Pioglitazone preserves pancreatic islet structure and insulin secretory function in three murine models of type 2 diabetes

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Pioglitazone preserves pancreatic islet structure and insulin secretory function in three murine models of type 2 diabetes. Am J Physiol Endocrinol Metab 286: E116–E122, 2004. First published October 7, 2003; 10.1152/ajpendo.00331.2003.—Thiazolidinediones may slow the progression of type 2 diabetes by preserving pancreatic β-cells. The effects of pioglitazone (PIO) on structure and function of β-cells in KKA(y), C57BL/6j ob/ob, and C57BL/KsJ db/db mice (genetic models of type 2 diabetes) were examined. ob/ob (n = 7) and db/db (n = 9) mice were randomly assigned to 50–125 mg·kg body wt⁻¹·day⁻¹ of PIO in chow beginning at 6–10 wk of age. Control ob/ob (n = 7) and db/db mice (n = 9) were fed chow without PIO. KKA(y) mice (n = 15) were fed PIO daily at doses of 62–144 mg·kg body wt⁻¹·day⁻¹. Control KKA(y) mice (n = 10) received chow without PIO. Treatment continued until euthanasia at 14–26 wk of age. Blood was collected at baseline (before treatment) and just before euthanasia and was analyzed for glucose, glycated hemoglobin, and plasma insulin. Some of the splenic pancreas of each animal was resected and partially sectioned for light or electron microscopy. The remainder of the pancreas was assayed for insulin content. Compared with baseline and control groups, PIO treatment significantly reduced blood glucose and glycated hemoglobin levels. Plasma insulin levels decreased significantly in ob/ob mice treated with PIO. All groups treated with PIO exhibited significantly greater β-cell granulation, evidence of reduced β-cell stress, and 1.5- to 15-fold higher levels of pancreatic insulin. The data from these studies suggest that comparable effects would be expected to slow the progression of type 2 diabetes, either delaying or possibly preventing progression to an insulin-dependent state.

β-cells; animal models; thiazolidinediones

The natural history of type 2 diabetes mellitus is characterized by progression of disease severity, eventually leading to reliance on exogenous insulin (38). In the earliest stages of disease, patients generally exhibit reduced sensitivity to insulin; this condition commonly progresses to lower glucose disposal and impaired glucose tolerance, eventually leading to clinically overt type 2 diabetes (27). However, progression of disease does not stop with the diagnosis of overt diabetes. In the United Kingdom Prospective Diabetes Study (UKPDS), patients receiving intensive treatment with either insulin or sulphonylureas continued to experience worsening of their glycemic control (28, 44). After 3 yr, ~50% of patients could maintain a glycosylated hemoglobin level below 7% with oral drug monotherapy, and by 9 yr, this declined to 25% (29, 43).

At the time of initial diagnosis, most patients with type 2 diabetes have high levels of fasting and postprandial plasma insulin, indicating that their pancreatic β-cells are still producing the hormone in an attempt to overcome peripheral insulin resistance. As the disease progresses, most patients eventually exhibit evidence of β-cell dysfunction, often leading to nearly complete loss of insulin secretion. Indeed, individuals with type 2 diabetes have fewer β-cells on average at death compared with nondiabetic individuals (6). Although dysfunction of the β-cells may contribute to the underlying causes of disease, the continuing demand for high insulin output, resulting from insulin resistance of peripheral tissues and from hyperglycemia, may also contribute to the decline in function of β-cells (6, 9, 20, 44). If this is the case, improvements in peripheral insulin sensitivity would be expected to preserve β-cell function. Indeed, clinical studies of peroxisome proliferator-activated receptor-γ (PPARγ) agonists have demonstrated that treatment with these agents is associated with improvements in β-cell function, as determined by homeostatic model assessment (HOMA) (14, 28, 30).

Studies in diabetic rats have shown that the acute insulin response to a glucose challenge is improved in animals receiving pioglitazone (PIO) (12). Furthermore, progressive structural and ultrastructural changes to pancreatic islets, and to β-cells in particular, in diabetic animals are slowed or prevented by treatment with Rosiglitazone, troglitazone, or PIO in the early stages of disease (16, 18, 39, 45). In some cases, these effects were associated with a lower likelihood of progression from a prediabetic to an overtly diabetic state. The prevention of β-cell degeneration by thiazolidinediones is linked to their anti-inflammatory action against glucose-mediated IL-1β and to their ability to decrease islet triglyceride content, which, if left untreated, in turn generates nitric oxide, leading to both diminished islet cell viability and mitochondrial biogenesis (26, 33, 39).

Type 2 diabetes has a complex etiology involving multiple genetic and environmental factors; because of this complexity, we chose to study PIO in three strains of inbred mice with different genetic mutations that lead to insulin resistance and diabetes. Mice with the ob/ob mutation are obese, hyperinsulinemic, and hyperglycemic (9). The ob gene encodes for the leptin protein (35). The db/db mutation confers a similar but more severe phenotype (9), and the db gene encodes for the leptin receptor (8). The KKA(y) mouse, another genetic model, carries the yellow obese and diabetes genes. KKA(y) mice exhibit early-onset hyperinsulinemia and hyperglycemia (19). Even with their differing genetic backgrounds, each of these genetic models of type 2 diabetes has been shown to exhibit
similar pathological changes to pancreatic islets and to β-cells in particular (3, 10, 13, 23, 24, 41). In addition, islets have an altered ability to secrete and store insulin. In an attempt to make our results as applicable to human disease as possible, we used these three strains of mice to study the effects of the PPARγ agonist PIO on the structure and function of pancreatic islets.

MATERIALS AND METHODS

Experiment 1. Fourteen male C57BL/6J-ob/ob mice and 18 male C57BL/KsJ-db/db mice (all animals 4 wk old) were purchased from Jackson Laboratories (Bar Harbor, ME), housed individually in a metabolic cage under a 12:12-light-dark cycle commencing at 6:00 AM, and administered Purina Mouse Chow no. 50-15 and water ad libitum. At 6 wk of age, one-half of the animals from each strain were randomly chosen to receive PIO, which was incorporated into mouse chow. The other one-half (controls) continued to receive mouse chow without the drug. The dosage of PIO for the ob/ob mice was gradually increased to 50 mg/kg body wt \(^{-1}\)day \(^{-1}\) and maintained at that level for the last 55 days of the study. For the db/db mice, the dosage was gradually increased to 125 mg/kg body wt \(^{-1}\) day \(^{-1}\), then decreased to 110 mg/kg body wt \(^{-1}\) day \(^{-1}\) (for reasons of hypoglycemia), and maintained for the last 42 days of the study. Dosages of PIO were derived from previous dose-ranging studies in ob/ob and db/db mice. The mice were killed at 8 wk (db/db mice) or 11 wk (ob/ob mice) after they were assigned to a treatment group (ages 14 and 17 wk, respectively).

Experiment 2. Twenty-five male KKA(y) mice (7–10 wk old) from a colony maintained at Upjohn Pharmaceutical Research and Development (Kalamazoo, MI) were divided into three groups and assigned to receive no treatment \((n = 10)\), low-dose PIO (average dose of 142 mg/kg body wt \(^{-1}\) day \(^{-1}\) for the first 3 days, 96 mg/kg body wt \(^{-1}\) day \(^{-1}\) for the next 6 days, and 62 mg/kg body wt \(^{-1}\) day \(^{-1}\) for the remainder of the study; \(n = 10\)) or high-dose PIO (average dose of 144 mg/kg body wt \(^{-1}\) day \(^{-1}\) for the first 7 days and 115 mg/kg body wt \(^{-1}\) day \(^{-1}\) for the duration of the study; \(n = 5\)). These low and high doses were derived from previous dose-ranging studies in KKA(y) mice. High-dose PIO and low-dose PIO were incorporated into the mouse chow. Control KKA(y) mice received chow without PIO. Animals were killed at 26 wk of age. All animal protocols were reviewed and approved by the animal rights committee at Upjohn Laboratories.

Glucose and glycosylated hemoglobin. Whole blood samples were obtained from the periorbital venous plexus in all fasted animals both before treatment allocation and after they were killed. Samples were analyzed for blood glucose by using a fluorometer and autoanalyzer, as previously described (25). Total glycosylated hemoglobin was measured in columns obtained from Isolab (Akron, OH). Glycosylated hemoglobin levels are ≤5% in normal mice (11).

Serum and pancreatic insulin determinations. The pancreases of each animal was removed and the splenic region used for histological analysis. The remainder of the pancreas was assayed to determine insulin content. Extraction and assay of insulin were conducted on the remainder of the pancreas as previously described (7). Plasma insulin was measured by radioimmunoassay (46).

Light and electron microscopy. For light microscopy, part of the splenic region of the pancreas was fixed in Bouin’s solution and embedded in paraffin blocks. The blocks were serially sectioned at a thickness of 6 μm by using an American Optical rotary microtome. Sections were adhered to slides coated with poly-l-lysine and then stained with Gomori’s aldehyde-fuchsin. Slides were examined under bright-field illumination by use of a Zeiss Photomicroscope III. The staining section of highest quality on each slide was chosen for analysis on the basis of lack of artifacts and staining. Each islet in that section was examined by an observer who was unaware of the animal’s treatment allocation. Each islet was given a score between 1 (least) and 4 (greatest) for β-cell granulation observed with aldehyde-fuchsin staining. The sections from db/db and KKA(y) mice were also examined for the presence of exocrine cells within the islets.

For electron microscopy, portions of the splenic region of each pancreas were minced into 1-mm cubes, fixed (2% paraformaldehyde and 2.5% glutaraldehyde in 0.04 M cacodylate buffer), postfixed in 1% osmium tetroxide, and rinsed in 0.02 M cacodylate buffer. They were then dehydrated in ascending ethanols and embedded in Polybed 812 resin. The resin blocks were assigned code numbers so they could be examined and scored by an observer who was blinded to the treatment allocation. The blocks were then thick-sectioned at 1 μm, stained with toluidine blue, and examined under bright-field light microscopy for the presence of islets. Two islets from two different resin blocks were identified from each animal. Ultrathin sections (80–90 nm) of each islet were cut with an LKB Nova ultramicrotome, collected on 50-mesh nitrocellulose-coated grids, and conventionally stained with uranyl acetate and lead citrate. Each islet was examined by using a JEOL 1200 EX electron microscope and photographed (×5,000).

Statistics. Metabolic and morphological data are expressed as means ± SD. Differences in parameters were analyzed with the Wilcoxon rank sum test and were considered significant when \(P < 0.05\).

RESULTS

Two control db/db mice and one control KKA(y) mouse died during the course of the study. Values from these animals are included in baseline averages but not in terminal averages.

Glycemic control and plasma insulin levels. Mean blood glucose, glycosylated hemoglobin, and plasma insulin values are shown in Table 1 for ob/ob and db/db mice and in Table 2 for the three groups of KKA(y) mice. Animals receiving PIO had significantly lower blood glucose and glycosylated hemoglobin levels at the termination of the study compared with nontreated diabetic animals. Three of the eight PIO-treated db/db mice displayed high blood glucose and glycosylated hemoglobin values at termination. These mice were considered refractory to PIO treatment and increased the variability of the data. Plasma insulin levels exhibited a more complex behavior. In nontreated ob/ob mice, plasma insulin levels increased nearly 10-fold from baseline. In PIO-treated ob/ob mice, plasma insulin levels did not rise and were significantly lower than in nontreated animals at termination. In nontreated db/db mice, plasma insulin levels decreased almost ninefold from baseline. Plasma insulin levels decreased about fourfold from baseline. Nontreated NA 8.0 NA 13.6 NA 26.2 517.3 2483.4 56 = 26

Table 1. Metabolic parameters in ob/ob and db/db mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ob/ob Mice</th>
<th>db/db Mice</th>
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<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Terminal</td>
</tr>
<tr>
<td></td>
<td>(n = 10)</td>
<td>(n = 10)</td>
</tr>
<tr>
<td>Blood glucose, mg/dl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nontreated</td>
<td>313 ± 99</td>
<td>181 ± 59</td>
</tr>
<tr>
<td>PIO</td>
<td>316 ± 91</td>
<td>120 ± 15*</td>
</tr>
<tr>
<td>Glycosylated Hb A1c, %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nontreated</td>
<td>NA</td>
<td>8.0 ± 0.8</td>
</tr>
<tr>
<td>PIO</td>
<td>NA</td>
<td>4.4 ± 0.1‡</td>
</tr>
<tr>
<td>Plasma insulin, μU/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nontreated</td>
<td>583 ± 262</td>
<td>517.3 ± 2483.4</td>
</tr>
<tr>
<td>PIO</td>
<td>552 ± 347</td>
<td>249 ± 137‡</td>
</tr>
</tbody>
</table>

Values are means ± SD; \(n\), no. of mice/group; PIO, pioglitazone; NA, not available or not measured. *\(P < 0.05\); †\(P < 0.005\); ‡\(P < 0.001\) vs. nontreated mice.
baseline in PIO-treated \(db/db\) mice. At termination, plasma insulin levels were significantly higher in PIO-treated compared with nontreated \(db/db\) mice. In the KKA(\(y\)) mice, plasma insulin levels increased approximately fivefold from baseline in untreated and low- and high-dose PIO groups. At termination, plasma insulin levels of both PIO groups were unchanged compared with nontreated KKA(\(y\)) mice.

**Islet morphology and pancreatic insulin content.** Diabetes is associated with characteristic and progressive changes in the structure of pancreatic islets. Such changes include depletion of insulin-containing secretory granules in \(\beta\)-cells, loss of definition of the islet boundary, and displacement of exocrine cells into the islet tissue. We used light microscopy to examine the \(\beta\)-cell granulation and exocrine cell displacement in islets isolated from the splenic region of the pancreas of our experimental animals. In addition, the remainder of the pancreas was used for biochemical measurements of insulin content.

\(\beta\)-Cell granulation was scored on a scale of 1 (lowest) to 4 (highest) by an observer who was unaware of treatment allocation. A mean \(\beta\)-cell granulation score was obtained for each animal on the basis of all of the islets scored for that animal. Mean \(\beta\)-cell granulation scores and pancreatic insulin content for each group of animals are shown in Table 3. All three strains exhibited significantly higher \(\beta\)-cell granulation scores and levels of pancreatic insulin when treated with PIO compared with those not treated. Overall, these results support the conclusion that PIO treatment reduced the demand for insulin secretion, leading to an increase in the amount of insulin contained in secretory granules within the \(\beta\)-cells. Representative aldehyde-fuchsin-stained sections from nontreated and treated \(ob/ob\) and \(db/db\) mice are shown in Figs. 1 and 2, respectively.

### Table 3. \(\beta\)-Cell granulation scores

<table>
<thead>
<tr>
<th>Group</th>
<th>(\beta)-Cell Granulation</th>
<th>Pancreatic Insulin Content, (\mu)g</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Score, Scale of 1–4</td>
<td></td>
</tr>
<tr>
<td>(ob/ob) Mice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nontreated</td>
<td>1.3±0.4</td>
<td>8.9±5.8</td>
</tr>
<tr>
<td>PIO</td>
<td>3.6±0.3*</td>
<td>14.9±6.6†</td>
</tr>
<tr>
<td>(db/db) Mice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nontreated</td>
<td>1.1±0.2</td>
<td>0.4±0.2</td>
</tr>
<tr>
<td>PIO</td>
<td>2.4±1.1†</td>
<td>6.2±5.3†</td>
</tr>
<tr>
<td>KKA((y)) mice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1 (nontreated)</td>
<td>2.2±0.4</td>
<td>5.1±2.3</td>
</tr>
<tr>
<td>Group 2 (low-dose PIO)</td>
<td>3.5±0.5*</td>
<td>19.7±10.3*</td>
</tr>
<tr>
<td>Group 3 (high-dose PIO)</td>
<td>3.3±0.6†</td>
<td>18.5±7.2*</td>
</tr>
</tbody>
</table>

Values are means ± SD. *\(P < 0.001\) vs. group 1.

Islet integrity and morphology were assessed in \(db/db\) and KKA(\(y\)) mice by quantifying the displacement of exocrine cells into the islets at termination of the study. The percentage of islets with exocrine cell displacement was measured as the number of islets with exocrine cell displacement divided by the total number of islets (with and without exocrine cell displacement) examined per animal. The aldehyde-fuchsin-stained sections were used to measure exocrine cell displacement. When expressed as percentages of exocrine cell displacement, the differences between nontreated and PIO-treated animals were not significant. However, only four of nine PIO-treated \(db/db\) mice showed exocrine cell displacement, whereas all six of the nontreated animals showed such evidence. In the KKA(\(y\)) mice, five of the nine nontreated animals exhibited exocrine cell displacement, but only one of 10 animals in the low-dose PIO group and one of 5 in the high-dose PIO group exhibited this effect.

**Ultrastructure of \(\beta\)-cells.** Previous studies have also described ultrastructural changes within \(\beta\)-cells that are associated with diabetes (5, 18, 24, 40). These changes include proliferation and infrequent hypertrophy of mitochondria, hypertrophy of Golgi complexes, and reduction of the number of dense-core vesicles (most likely insulin secretory granules). We have observed similar derangements in the \(\beta\)-cells of control \(ob/ob\) and \(db/db\) mice (Fig. 3, A and B, respectively). \(\beta\)-Cells from these animals showed evidence of stress induced by demands to synthesize and secrete insulin. Such evidence included proliferative Golgi apparatus and rough endoplasmic reticulum, depletion of secretory granules, and infrequent hypertrophy of mitochondria. In the \(\beta\)-cells of control \(db/db\) mice (Fig. 3B), aggregates of lysosomes were also observed, a common feature of cells in the early stages of necrosis. \(\beta\)-Cells from PIO-treated animals exhibited much less evidence of stress (Fig. 3, C and D). In both \(ob/ob\) and \(db/db\) mice that received PIO treatment, \(\beta\)-cells were more densely granulated and had no lysosomes indicative of necrosis. PIO-treated cells from \(ob/ob\) mice had unremarkable organelles, except for rare cases showing modest proliferation of Golgi bodies or endoplasmic reticulum. These effects were more common in \(\beta\)-cells from treated \(db/db\) mice, although \(\beta\)-cells from these animals were well granulated.

**DISCUSSION**

Previous studies have shown that PPAR\(\gamma\) agonists can slow or prevent the degeneration of pancreatic islets and \(\beta\)-cells, in part by reducing the triglyceride content that accompanies type 2 diabetes in specific animal models of the disease (5, 16, 18, 39, 40). We have extended those studies to include another PPAR\(\gamma\) agonist, PIO, and have shown that the protective effects of this agent are evident in three strains of mice, each
with different genetic defects leading to disease. In all three strains, therapy with PIO reduced levels of blood glucose and glycosylated hemoglobin (Tables 1 and 2), increased pancreatic insulin content (Table 3), preserved islet structure (Figs. 1–3), and restored granulation and alleviated stress of β-cells (Figs. 1–3).

The effects of prolonged PIO therapy on plasma insulin levels were not directly related to changes in glucose and glycosylated hemoglobin. In our studies, diabetic ob/ob mice exhibited hyperinsulinemia that was reduced by PIO treatment. Plasma insulin levels decreased from baseline in both non-treated and PIO-treated db/db mice due to progressive loss of β-cells in this model. However, PIO-treated db/db mice displayed significantly higher plasma insulin levels at termination. This finding suggests that PIO treatment may have attenuated death of β-cells in the db/db mouse. PIO did not significantly lower terminal plasma insulin levels in KKA(y) mice, although both glucose and glycosylated hemoglobin levels were significantly reduced. These incongruent plasma insulin data suggest that PIO may have a different mechanism of action in ob/ob, db/db, and KKA(y) mice. In all three strains of diabetic mice, PIO treatment was associated with significant increases in insulin content and with preservation of islet and β-cell architecture and granulation. Similar results in morphology and granulation have been observed in db/db mice treated with PIO.

Fig. 1. A: pancreatic islet from a nontreated ob/ob mouse. Note the severe degranulation of most β-cells in this islet. Aldehyde-fuchsin, ×330. B: pancreatic islet from an ob/ob mouse treated with pioglitazone (PIO). Marked regranulation of β-cells is observed in this islet compared with that in A. Aldehyde-fuchsin, ×330.

Fig. 2. A: pancreatic islet from a nontreated db/db mouse. Note the sparsely granulated β-cells in the islet. Aldehyde-fuchsin, ×330. B: pancreatic islet from a nontreated db/db mouse. A combination of degranulated endocrine (E arrowhead) and heavily granulated acinar (A arrowhead) cells are located within the degenerate islet. The presence of isolated, invasive acinar cells among the endocrine cells suggests that this is a degenerate islet. Arrowheads indicate the probable outline of the islet. Aldehyde-fuchsin, ×330. C: pancreatic islet from a db/db mouse treated with PIO. Note the pronounced regranulation of the β-cells and the unremarkable morphology of this islet compared with those of A and B. Unstained cells (U arrowhead) around the periphery of this islet are probably glucagon, somatostatin, and pancreatic polypeptide cells. Aldehyde-fuchsin, ×330.
as well as in other animal models of diabetes with rosiglitazone (5, 15, 42) and troglitazone (18, 39, 40).

Before the widespread use of PPAR agonists, most patients with type 2 diabetes eventually failed to respond adequately to oral antidiabetic agents (sulfonylureas or metformin). For example, the UKPDS found that 40–50% of patients receiving a sulfonylurea required an additional therapy after 6 years (29). This decline in efficacy is accompanied by gradual increases in blood glucose and glycosylated hemoglobin levels and by declines in β-cell function (6, 44). Although the mechanisms responsible for decline of β-cell function in type 2 diabetes are still being debated, it has been recognized that preservation of β-cell function through decreased apoptosis is possible and is critical for slowing or halting the progression of disease (6, 28).

In addition to improving β-cell function, as determined with HOMA analysis (15, 22, 36), PPARγ agonists also improve numerous other factors associated with declines in β-cell function, such as hyperglycemia (1, 15, 22, 36), serum triglycerides (21, 36, 37), serum free fatty acids (17, 30, 31), and intra-abdominal and hepatic fat content (2, 32). The present study, along with previous studies of other PPARγ agonists, directly demonstrates that these agents preserve β-cell and islet structure and function in animal models. Our data also suggest that this effect appears to be independent of any reduction in plasma insulin concentration. Because human islets contain PPARγ receptors similar to those found in rodent islets, it is likely that longer-term clinical studies with PPARγ agonists may show improved β-cell preservation in humans through a reduction in β-cell apoptosis (4, 6, 14). These effects of the PPARγ agonists are likely to have important clinical implications for slowing the rate and severity of disease progression in patients with type 2 diabetes.

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DISCLOSURES

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M. Khan is an employee of Takeda Pharmaceuticals North America (TPNA) Inc. F. T. Murray was an employee of TPNA when this manuscript was written.

REFERENCES


