Structural and functional analysis of pancreatic islets
preserved by pioglitazone in db/db mice

Fumiko Kawasaki, Masafumi Matsuda, Yukiko Kanda, Hiroshi Inoue, and Kohei Kaku

Diabetes and Endocrine Division, Kawasaki Medical School, Kurashiki-shi, Okayama, Japan

Submitted 17 March 2004; accepted in final form 1 November 2004

Hyperglycemia and hyperlipidemia have attracted attention recently as factors that affect the mechanism of insulin secretion. Impaired glucose-mediated insulin secretion (26) from pancreatic β-cells leads to insulin insufficiency and thus hyperglycemia and lipid metabolism abnormalities. In the long term, hyperglycemia leads to glucose toxicity (glucotoxicity) and worsening of the impaired insulin secretion. One mechanism of glucotoxicity on pancreatic β-cells is the overwork hypothesis of Leahy (21). The hyperglycemic state leads to overworking of pancreatic β-cells and a decreased ability to secrete insulin. The glucotoxicity-mediated pancreatic β-cell dysfunction is reversible to some degree (20, 24).

Lee et al. (22) have also proposed the concept of lipotoxicity, in which increases of nonesterified fatty acids released from adipocytes lead to decreased insulin action on the skeletal muscles and impaired insulin secretion by pancreatic β-cells. Zucker diabetic fatty (ZDF) rats show increased fatty acids in the plasma and increased triglyceride content in the pancreatic islets before any increases in the blood glucose level. When normal rat pancreatic islets are cultured in the presence of fatty acids, the triglyceride content in islets increases and the glucose-dependent insulin secretion decreases. These findings strongly suggest a relationship between lipotoxicity and impaired pancreatic β-cell function (34).

Spontaneous mutant strain C57BL/KsJ db/db mice have the db mutation, a splicing mutation caused by a point mutation in the downstream intron of the leptin receptor gene, and are unresponsive to leptin. Leptin is a peptide hormone secreted by adipocytes and is involved in eating behavior and energy homeostasis. For this reason, the homozygotes (db/db) after birth have unrepressed eating behavior, become obese, and develop severe insulin resistance associated with hyperinsulinemia, hyperglycemia, and hypertriglyceridemia, so that by 3–6 mo after birth, the pancreatic islet β-cells reduce their mass, resulting in severe insufficiency of insulin secretion (5, 16, 22, 28). The C57BL/KsJ db/db mice, with severe insulin resistance and impaired insulin secretion, are widely used as experimental models of obese type 2 diabetes mellitus. There are reports suggesting genetic control over the pathogenetic mechanisms leading to insufficient insulin secretion (17), but this process, including the possible involvement of glucotoxicity and lipotoxicity, is not well understood.

Pioglitazone, a thiazolidinedione derivative, promotes glucose utilization by the tissues targeted by insulin and lowers the blood glucose as well as the plasma insulin level. The efficacy of this compound is particularly prominent in patients with strong insulin resistance, and it is widely used to treat type 2 diabetes mellitus.

Kawasaki, Fumiko, Masafumi Matsuda, Yukiko Kanda, Hiroshi Inoue, and Kohei Kaku. Structural and functional analysis of pancreatic islets preserved by pioglitazone in db/db mice. Am J Physiol Endocrinol Metab 288: E510–E518, 2005. First published November 2, 2004; doi:10.1152/ajpendo.00128.2004.—To evaluate preventive effects of pioglitazone on pancreatic β-cell damage in C57BL/KsJ db/db mice, an obese diabetic animal model, the pancreatic islets were compared morphologically between pioglitazone-treated (100 mg/kg daily po) and untreated db/db mice (n = 7 for each) after a 12-wk intervention (6–18 wk of age). The fasting blood glucose level was significantly improved by the treatment with pioglitazone (260 ± 12 vs. 554 ± 62 mg/dl, P < 0.05). The islet mass in the pancreas was significantly greater in pioglitazone-treated mice than in untreated mice (10.2 ± 1.1 vs. 4.6 ± 0.2 mg, P < 0.01). Subsequently, biochemical and physiological analyses of the β-cell function were employed using pioglitazone-treated and untreated db/db mice (n = 6 for each) and pioglitazone-treated and untreated db/+ mice (n = 6 for each). After 2 wk of treatment (10–12 wk of age), the plasma levels of triglyceride and free fatty acid were significantly decreased, whereas the plasma adiponectin level increased significantly compared with the untreated group (65.2 ± 18.0 vs. 18.3 ± 1.3 μg/ml, P < 0.05). Pioglitazone significantly reduced the triglyceride content in the islets (43.3 ± 3.6 vs. 65.6 ± 7.6 ng/islet, P < 0.05) with improved glucose-stimulated insulin secretion. Pioglitazone showed no significant effects on the biochemical and physiological parameters in db/+ mice. The present study first demonstrated that pioglitazone prevents β-cell damage in an early stage of the disease progression in db/db mice morphologically and physiologically. Our results suggest that pioglitazone improves glucolipotoxicity by increasing insulin sensitivity and reducing fat accumulation in the pancreatic islets.

pancreatic β-cells; adiponectin; type 2 diabetes mellitus

DIABETES MELLITUS induces a variety of metabolic abnormalities because of insufficient insulin action. Of these, abnormalities in glucose metabolism are the most specific and are manifested clinically as hyperglycemia after glucose ingestion. In type 2 diabetes mellitus, which affects the majority of patients with diabetes mellitus, the factors involved in the pathogenesis and the progression of the disease are insufficient insulin secretion and decreased insulin sensitivity (insulin resistance) (7, 33). The relationship between insufficient insulin secretion and diabetes mellitus was underscored by the discovery of the causative gene for maturity onset diabetes of the young (9), and abnormalities in insulin secretion are considered to be particularly important (3). Prevention of the progression of pancreatic β-cell dysfunction in subjects with diabetes mellitus should be a key in the long-term management of this disease.

Address for reprint requests and other correspondence: K. Kaku, Diabetes and Endocrine Division, Dept. of Medicine, Kawasaki Medical School, 577 Matsushima, Kurashiki-shi, Okayama-ken 701-0192, Japan (E-mail: kkaku@med.kawasaki-m.ac.jp).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
diabetes mellitus. It has been reported that troglitazone, a thiazolidinedione class of compound, improves glucose tolerance and prevents pancreatic β-cell destruction in the ZDF rat, an animal model of obese diabetes mellitus (12, 13). Pioglitazone also has been shown to preserve insulin secretion and pancreatic morphology in some animal models of diabetes mellitus (8). However, it is unknown how thiazolidinediones, whose in vivo molecular target is the nuclear hormone receptor-type transcription factor peroxisome proliferator-activated receptor (PPAR)γ, improve insulin sensitivity and preserve pancreatic β-cell function.

To characterize possible prevention of the progression of diabetes mellitus in an animal model of obese type 2 diabetes mellitus, C57BL/KsJ db/db mice, we initiated pioglitazone early in the course of the disease in a long-term intervention (12 wk) to compare the morphological changes in the pancreatic islets and to assess the prevention of the pancreatic β-cell dysfunction (experiment 1). In a short-term (2 wk) intervention study, we analyzed pancreatic islets physiologically and biochemically to elucidate the way pancreatic β-cell dysfunction is prevented by this drug (experiment 2). Through this analysis of pioglitazone administration, with respect to its effects on preserving pancreatic β-cell function, it was hoped that this study would clarify the mechanism of the progression of pancreatic β-cell dysfunction in the db/db mice and provide support for preventive strategies in patients with type 2 diabetes mellitus.

ANIMALS AND METHODS

Preparation of C57BL/KsJ db/db Mice

After birth, the spontaneous C57BL/KsJ db/db mutant mice have uncontrolled eating behavior, become obese, and develop insulin resistance and diabetes mellitus. To obtain the db/db mice, nonobese heterozygotes (db/+ ) that were maintained in repulsion with misty coat color were obtained from Jackson Laboratory (Bar Harbor, ME) and mated and housed in the animal care facility of the Kawasaki Medical School. The misty coat color mutation is commonly maintained in linkage disequilibrium with the obesity mutation diabetes (Lepr db) to serve as a marker. On the basis of the misty coat color, the db/+ and db/db mice were identified, and db/db mice were confirmed based on their obesity and characteristic body habitus. The animals were provided free access to standard feed (MF; Oriental Yeast, Tokyo, Japan) and tap water and maintained at 25°C. These studies were approved by the Animal Use Committee of the Kawasaki Medical School (no. 01-080) and were conducted in compliance with the Animal Use Guidelines of the Kawasaki Medical School.

Experiment 1

The following experiment was conducted to study the mechanisms involved in the prevention of pancreatic β-cell dysfunction by drug intervention through an assessment of morphological changes.

Intervention protocol. Six-week-old db/db mice were divided into two groups: group P (n = 7) received pioglitazone (100 mg·kg body wt⁻¹·day⁻¹, oral), and group C (n = 7) received vehicle administration. Drug intervention was continued for 12 wk until the age of 18 wk. For comparison, lean nondiabetic db/+ mice of the same age were used: group LC (n = 7) received vehicle, and group LP (n = 7) received pioglitazone (100 mg·kg body wt⁻¹·day⁻¹, oral). Pioglitazone was provided by Takeda Pharmaceuticals (Osaka, Japan) and was prepared as an emulsion in distilled water containing 0.5% carboxymethylcellulose. The drugs were administered orally using a stomach probe. The drug dosages were chosen on the basis of prior published reports (32). During the intervention period, body weight and blood glucose were measured at weekly intervals. For blood glucose measurements, the mice were placed under ether inhalational anesthesia, and the blood was collected into hematocrit tubes from the retroorbital fossa.

Morphological assessment of the pancreatic islets. At 6 wk (in 12-wk-old animals) and at 12 wk (in 18-wk-old animals) after the start of drug intervention, the pancreas was isolated, fixed in formalin, and embedded in paraffin. The specimens were thin-sectioned into 4-μm sections for slide preparation. We examined 10 consecutive slices in 2 isolated parts in the head, center, and tail area per pancreas, and at least 3 different pancreases were used in each group. The examination of a total of 30 sections in a rat covered 6 different volumes in a pancreas. For standard stains, hematoxylin-eosin stain and azan stain were used. Immunohistochemical staining was conducted using an anti-insulin antibody and a mix of three anti-pancreatic hormone antibodies (glucagon, somatostatin, and pancreatic polypeptide).

Immunostaining was performed by immunohistochemistry. The paraffin-embedded pancreatic sections were deparaffinized in Lemosol (Wako Pure Chemical Industries, Osaka, Japan) and ethanol, and endogenous peroxidase activity was blocked by immersion in 3% hydrogen peroxide in methanol for 10 min. After being washed in PBS (10 mM, pH 7.0), the sections were incubated with guinea pig anti-insulin polyclonal antibody (Nichirei, Tokyo, Japan) as the primary antibody at 25°C for 20 min. After another washing in PBS, Simple Stain MAX-PO (Nichirei) was added, and the reaction was allowed to proceed at 25°C for 30 min. After a third washing in PBS, Simple Stain DAB solution (Nichirei) was added, and the color reaction was allowed to proceed at 25°C for 7 min. The adjacent sections were similarly immunostained with an antibody mix of three anti-pancreatic hormone antibodies (rabbit anti-glucagon antibody-rabbit anti-somatostatin-rabbit anti-pancreatic polypeptide, 1:1:1; all from Nichirei) as the primary antibody. Hematoxylin was used as the counterstain. The image analysis software NIH Image v. 1.61 was used to calculate the total pancreatic tissue area, pancreatic islet area, and insulin-positive and -negative cells in the pancreatic islets to determine the percent pancreatic islet area (relative to the pancreatic area) and the percent β-cell count (relative to the pancreatic islets). Islet mass was estimated by multiplying the average of the ratio of total islet area to total pancreatic area by the pancreatic wet weight.

Experiment 2

The following experiment was performed to characterize the biochemical events occurring during the prevention of pancreatic β-cell dysfunction by drug intervention.

Intervention protocol. Ten-week-old db/db mice were divided into group P (n = 6), treated with pioglitazone (100
mg·kg body wt\(^{-1}\)·day\(^{-1}\), oral), and group \(C (n = 6)\), treated with vehicle only. Ten-week-old \(db/db\) mice were also divided, into group \(LP (n = 6); \) treated with pioglitazone at 100 mg·kg body wt\(^{-1}\)·day\(^{-1}\), oral) and group \(LC (n = 6); \) treated with the vehicle only). Animals underwent 2 wk of drug intervention. Before and after the intervention period, blood was collected by retroorbital venipuncture from the mice under diethyl ether inhalational anesthesia, and the blood glucose level was immediately measured. The plasma was then separated and stored. Frozen at \(-80^\circ\)C until it was assayed for insulin, triglycerides (TG), nonesterified fatty acids (NEFA), and adiponectin. At the end of the intervention, an insulin sensitivity assay was performed, and glucose-stimulated insulin secretion from the isolated pancreatic islets and pancreatic islet TG content assay were analyzed.

Blood biochemical marker measurements. Blood glucose was measured by an enzyme electrode method, using the Dexter-Z system (Bayer Medical, Tokyo, Japan). The insulin level was measured by an ELISA, using an insulin assay kit (Morinaga Institute of Biological Sciences, Yokohama, Japan). The plasma TG level and NEFA level were measured enzymatically with the Triglyceride G-Test Wako and NEFA \(C\)-Test Wako (both from Wako Pure Chemical Industries).

Adiponectin was measured by a Western blot on the basis of the method of Maeda et al. (25). The plasma sample diluted 250-fold with SDS buffer was heat treated at 100°C for 5 min, separated by 12.5% polyacrylamide gel electrophoresis, and blotted onto polyvinylidene difluoride membranes (Bio-Rad). The fluorescent signal was followed by fluorescent light emission using the enhanced chemiluminescence Western blotting detection reagent (Amer sham Biosciences, Piscataway, NJ). The fluorescent reaction was performed at 25°C for 120 min. After a washing, 2,000-fold diluted horseradish peroxidase-labeled anti-rabbit IgG antibody (Cell Signaling Technologies, Beverly, MA) was used as the primary antibody, and the primary antibody reaction was performed at 25°C for 120 min. After a washing, the secondary antibody reaction, followed by fluorescent light emission using the enhanced chemiluminescence Western blotting detection reagent (Amer sham Biosciences, Piscataway, NJ). The fluorescent signal was detected with a Lumino Image Analyzer LAS-1000 plus (Fuji Photo Film, Tokyo, Japan) and semiquantitatively analyzed using Image Gauge v. 3.4 (Fuji Photo Film). Plasma adiponectin concentration was also confirmed by an ELISA kit (Otsuka Pharmaceutical, Tokyo, Japan).

Insulin sensitivity test. To determine sensitivity to insulin, blood glucose levels were measured before and after insulin loading. After an overnight fast, the mice were administered intraperitoneally with human regular insulin at 1 U/kg body wt. Blood was sampled from the tail vein before and 90 min after insulin administration, and the blood glucose level was measured.

Isolation of pancreatic islets. The pancreatic islets were isolated by the collagenase digestion method. The collagenase solution (4 mg/ml collagenase XI; Sigma Chemical, St. Louis, MO) was prepared in HEPES-Krebs buffer (HKB; 119 mM NaCl, 4.75 mM KCl, 2.54 mM CaCl\(_2\), 1.2 mM MgSO\(_4\), 1.2 mM KH\(_2\)PO\(_4\), 5 mM NaHCO\(_3\), 20 mM HEPES, pH 7.4). The mice were anesthetized by diethyl ether inhalation, and after the abdominal cavity was opened, the animals were killed by severing the carotid artery. A 25-gauge needle and syringe were used to inject small amounts of collagenase solution throughout the pancreatic tissue. The pancreatic tissue was then isolated and transferred to a small plastic bag, to which additional collagenase solution was added, and the tissue was shaken continuously at 37°C for 5 min. The pancreatic tissue was transferred to a 50-ml conical tube, completely digested by mixing on a vortex mixer, transferred to a tall glass test tube, and made into a suspension by adding HKB. When allowed to settle, the pancreatic islets sediment to the bottom of the test tube within 90 s. The HKB supernatant containing the pancreatic exocrine cells was removed by pipetting. This operation was repeated three times, and the final pancreatic islet preparation was transferred to a glass dish. Dithizone solution at 0.25 mg/ml in dimethyl sulfoxide (Wako Pure Chemicals) was added, and the pink-staining pancreatic islets were recovered.

Fig. 1. A: during the intervention period, body weight was measured at weekly intervals in experiment 1 (see ANIMALS AND METHODS for experiment details). Group \(P\), \(db/db\) mice treated with 100 mg·kg body wt\(^{-1}\)·day\(^{-1}\) pioglitazone \((\bullet)\); group \(C\), \(db/db\) mice treated with vehicle \((\odot)\); group \(LC\), untreated lean nondiabetic \(db/+\) mice \((\triangle)\); group \(LP\), \(db/+\) mice treated with pioglitazone \((\bigtriangleup)\). Data are means ± SE. *\(P < 0.05\) vs. groups \(LP, LC,\) and \(C\).
by pipetting under an SMZ-1000 stereomicroscope (Nikon, Tokyo, Japan).

Glucose-stimulated insulin secretion from isolated pancreatic islets. Size-matched pancreatic islets were prepared in small test tubes (4 pancreatic islets/tube) and preincubated in a 3 mM glucose solution (HKB containing 5 mg/ml BSA, 37°C, 60 min). The supernatant was replaced with a fresh 3 or 16.7 mM glucose solution, and the mixture was further incubated (37°C, 60 min). On completion of the reaction, the supernatant was recovered and stored at −80°C until analysis for insulin by ELISA.

Measurement of TG content in pancreatic islets. The TG content in pancreatic islets was measured using the method of Lee et al. (22). Isolated pancreatic islets (32–65 pieces) were washed twice in PBS, to which 50 μl of high-salt buffer (2 M NaCl, 2 mM EDTA, 50 mM sodium phosphate) were added, followed by sonication for 1 min to disrupt the pancreatic islets. After centrifugation at 12,000 rpm for 5 min, 10 μl of the supernatant were mixed with 10 μl of r-butanol plus 50 μl of Triton X-100-methyl alcohol (1:1). The TG content in the pancreatic islets was measured using a TG kit (GPO-Trinder, Sigma Diagnostics).

Statistics

All data are expressed as means ± SE. For a comparison of groups, we used ANOVA, and if the difference was significant, Student’s t-test was used to compare two groups. P < 0.05 was considered significant. Statistical analysis was conducted with StatView v. 5 (SAS).

RESULTS

Experiment 1

Changes in body weight and fasting blood glucose level. The db/db mice began to exhibit obesity by 5 wk of age, and body weight increased rapidly with age (Fig. 1A). Eighteen-week-old animals weighed twice as much as the control db/+ mice (50 ± 1 vs. 26 ± 1 g, P < 0.001). The external appearance of the mice in drug intervention group P immediately after intervention was not particularly different from that of the mice in group C. However, group P at 2 wk after the start of the intervention showed an increase in body weight compared with the control group (P < 0.01). At the end of the intervention period, the mean body weight of the animals in
group P was 22% greater than in group C (61 ± 2 vs. 50 ± 1 g, \( P < 0.01 \)).

The fasting blood glucose levels (Fig. 1B) in group P were lower than in group C at week 6 of intervention and beyond. At the end of intervention, the fasting blood glucose level was 554 ± 62 mg/dl in group C and 260 ± 12 mg/dl in group P (\( P < 0.05 \) vs. group C).

Morphological changes in pancreatic islets. Figure 2 shows typical immunostaining patterns of pancreatic islet tissues in the nondiabetic nonobese \( db/+ \) mice and diabetic obese \( db/db \) mice in group C and group P. A comparison of \( db/+ \) mice vs. \( db/db \) mice indicated that the latter animals have generally hypertrophied pancreatic islets, of various sizes and morphology, and a mixture of \( \beta \)-cells and non-\( \beta \)-cells in the islets. In groups LC, P, and LP, \( \beta \)-cells occupied most parts of the islets and existed in the center, whereas non-\( \beta \)-cells existed peripherally. There was no overlap of \( \beta \)-cells and non-\( \beta \)-cells. There was a slight but not significant increase in the number of islets in a slice obtained from a pancreas in rats treated with pioglitazone in \( db/db \) mice (group P at 12 wk, 32.4 ± 4.9; group C at 12 wk, 29.3 ± 2.4; group P at 16 wk, 23.7 ± 3.8; group C at 16 wk, 16.7 ± 3.1; \( n = 3 \) in each group).

The islet mass in untreated \( db/+ \) mice was 1.32 ± 0.21 mg in the 12-wk-old animals and 1.83 ± 0.11 mg in the 18-wk-old animals, indicating a trend toward an increase with age (Fig. 3). On the other hand, the islet mass in the \( db/db \) mice (group C) was 6.46 ± 0.49 mg at 12 and 4.64 ± 0.24 mg at 18 wk of age, indicating a significant decrease with age (\( P < 0.01 \)). The islet mass in the \( db/db \) mice was significantly higher than that in the nonobese \( db/+ \) mice during the experiment period (all \( P < 0.001 \)). The islet masses in the pioglitazone-treated animals (group P) were 14.6 ± 10.2 mg at 12 wk and 10.2 ± 1.1 mg at 18 wk of age and significantly larger compared with those of animals of the same age in group C (all \( P < 0.001 \)).

The ratio of \( \beta \)-cells to total islet cells in a pancreatic islet in the 12-wk-old animals appeared to be greater in the \( db/db \) mice (82.2 ± 12% in group C and 82.8 ± 0.9% in group P) than in untreated \( db/+ \) mice (76.4 ± 3.4%) but was not statistically significant (Table 1). Among the 18-wk-old animals, the \( \beta \)-cell ratio was 77.8 ± 3.6% in the \( db/+ \) mice (group LC), 73.4 ± 2.2% in group C, and 80.6 ± 12% in group P, so that group C showed a significant decrease in the \( \beta \)-cell ratio with age (\( P < 0.01 \)). Among the 18-wk-old animals, comparisons among the groups showed that the \( \beta \)-cell ratio of group P was significantly greater than that of group C (\( P < 0.01 \)).

There were no significant differences in the islet mass and the ratio of \( \beta \)-cells to total islet cells in a pancreatic islet between group LC and group LP (Fig. 3 and Table 1).

Experiment 2

Blood biochemistry markers. The preintervention (10-wk-old animals) and postintervention (12-wk-old animals) blood glucose level and plasma levels of insulin, TG, NEFA, and adiponectin at the fasted state were compared (Figs. 4 and 5). The 10-wk-old (preintervention) \( db/db \) mice had a fasting blood glucose level of 223 ± 27 mg/dl, whereas the 12-wk-old animals in group C had a level of 256 ± 25 mg/dl, indicating an increasing trend [not significant (NS) vs. preintervention]. On the other hand, 12-wk-old animals in group P had a fasting blood glucose level of 198 ± 27 mg/dl (NS vs. preintervention or group C).

The plasma insulin level was 3.5 ± 0.4 ng/ml in 10-wk-old animals and 3.3 ± 0.4 ng/ml in group C and 2.8 ± 0.6 ng/ml in group P after 2 wk. The baseline plasma TG level was 119 ± 6 mg/dl and increased to 171 ± 15 mg/dl in group C after 2 wk (\( P = 0.003 \)). Pioglitazone restored the TG level (117 ± 23 mg/dl, \( P < 0.01 \) vs. group C). The baseline plasma NEFA levels were 1.5 ± 0.1 and 1.9 ± 0.2 meq/l in group C after 2 wk. Pioglitazone significantly lowered the NEFA level (1.3 ± 0.2 meq/l, \( P < 0.05 \) vs. group C).

When the plasma adiponectin level in 10-wk-old animals was taken as 100% in Western analysis, this level was 61 ± 7% in 12-wk-old group C animals and 226 ± 20% in 12-wk-old group P animals. The plasma adiponectin levels were confirmed by ELISA (65.2 ± 18.0 in group P vs. 18.3 ± 1.3

<p>| Table 1. ( \beta )-Cells per islet |</p>
<table>
<thead>
<tr>
<th>Groups</th>
<th>Week 12</th>
<th>Week 18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group C</td>
<td>82.2±1.2</td>
<td>73.4±2.2</td>
</tr>
<tr>
<td>Group P</td>
<td>82.8±0.9</td>
<td>80.6±1.2*</td>
</tr>
<tr>
<td>Group LC</td>
<td>76.4±3.4</td>
<td>77.8±3.6</td>
</tr>
<tr>
<td>Group LP</td>
<td>78.6±1.8</td>
<td>81.5±1.3</td>
</tr>
</tbody>
</table>

Values are means ± SE. See ANIMALS AND METHODS for group details. *\( P < 0.01 \) vs. group C.
μg/ml in group C, P < 0.05; Fig. 5). The adiponectin level appeared to be higher in group LP at 12 wk compared with group LC, but the difference was not statistically significant (28.4 ± 9.0 vs. 20.0 ± 2.6 μg/ml, NS).

Insulin sensitivity. The blood glucose level at 90 min after insulin injection (1 IU/kg body wt) compared with the baseline blood glucose level (198 ± 94 mg/dl in group P and 256 ± 60 mg/dl in group C) was 46 ± 4% in group P and 102 ± 8% in group C (P < 0.01; Fig. 6).

Insulin secretion in response to glucose in isolated pancreatic islets. With the addition of 3 mM glucose, the basal insulin secretion rate showed no difference between group C and group P. With the addition of glucose at a high concentration of 16.7 mM, insulin secretion was higher in group P than in group C. Within each group, when compared against 3 mM glucose, secretion was increased 8.5-fold in group C and 18-fold in group P (Table 2). The islet responses to glucose were not different between group LP and group LC.

TG content in isolated pancreatic islets. The baseline TG content in the pancreatic islet was 58.9 ± 7.3 ng/islet. This content slightly increased in group C (65.6 ± 7.6 ng/islet) after 2 wk, but the intervention with pioglitazone significantly lowered the islet TG level (43.3 ± 3.6 ng/islet, P < 0.05 vs. group C; Fig. 7).

**DISCUSSION**

In this study C57BL/KsJ db/db mice, an animal model of obese diabetes mellitus caused by overeating, inappropriate insulin secretion, and hyperglycemia, were treated with pioglitazone for 12 wk starting at 6 wk of age, when blood glucose elevation begins to become apparent, to study prevention of the progression of impaired pancreatic β-cell function. The results indicate that, in untreated db/db mice, the islet area per pancreas and β-cell ratio to total islet cells in the islet decreased over time, with obvious heterogeneity in the sizes of the islets and a decrease overall. The present study clearly demonstrated that a deranged morphology of pancreatic islet was significantly preserved by an intervention with pioglitazone initiated at an early stage of the disease pathogenesis of diabetes mellitus. On the other hand, the islet morphology and its parameters in nondiabetic db/+ mice were not altered by pioglitazone treatment, indicating no evident effect of this drug on the pancreatic β-cell proliferation. Thus our results sug-
effects of this drug on the development of impaired β-cell loss but not to stimulation of cell growth. The preventive effect of pioglitazone on the islet morphology in db/db mice was probably due to reduction of β-cell loss but not to stimulation of cell growth. The preventive effects of this drug on the development of impaired β-cell function were confirmed by a glucose-stimulated insulin secretion test using isolated islets. It is known that troglitazone preserved pancreatic β-cell function and prevented the incidence of type 2 diabetes in subjects with insulin resistance (2), and pioglitazone improved insulin secretion as well as insulin sensitivity (27). Thus our results may support, theoretically, clinical evidence for the anti-diabetic effects of thiazolidinediones.

Finegood et al. (10) reported that an intervention of rosiglitazone in Zucker diabetic fatty rats enabled improvement of glucotoxicity and insulin resistance. In addition, because thiazolidinediones increase the fat weight significantly in db/db mice (32), it is crucial to determine TG content in pancreatic islets. Increased plasma fatty acids and TG content in pancreatic islets in untreated db/db mice were significantly reduced by treatment with pioglitazone, suggesting the involvement of lipotoxicity even in an early stage of the disease pathogenesis of diabetes mellitus in db/db mice.

Table 2. Glucose-stimulated insulin secretion

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>3.0 mM Glucose</th>
<th>16.7 mM Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group C</td>
<td>4</td>
<td>0.19±0.05</td>
<td>1.61±0.31†</td>
</tr>
<tr>
<td>Group P</td>
<td>4</td>
<td>0.23±0.08</td>
<td>4.14±0.87†</td>
</tr>
<tr>
<td>Group LC</td>
<td>4</td>
<td>0.73±0.20</td>
<td>6.24±0.54†</td>
</tr>
<tr>
<td>Group LP</td>
<td>4</td>
<td>0.56±0.15</td>
<td>8.70±2.47†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of mice. See ANIMALS AND METHODS for group details. *P < 0.001 vs. 3.0 mM glucose. †P < 0.05 vs. group C.

With respect to a mechanism of pioglitazone effects on the preservation of pancreatic β-cell morphology and function in the db/db mice, several hypotheses are suggested, as follows: reduction of glucose toxicity and lipid toxicity (glucolipotoxicity), reduction of β-cell overwork due to decreasing insulin resistance, an indirect mechanism on islet cells via modulation of plasma adipocytokine levels, and a direct effect via PPAR receptors in the islet cells. In general, glucolipotoxicity has attracted attention as a mechanism of pancreatic β-cell dysfunction in diabetes mellitus (29, 37). The present study clearly showed that pioglitazone decreased fasted plasma glucose level and increased insulin sensitivity in the db/db mice, suggesting improvement of glucotoxicity and insulin resistance. In addition, because thiazolidinediones increase the fat weight significantly in db/db mice (32), it is crucial to determine TG content in pancreatic islets. Increased plasma fatty acids and TG content in pancreatic islets in untreated db/db mice were significantly reduced by treatment with pioglitazone, suggesting the involvement of lipotoxicity even in an early stage of the disease pathogenesis of diabetes mellitus in db/db mice.
have been several reports that troglitazone causes a decrease in TG and NEFA levels in the pancreatic islets (13, 30). The extent of impaired insulin secretion has been reported to be related to the TG content in pancreatic islets (31). However, the relationship between lipotoxicity and impaired insulin secretion is still not fully understood.

In this study, pioglitazone caused a significant increase in the plasma adiponectin level. The physiological function of adiponectin in the pancreatic islets remains poorly understood. Recent reports have demonstrated that adiponectin is a novel adipocytokine with antidiabetic and antiatherogenic effects (6, 11, 14, 15, 36). Very recently, the significant expression of mRNAs for the adiponectin receptors AdipoR1 and AdipoR2 in human and rat pancreatic β-cells was shown (18). Although the role of adiponectin in the pancreatic islet is not known at all, the existence of receptors suggests that pioglitazone may act on the pancreatic β-cells through regulation of the plasma level of this adipocytokine.

For investigative approaches aimed at preventing the progression of type 2 diabetes mellitus, the question of how to preserve the pancreatic β-cell function is one of the most important topics. Large-scale clinical trials that sought to prevent type 2 diabetes mellitus showed that drug intervention before the onset of the disease can retard the worsening of glucose tolerance (1, 4, 19). It is anticipated that early drug intervention to prevent the progression of type 2 diabetes mellitus might become important in the future. The current study employed an animal model of obese type 2 diabetes (db/db mice) to investigate the effect of intervention with pioglitazone on the morphological and functional changes during the early stage of progression of diabetes associated with the development of pancreatic β-cell dysfunction, and demonstrated that pioglitazone prevented morphological damage in the pancreatic islets and preserved β-cell function. Thus the present findings provide theoretical support for evidence observed in clinical studies.

ACKNOWLEDGMENTS

We thank Yuii Matsuzawa, Department of Internal Medicine and Molecular Science, Graduate School of Medicine, Osaka University (Osaka, Japan), for the gift of the anti-adiponecin antibody; Ichiro Niki, Department of Pharmacology, Oita Medical University (Oita, Japan), for instructions on the isolation of pancreatic islets; and Takeda Chemical Industries (Osaka, Japan) for the gift of pioglitazone.

A portion of this article was presented at the 44th Annual Meeting of the Japan Diabetes Society (April, 2001, Kyoto, Japan), the 45th Annual Meeting of the Japan Diabetes Society (May, 2002, Tokyo, Japan), the 37th Annual Meeting of the European Association for the Study of Diabetes (September, 2001, Glasgow, UK), and the 38th Annual Meeting of the European Association for the Study of Diabetes (September, 2002, Budapest, Hungary).

GRANTS

This work was supported by a Grant-in-Aid for Scientific Research (principal investigator K. Kaku; no. 15590962) from the Japan Society for the Promotion of Science and Research Project Grants (principal investigator K. Kaku; nos. 11-403, 12-506, 12-507, 13-503, 13-507) from Kawasaki Medical School.

REFERENCES


