress after the treatment with PIO and/or LIRA. We evaluated ER stress-related gene expression. Chop and Bip expressions were markedly suppressed by Comb treatment in an early stage (Fig. 3s, t), which was not observed in an advanced stage (Fig. 3u, v).
3.5. Evaluation of β-cell ratio and mass

We next employed morphological investigations of pancreatic islets after various treatments in an early and advanced stage of diabetes. Double immunostaining with antibodies against insulin and glucagon was carried out. There was a significant difference in β-cell ratio between an early and advanced stage of CTL group. In an early stage, β-cell mass was increased in PIO- and LIRA-treated mice, and the most significant effect was observed in the Comb group (Fig. 4a-c). In an advanced stage, on the other hand, the effect of these drugs on β-cell mass was not significant statistically (Fig. 4d-f). These results suggest that protective effects of pioglitazone and/or liraglutide on pancreatic β-cell mass were more powerful in an early stage of diabetes compared to those in an advanced stage.

3.6. Evaluation of β-cell proliferation and apoptosis

Histological sections of the pancreas were stained by antibody specific for Ki67 and the proportion of antibody-positive cells was measured to evaluate the proliferative effect of pioglitazone and liraglutide upon pancreatic islets. These drugs increased the ratio of Ki67 positive cells in an early stage (Fig. 5a, b), but there was no significant changes among the 4 groups in an advanced stage (Fig. 5d, e). These results suggest that the decreased effects of pioglitazone and liraglutide on β-cell proliferation capacity with duration of diabetes (Fig. 5a, b, d, e) explain, at least in part, the decreased effects of such drugs on β-cell mass (Fig. 4a-f).

Apoptotic cell death, analyzed by TUNEL assay, was suppressed by pioglitazone or liraglutide, and further enhanced by combination treatment in each early (Fig. 5c) and advanced stage (Fig. 5f). These results suggest that pioglitazone and liraglutide preserve β-cell mass by suppressing apoptosis in both early and advanced stages.

In addition, to examine islet fibrosis, we performed Azan staining. Fibrosis area in the islets was decreased by the treatment in an early stage (Fig. 6a). Larger area was stained in advanced model compared to early model, but there was no significant difference among the 4 groups in an advanced stage (Fig. 6b).

3.7. Gene expression profiles related with β-cell mass

To examine how β-cell mass was preserved by such treatment, we evaluated gene expression profiles related with β-cell mass, proliferation and apoptosis. As shown in Fig. 7a, d, CyclinD expression was augmented by several treatments in an early stage, but not in an advanced stage, although it did not reach a statistical significance. Next, we evaluated fibrosis-related gene expression. As shown in Fig. 7b, c, e, f, collagen I and III were markedly suppressed by Comb treatment in an early stage (Fig. 7b, c) which was not so marked in an advanced stage (Fig. 7e, f). On the other hand, in both early and advanced stages, expression levels of pro-apoptotic genes, such as caspase 3 and 8, were suppressed in PIO- and LIRA-treated groups (Fig. 7g, h, j, k). Anti-apoptotic factor Bcl-2 level was up-regulated by such drug treatment in both an early and advanced stage (Fig. 7l).

3.8. Evaluation of insulin resistance

To examine the effects of anti-diabetic treatment on insulin resistance in an early and advanced stage of diabetes, we performed intraperitoneal insulin tolerance test (IPITT). Insulin sensitivity was improved in PIO and Comb groups in an early stage (Fig. 8a, b) and advanced stage (Fig. 8c, d).

4. Discussion

Numerous studies have shown that thiazolidines and incretin-related drugs preserve both β-cell function and mass in animal model of diabetes (Hamamoto et al., 2013; Kanda et al., 2010; Kawasaki et al., 2005; Shimoda et al., 2011; Xu et al., 2013). But many of these reports showed the results examined in the relatively early stage
Fig. 3. (a–f) Expression levels of insulin and insulin gene transcription in an early (a–d) and advanced stage of diabetes (e–h) treated for 2 weeks with vehicle (white), pioglitazone (light gray), liraglutide (dark gray), or combination (black). (b, f) MafA, (c, g) Pdx-1, (d, h) NeuroD. (i–n) Expression levels of genes involved in glucose-stimulated insulin secretion in an early (i–k) and advanced stage of diabetes (l–n). (i, l) GLUT2, (j, m) Glucokinase, (k, n) GLP-1 receptor. (o–r) Expression levels of genes involved in lipid synthesis in an early (o, p) and advanced stage of diabetes (q, r). (o, q) Fas, (p, r) SREBP-1c. (s–v) Expression levels of genes involved in ER stress in an early (s, t) and advanced stage of diabetes (u, v). (s, u) CHOP, (t, v) Bip. Data are presented as mean ± S.E. n = 5 for each group. *: p < 0.05.
of diabetes (Hamamoto et al., 2013; Kanda et al., 2010; Shimoda et al., 2011), and thus the effects of these drugs in severe diabetic morbidity remained unclear. In this study, we demonstrated that insulin biosynthesis and secretion were attenuated with the progression of diabetes and that pioglitazone and liraglutide increased β-cell function and mass in an early stage of diabetes but these effects were attenuated in an advanced stage. In an early stage insulin biosynthesis and secretion were markedly increased by PIO...
and LIRA which was not clearly observed in an advanced stage (Fig. 2). In concomitant with such phenomena, expression levels of various insulin gene transcription factors were up-regulated by PIO and LIRA in an early stage, but not in an advanced stage (Fig. 3). We think that the increased expression of such factors in an early stage explains the increased insulin biosynthesis and secretion in an early stage. In addition, the increased expression of GLUT2 as well as glucokinase could explain the augmentation of glucose-stimulated insulin secretion in an early stage (Bae et al., 2010; Hay and Docherty, 2011; Kim et al., 2012; Robertson et al., 2013; Cantley et al., 2007; Kubota Buteau et al., 1999, 2003; Frodin et al., 2004). The binding of GLP-1 to its receptors activates adenylyl cyclase and the cyclic AMP/protein kinase A signaling pathway (Buteau et al., 1999, 2003; Frodin et al., 1995; Jhala et al., 2003; Park et al., 2006). In this study, the expression of GLP-1 receptor was down-regulated in an advanced stage compared with an early stage. Therefore, we assume that the down-regulation of some signal such as PKA downstream of GLP-1 receptor (Takeda et al., 2011) is involved in the ineffectiveness of anti-diabetic drugs on insulin secretion in an advanced stage (Awazawa et al., 2011; Costes et al., 2006; Park et al., 2006; Pugazhenthi et al., 2000). In addition, we think that the effects of pioglitazone are not necessarily related to the GLP-1 receptor signaling but that some other mechanisms are also involved in the effects of pioglitazone.

In the advanced stage model, the improvement of blood glucose levels was observed in combination group slightly but significantly. We assume that the reduction of insulin resistance largely explains the improvement of glycemic control although suppression of glucagon and slight improvement of GSIS are also involved in the amelioration of glycemic control. In addition, it is known that GLP-1 can suppress macrophage infiltration into adipocytes and directly inhibit adipocyte inflammation. Therefore, we think that the combined use of both drugs is effective for the improvement of glycemic control.

It is well established that chronically elevated glucose, lipids and inflammatory cytokines induce not only oxidative stress but also endoplasmic reticulum (ER) stress (Kim et al., 2012; Robertson et al., 2004). Moreover, it has been reported that oxidative and ER stresses contribute to the inhibition of cell proliferation and the induction of apoptosis by suppressing PI3K signal or Bcl2 family expression. The reduction of ER stress by PIO and LIRA was more evident in an early model compared with that of an advanced model (Fig. 3). Decreased expressions of these genes were associated with the improvement of metabolic state in an early stage of diabetes treated with PIO and LIRA. Thus, we assume that the suppressive effect of these drugs against ER stress is mediated by the improvement of glycemic and lipid metabolism.

In addition, we showed that the influence of such drug intervention on β-cell proliferation capacity varied according to the degree of diabetic morbidity (Fig. 4). The ratio of Ki67 positive β-cells was significantly increased by drug intervention only in an early stage, but not in an advanced stage (Fig. 5) (Cantley et al., 2007; Kubota et al., 2000; Maedler et al., 2004; Pieczynik and Neustadt, 2007; Withers et al., 1998). In addition, the expression of GLP-1 receptor was down-regulated in advanced stage compared with an early stage. Therefore, we assume that the down-regulation of incretin signaling is involved in the ineffectiveness of anti-diabetic drugs on β-cell mass in an advanced stage. In contrast, the inhibition of apoptosis in intervention groups was similarly observed both in an early and advanced stage (Fig. 5). Bcl-2 gene expression was increased and caspase 3 and caspase 8 gene expressions were decreased by PIO and/or LIRA in both stages (Fig. 7) (Augstein et al., 2009; Xu et al., 2011).
We assume that the alteration of such gene expression explains, at least in part, the reason why PIO and/or LIRA preserved proliferation capacity without influencing the ratio of apoptosis (Dalboge et al., 2013).

It remains unknown whether a longer treatment strategy would exert beneficial effects in an advanced model. In the advanced stage, β-cell function or mass was not protected by 2-week intervention. The improvement of blood glucose levels was observed by 1-week

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**Fig. 5.** Effect of 2-week intervention on β-cell proliferation and apoptosis in an early (a–c) and advanced stage of diabetes (d–f). (a, d) Immunofluorescence staining with antibody against Ki67. Quantification of β-cell proliferation (b, e) and apoptosis (c, f). n = 5 for each assay. Scale bars 50 μm. Control (gray), pioglitazone (light gray), liraglutide (dark gray), combination (black). Scale bars 50 μm. Data are presented as mean ± S.E. n = 5 for each assay. *: p < 0.05.

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**Fig. 6.** Effect of 2-week intervention on islet fibrosis in an early (a) and advanced stage of diabetes (b). (a, b) Azan staining was performed to estimate islet fibrosis. Scale bars 50 μm.
Fig. 7. (a–f) Expression levels of genes involved in cell proliferation and fibrosis in an early (a–c) and advanced stage of diabetes (d–f) treated with vehicle (white), pioglitazone (light gray), liraglutide (dark gray) or combination (black). (a, d) Cyclin D, (b, e) collagen I, (c, f) collagen III. (g–l) Expression of genes involved in pro- and anti-apoptosis in an early (g–i) and advanced stage model (j–l). (g, j) caspase 3, (h, k) caspase 8, (i, l) Bcl-2. Data are presented as mean ± S.E. n = 5 for each group. *: p < 0.05.
intervention presumably due to the reduction of food consumption in LIRA group, but after 2-week intervention there was no difference in blood glucose levels between LIRA and CTL groups. Blood glucose levels were not altered at all in PIO group. In consideration of these data, it is likely that longer pharmaceutical intervention with LIRA or PIO would not exert β-cell protective effect in the presence of very strong gluco-lipotoxicity. On the other hand, blood glucose levels were improved by the combination therapy. We assume that this is due not only to the direct effect of such drugs but also the secondary effect through the improvement of glycemic control and β-cell function. It seems that when intervention is continued from 7 week to 18 week, more beneficial effects for β-cell protection would be observed at 18 weeks of age, although not examined in this study. In addition, considering from the life span of mice (2–3 years), 18 weeks of age would correspond to childhood or adolescence in humans. Therefore, we think that the data in this study were due to the influence of morbidity progress of diabetes rather than aging.

In conclusion, protective effects of pioglitazone and/or liraglutide on pancreatic β-cells were more powerful in an early stage of diabetes compared to those in an advanced stage. These results suggest that the pharmacological intervention to type 2 diabetes would be “the earlier, the better”.

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References

Augstein, P., Heinke, P., Schober, C., Salzsieder, E., 2009. Impact of cytokine- and FasL-induced apoptosis in the beta-cell line NIT-1. Horm. Metab. Res. 41, 207–212.

Awazawa, M., Ueki, K., Inabe, K., Yamauchi, T., Kubota, N., Kameko, K., et al., 2011. Adiponectin enhances insulin sensitivity by increasing hepatic IRS-2 expression via a macrophage-derived IL-6-dependent pathway. Cell Metab. 13, 401–412.

Bae, J.S., Kim, T.H., Kim, M.Y., Park, J.M., Ahn, Y.H., 2010. Transcriptional regulation of glucose sensors in pancreatic beta-cells and liver: an update. Sensors (Basel) 10, 5031–5053.

Buteau, J., Roduit, R., Susini, S., Prentki, M., 1999. Glucagon-like peptide-1 promotes DNA synthesis, activates phosphatidylinositol 3-kinase and increases transcription factor pancreatic and duodenal homeobox gene 1 (PDX-1) DNA binding activity in beta (INS-1)-cells. Diabetologia 42, 856–864.

Buteau, J., Foisy, S., Joly, E., Prentki, M., 2003. Glucagon-like peptide 1 induces pancreatic beta-cell proliferation via transactivation of the epidermal growth factor receptor. Diabetes 52, 124–132.

Butler, A.E., Janson, J., Soeller, W.C., Butler, P.C., 2003. Increased beta-cell apoptosis prevents adaptive increase in beta-cell mass in mouse model of type 2 diabetes: evidence for role of islet amyloid formation rather than direct action of amyloid. Diabetes 52, 2304–2314.

Cantley, J., Choudhury, A.I., Asare-Anane, H., Selman, C, Lingard, S., Haffron, H., et al., 2007. Pancreatic deletion of insulin receptor substrate 2 reduces beta and alpha cell mass and impairs glucose homeostasis in mice. Diabetologia 50, 1248–1256.

Costes, S., Broca, C., Bertrand, G., Lajoix, A.D., Bataille, D., Bockaert, J., et al., 2006. ERK1/2 control phosphorylation and protein level of CAMP-responsive element-binding protein: a key role in glucose-mediated pancreatic beta-cell survival. Diabetes 55, 2220–2230.
Dalboge, L.S., Almholt, D.L., Neerup, T.S., Vassiliades, E., Vrang, N., Pedersen, L., et al., 2013. Characterization of age-dependent beta cell dynamics in the male db/db mice. PLoS ONE 8, e82813.

Frodin, M., Sekine, N., Roche, E., Filouos, P., Pretkni, M., Wollheim, C.B., et al., 1995. Glucose, other secretagogues, and nerve growth factor stimulate mitogen-activated protein kinase in the insulin-secreting beta-cell line, INS-1. J. Biol. Chem. 270, 7882–7889.

Hamamoto, S., Kanda, Y., Shimoda, M., Tatsumi, F., Kohara, K., Tawaramoto, K., et al., 2013. Vildagliptin preserves the mass and function of pancreatic beta cells via the developmental regulation and suppression of oxidative and endoplasmic reticulum stress in a mouse model of diabetes. Diabetes Obes. Metab. 15, 153–163.

Hay, C.W., Docherty, K., 2006. Comparative analysis of insulin gene promoters: implications for diabetes research. Diabetes 55, 3201–3213.

Jhala, U.S., Canettieri, G., Screaton, R.A., Kulkarni, R.N., Krajewski, S., Reed, J., et al., 2003. AMP promotes pancreatic beta-cell survival via CREB-mediated induction of IRS2. Genes Dev. 17, 1575–1580.

Jonsson, J., Carlsson, L., Edlund, T., Edlund, H., 1994. Insulin-promoter-factor 1 is required for pancreas development in mice. Nature 371, 606–609.

Kanda, Y., Shimoda, M., Hamamoto, S., Tawaramoto, K., Kawasaki, F., Hashimarto, M., et al., 2010. Molecular mechanism by which pioglitazone preserves pancreatic beta-cells in obese diabetic mice: evidence for acute and chronic actions as a PPAR gamma agonist. Am. J. Physiol. Endocrinol. Metab. 298, E278–E286.

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Matsuoka, T., Kajimoto, Y., Watada, H., Kaneto, H., Kishimoto, M., Umayahara, Y., et al., 2000. Disruption of IRS-2 causes type 2 diabetes because of liver insulin resistance and lack of compensatory beta-cell hyperplasia. Diabetes 49, 1880–1889.

Leonard, J., Peers, B., Johnson, T., Krajewski, S., Reed, J., et al., 2003. Members of the large Maf transcription family regulate insulin gene transcription in islet beta cells. Mol. Cell. Biol. 23, 6049–6062.

Miller, C.P., McGehee, R.E., Jr., Habener, J.F., 1994. IDX-1: a new homeodomain transcription factor expressed in rat pancreatic islets and duodenum that transactivates the somatostatin gene. EMBO J. 13, 1145–1156.

Obukogo, Y., Kishikawa, H., Araki, E., Miyata, T., Isami, S., Motoyoshi, S., et al., 1995. Intensive insulin therapy prevents the progression of diabetic microvascular complications in Japanese patients with non-insulin-dependent diabetes mellitus: a randomized prospective 6-year study. Diabetes Res. Clin. Pract. 28, 103–117.

Ohsimson, H., Karlsson, K., Edlund, T., 1993. IFP1, a homeodomain-containing transactivator of the insulin gene. EMBO J. 12, 4251–4259.

Park, S., Dong, X., Fisher, T.L., Dunn, S., Omer, A.K., Weir, G., et al., 2006. Exendin-4 uses Irs2 signaling to mediate pancreatic beta cell growth and function. J. Biol. Chem. 281, 1159–1168.

Pieczenik, S.R., Neustadt, J., 2007. Mitochondrial dysfunction and molecular pathways of disease. Exp. Mol. Pathol. 83, 84–92.

Pugazhenthi, S., Nesterova, A., Sable, C., Heidenreich, K.A., Boxer, L.M., Heasley, L.E., et al., 2000. Akt/protein kinase B up-regulates Bcl-2 expression through cAMP-response element-binding protein. J. Biol. Chem. 275, 10761–10766.

Rankin, M.M., Kushner, J.A., 2009. Adaptive beta-cell proliferation is severely restricted with advanced age. Diabetes 58, 1365–1372.

Robertson, R.P., 2004. Chronic oxidative stress as a central mechanism for glucose toxicity in pancreatic islet beta cells in diabetes. J. Biol. Chem. 279, 42351–42354.

Robertson, R.P., Harmon, J., Tran, P.O., Tanaka, Y., Takahashi, H., 2003. Glucose toxicity in beta-cells: type 2 diabetes, good radicals gone bad, and the glutathione connection. Diabetes 52, 581–587.

Robertson, R.P., Harmon, J., Tran, P.O., Poitout, V., 2004. Beta-cell glucose toxicity, lipotoxicity, and chronic oxidative stress in type 2 diabetes. Diabetes 53 (Suppl. 1), S119–S124.

Shimoda, M., Kanda, Y., Hamamoto, S., Tawaramoto, K., Hashimarto, M., Matsuiki, M., et al., 2011. The human glucagon-like peptide-1 analogue lixisenatide preserves pancreatic beta cells via regulation of cell kinetics and suppression of oxidative and endoplasmic reticulum stress in a mouse model of diabetes. Diabetologia 54, 1098–1108.

Takeda, Y., Amano, A., Noma, A., Nakamura, Y., Fujimoto, S., Inagaki, N., 2011. Systems analysis of GLP-1 receptor signaling in pancreatic beta-cells. Am. J. Physiol. Cell Physiol. 301, C792–C803.

Taylor, S.L., 1991. Deconstructing type 2 diabetes. Cell 97, 9–12.

The Diabetes Control and Complications Trial Research Group, 1993. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. N. Engl. J. Med. 329, 977–986.

UK Prospective Diabetes Study (UKPDS) Group, 1998. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). Lancet 352, 837–853.

Waebener, G., Thompson, N., Nicod, P., Bonny, C., 1996. Transcriptional activation of the GLUT 2 gene by the IFP-1/STF-1/IDX-1 homeobox factor. Mol. Endocrinol. 10, 1327–1334.

Weir, G.C., Laybutt, D.R., Kaneto, H., Bonner-Weir, S., Sharma, A., 2001. Beta-cell adaptation and decompensation during the progression of diabetes. Diabetes 50 (Suppl. 1), S154–S159.

Welters, D.J., Gutierrez, J.S., Towery, H., Burks, D.J., Ren, J.M., Previs, S., et al., 1998. Disruption of IRS-2 causes type 2 diabetes in mice. Nature 391, 900–904.

Xu, G., Kaneto, H., Laybutt, D.R., Duvivier-Kali, V.F., Trivedi, N., Suzuma, K., et al., 2007. Downregulation of GLP-1 and GIP receptor expression by hyperglycaemia: possible contribution to impaired incretin effects in diabetes. Diabetes 56, 1551–1558.

Xu, P., Lou, X.L., Chen, C., Yang, Z.W., 2013. Effects of peroxisome proliferator-activated receptor-gamma activation on apoptosis in rats with acute pancreatitis. Dig. Dis. Sci. 58, 3516–3523.