Peroxisome Proliferator-Activated Receptor γ Activation Restores Islet Function in Diabetic Mice through Reduction of Endoplasmic Reticulum Stress and Maintenance of Euchromatin Structure

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The nuclear receptor peroxisome proliferator-activated receptor γ (PPAR-γ) is an important target in diabetes therapy, but its direct role, if any, in the restoration of islet function has remained controversial. To identify potential molecular mechanisms of PPAR-γ in the islet, we treated diabetic or glucose-intolerant mice with the PPAR-γ agonist pioglitazone or with a control. Treated mice exhibited significantly improved glycem control, corresponding to increased serum insulin and enhanced glucose-stimulated insulin release and Ca2+ responses from isolated islets in vitro. This improved islet function was at least partially attributed to significant upregulation of the islet genes Irx1, SERCA, Ins1/2, and Glut2 in treated animals. The restoration of the Ins1/2 and Glut2 genes corresponded to a two- to threefold increase in the euchromatin marker histone H3 dimethyl-Lys4 at their respective promoters and was coincident with increased nuclear occupancy of the islet methyltransferase Set7/9. Analysis of diabetic islets in vitro suggested that these effects resulting from the presence of the PPAR-γ agonist may be secondary to improvements in endoplasmic reticulum stress. Consistent with this possibility, incubation of thapsigargin-treated INS-1 β cells with the PPAR-γ agonist resulted in the reduction of endoplasmic reticulum stress and restoration of Pdx1 protein levels and Set7/9 nuclear occupancy. We conclude that PPAR-γ agonists exert a direct effect in diabetic islets to reduce endoplasmic reticulum stress and enhance Pdx1 levels, leading to favorable alterations of the islet gene chromatin architecture.

Type 2 diabetes mellitus results from a combination of insulin resistance and progressive islet dysfunction (46). In many individuals, β-cell failure may precede the clinical diagnosis of diabetes, and landmark studies such as the United Kingdom Prospective Diabetes Study have shown a continued decrement in β-cell function despite treatment intervention with sulfonylureas, metformin, and insulin (52). Thiazolidinediones are orally active agents used in the treatment of type 2 diabetes that act as agonists for the nuclear transcription factor peroxisome proliferator-activated receptor γ (PPAR-γ) (60). Although thiazolidinediones are classically thought to act as peripheral insulin sensitizers, there is growing evidence from studies of human and animal models that these agents may also act to preserve and/or enhance β-cell function in the setting of progressive type 2 diabetes and insulin resistance (3, 12). PPAR-γ is known to be expressed in the pancreatic islet (8, 48), and PPAR-responsive elements have been identified in the promoters of genes involved in glucose-stimulated insulin secretion, including Glut2, Gck, and Pdx1 (16, 21, 26, 27, 33). Reports from studies of β-cell lines, rodent models of progressive type 2 diabetes, and humans at risk for type 2 diabetes suggest that PPAR-γ agonist administration leads to preservation of islet mass and function (10, 13, 18, 22, 25, 33, 57, 58).

Whereas the studies noted above suggested a direct or indirect effect of PPAR-γ agonists on the biology of the islet, no studies to date have examined the molecular or epigenetic mechanisms whereby islet function is preserved or improved in response to PPAR-γ activation. Islet dysfunction in type 2 diabetes has been attributed to numerous etiologies, including amyloid deposition, oxidative stress, glucotoxicity, lipotoxicity, endoplasmic reticulum (ER) stress, and dedifferentiation (9, 46). Prior reports from our laboratory and others have suggested that a crucial component in the maintenance of normal islet gene transcription, and hence function, is the nature of the covalent modifications of histones H3 and H4, particularly Lys acetylation and methylation (4, 11, 35, 36). We therefore hypothesized that chronic daily administration of PPAR-γ agonist therapy would result in favorable changes at the level of gene transcription and, more specifically, at the level of histone modifications of those genes.

To test this hypothesis, we treated 8-week-old C57BLKS/J-db/db mice (henceforth referred to as db/db mice) or C57BLKS/J mice fed a high-fat diet (HFD) with the PPAR-γ agonist pioglitazone or with a vehicle control by daily oral gavage for 4 to 6 weeks. Our results showed that pioglitazone-treated mice displayed significantly improved whole-body glucose homeostasis, a finding attributable at least in part to improved insulin secretion and islet function. We show that
these improvements in islet function can be explained by an effect of pioglitazone directly upon β-cells to reduce ER stress and to maintain euchromatin structure at a subset of genes that regulate islet growth and glucose-stimulated insulin secretion. Our findings therefore suggest a novel model whereby PPAR-γ agonists may exert a direct effect for insulin-responsive tissues and for the β-cell to ensure efficient glucose disposal and adequate insulin secretion, respectively, in the setting of insulin resistance and diabetes.

MATERIALS AND METHODS

Mouse models. Male db/db mice and lean C57BLKS/J mice 8 weeks of age were obtained from Jackson Laboratories and maintained under protocols approved by the University of Virginia and Indiana University Animal Care and Use Committees. These mice therefore exhibited phenotypic features resembling those of progressive type 2 diabetes in humans. On four separate occasions, 12 db/db mice per group were treated either with pioglitazone, which was administered daily by oral gavage at a dose of 20 mg/kg of body weight in 400 μl of phosphate-buffered saline with daily gavage of a vehicle only (total, 48 mice per group). Treatment was initiated when the mice were 8 weeks of age, after the onset of hyperglycemia, and continued for 6 weeks.

For the HFD experiments, male C57BLKS/J mice were fed either a regular chow diet (17% of calories from fat) or a Western HFD containing 42% of calories from fat (Harlan Teklad). After the HFD group was gavaged daily with pioglitazone at 20 mg/kg as described above or with vehicle only. All mice were kept in a standard light-dark cycle and had access to a regular chow diet and water ad libitum.

For Pdx1 knockout studies, a genetically modified mouse strain in which Pdx1 expression can be inducibly eliminated by administration of doxycycline (“Tet-off” mice), Pdx1TmTm and TgPdx1 mice were obtained from Jackson Laboratories and bred to homozygosity to produce Pdx1tm/mice. In this condition, doxycycline was used to induce a single intraperitoneal dose of 100 mg/kg and was then continued in the drinking water at 0.5 mg/ml for 7 days to inducibly delete the Pdx1 gene as described previously (20).

Islet isolation and cell culture. Pancreatic islets were isolated from mice by collagenase digestion as described previously (15, 59), hand picked, and cultured in phenol-free low-glucose Dulbecco’s modified Eagle’s medium overnight prior to use. INS-1 (832/13) cells were cultured in RPMI 1640 with 11.1 mM glucose supplemented with 10% fetal bovine serum, 10 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 2 mM l-glutamine, 1 mM sodium pyruvate, and 50 μM/ml β-mercaptoethanol. Pioglitazone was used at a concentration of 10 μM for in vitro incubations in a final concentration of 0.1% dimethyl sulfoxide (DMSO). Thapsigargan was dissolved in DMSO and added to INS-1 cell cultures at a final concentration of 1 μM in 0.1% DMSO. Islets were fixed in 1% formaldehyde for 10 to 15 min before the experiment was begun. Excitation light from a xenon short-arc lamp was supplied to the preparation via a light pipe and filter wheel (Sutter Instrument Company). Images were taken sequentially under conditions of 28 mM versus 3 mM glucose. Data were analyzed with IP Lab software, version 4.0 (Scanalytics). The periods and amplitudes of [Ca2+]i oscillations were determined using the CLUSTER8 pulse detection algorithm (at settings of peak size = 2, nard size = 2, minimum = 0, and score = 2) or by direct visual inspection (41). Groups were compared using one-way ANOVA followed by the Tukey-Kramer posttest.

Measurement of glucose-stimulated insulin secretion and quantitation of total pancreatic insulin content. Approximately 50 islets per group were incubated in a 12-well dish for 1 h at 37°C in KREBS-HEPES-buffered solution containing 3 mM glucose. Islets were then transferred to KREBS-HEPES HEPES for an additional hour, after which the supernatant was collected for insulin measurement using a two-site immunospecific enzyme-linked immunosorbent assay (ELISA) (Alpco Diagnostics or Crystal Chem). The same islets were subsequently transferred to KREBS-HEPES-buffered solution containing 28 mM glucose, and insulin release into the medium was measured by ELISA after 1 h of incubation. Total pancreatic insulin content was measured by acid extraction as previously described (1). Pancreatic ChIP assay. Approximately 100 islets were fixed in 1% formaldehyde for 15 min, sonicated to shear DNA to obtain fragments in the range of 800 to 2,000 bp, and subjected to chromatin immunoprecipitation (ChIP) analysis as described previously (4, 5). Antibodies used included rabbit anti-human insulin [Santa Cruz] [1:500], Pdx1 (rabbit anti-mouse Pdx1 [Millipore, Billerica, MA]). ChIP assays were performed for at least three independent islet isolation experiments. For each experiment, samples were quantitated in triplicate by SYBR green I-based real-time PCR using forward and reverse primer sequences for the mouse Ins1/2 and Glut2 gene promoters and cycling parameters described previously (5).

Real-time RT-PCR. Total RNA (5 μg) from islets was reverse transcribed at 37°C for 1 h using 15 μg of random hexamers, 0.5 mM deoxynucleoside triphosphates, 5 μl first-strand buffer, 0.01 mM diithiothreitol, and 200 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen) in a final reaction volume of 20 μl. Real-time reverse transcription-PCR (RT-PCR) was performed for the Pdx1, NeuroD1, Nkx6.1, Pax6, Guk, Kcnj11, Glut2, Ins2/2, LAPP, Irs1, Irs2, IAPP, Pdx1, and Iperlin-chip assay described previously (23). The amplified product for each RT-PCR was subcloned into pcR2.1 A cloning vector (Invitrogen) and sequenced to confirm the identity of the amplified product. Primers used to amplify components of the ER stress pathway, and sarco(endo)plasmic/ER Ca2+ ATPase (SERCA) gene were used as previously described (28, 31). The cycle threshold (Ct) methodology (5) was used to calculate relative quantities of mRNA products from each sample; all samples were corrected for total input RNA by normalizing C values to the C value of the β-actin message.

Immunohistochemistry and immunocytochemistry. Pancreata from at least three mice per treatment group were fixed by cardiac perfusion with 4% paraformaldehyde, paraffin embedded, and sectioned at 5-μm intervals. INS-1 cells were cultured on coverslips with serum-free media and stained with rabbit anti-insulin [Santa Cruz] [1:5000], PDx1 (rabbit anti-mouse PDx1 [Millipore] [1:2000]), and Set7/9 (rabbit anti-human Set7/9 [Millipore] [1:800]) was stained for total insulin and glucagon (rabbit anti-human insulin and glucagon).
FIG. 1. Pioglitazone treatment results in greater weight gain but improved serum lipids in db/db mice. Male C57BLKS/J-db/db mice were treated with either vehicle (db/db) or pioglitazone (Pio-db/db) for 6 weeks and compared to age- and sex-matched lean C57BLKS/J mice. Following the treatment period, mice were evaluated for body weight (A), serum total cholesterol (B), serum triglycerides (C), and serum free fatty acids (D). Data represent the means ± standard errors of the results obtained with at least 12 mice per group. *, significantly (P < 0.05) different compared to C57BLKS/J mouse results; #, significantly (P < 0.05) different compared to db/db mouse results.

FIG. 2. Pioglitazone treatment improves glycemic control and insulin levels in db/db mice. Male db/db mice were treated with either vehicle (db/db) or pioglitazone (Pio-db/db) for 6 weeks and compared to age- and sex-matched lean C57BLKS/J mice. (A) Results of random blood glucose tests at the end of the 6-week treatment period; (B) results of intraperitoneal glucose tolerance tests at the end of the 6-week treatment period. Pio-db/db animals exhibited significantly improved glucose tolerance compared to db/db animals (P < 0.001 for the comparison by two-way ANOVA). (C) Random serum insulin levels at the end of the 6-week treatment period; (D) results of insulin tolerance tests. No differences in insulin tolerance were observed between the db/db and Pio-db/db groups, whereas both groups had significantly impaired insulin tolerance compared to the C57BLKS/J mouse group (P < 0.001 for the comparison by two-way ANOVA). Data represent the means ± standard errors of the results obtained with at least 12 animals per group. *, significantly (P < 0.05) different compared to C57BLKS/J mice; #, significantly (P < 0.05) different compared to db/db mice.

treated orally with pioglitazone. db/db mice on the C57BLKS/J background harbor a mutation of the leptin receptor and exhibit progressive obesity, insulin resistance, and islet dysfunction with age (22). At the start of the study, 8-week-old db/db mice weighed approximately 33.5 g (58% more than age-matched lean C57BLKS/J counterparts), consistent with the obese phenotype of these mice. The mice already exhibited a diabetic phenotype, as suggested by their average nonfasting blood glucose level of 268 mg/dl. At the completion of the 6-week study, vehicle-treated db/db mice exhibited approximately 33.5 g (58% more than age-matched lean C57BLKS/J counterparts) blood glucose level of 268 mg/dl. At the completion of the 6-week study, vehicle-treated db/db mice exhibited approximately 33.5 g (58% more than age-matched lean C57BLKS/J counterparts) blood glucose level of 268 mg/dl. At the completion of the 6-week study, vehicle-treated db/db mice exhibited approximately 33.5 g (58% more than age-matched lean C57BLKS/J counterparts) blood glucose level of 268 mg/dl.
mg/dl). Consistent with this improvement in nonfasting glucose levels, intraperitoneal glucose tolerance testing demonstrated that pioglitazone-treated mice exhibited enhanced glucose clearance compared to the controls, although the treatment did not completely normalize the results compared to those seen with the lean C57BLKS/J counterparts (Fig. 2B). Remarkably, the average nonfasting insulin levels for pioglitazone-treated mice were 90% higher than those for control animals (38 versus 20 ng/ml), suggesting that the improved nonfasting glucose levels and glucose tolerance in pioglitazone-treated animals could be at least partly explained by an enhanced islet insulin secretory capacity (Fig. 2C). This conclusion was further supported by the results of insulin tolerance testing, which showed that treated and untreated mice were equally insulin resistant after 6 weeks of pioglitazone treatment (Fig. 2D).

**Pioglitazone enhances islet function.** To determine whether pioglitazone treatment enhanced islet function, we first performed immunohistochemistry using pancreatic sections from control and pioglitazone-treated mice. As shown in Fig. 3A to C, it is apparent from hematoxylin and eosin staining that the islets of control- and pioglitazone-treated db/db mice were substantially larger than those of lean C57BLKS/J mice, consistent with the adaptive β-cell hyperplasia of insulin resistance (42). Given the increased islet size in treated mice compared to untreated controls, we next assessed proliferation by staining pancreatic sections with Ki67, insulin, and DAPI. There were no differences in Ki67 staining results observed between islets from treated and untreated animals (data not shown). Moreover, an extensive analysis of cell cycle genes by PCR array profiling revealed no notable differences between control and

FIG. 3. Islet architecture and insulin staining. Pancreata from male db/db mice, treated with either vehicle (db/db) or pioglitazone (Pio-db/db) for 6 weeks, were fixed and stained and compared to pancreata from age- and sex-matched lean C57BLKS/J mice. (A, B, and C) Hematoxylin and eosin staining of pancreatic sections from C57BLKS/J, db/db, and Pio-db/db mice; (D, E, and F) immunofluorescence staining of islets from C57BLKS/J, db/db, and Pio-db/db mice for insulin (red) and glucagon (green). Nuclei were counterstained with Hoechst dye (blue). The figure shows representative islets from three pancreata analyzed per group of mice.

FIG. 4. Pioglitazone treatment improves islet function in db/db mice. Islets from male db/db mice, treated with either vehicle (db/db) or pioglitazone (Pio-db/db) for 6 weeks, were isolated and compared to islets from age-matched lean C57BLKS/J mice. (A) Results of GSCa studies of isolated islets. The panel shows the continuous fura-2 AM fluorescence ratio (340/380 nm) as glucose in the incubation chamber was increased from 3 mM to 28 mM. Data represent the means ± standard errors of the results obtained with at least 30 islets from 12 different animals per group. (B) Data from panel A were used to calculate a GSCa index, which represents the fura-2 AM fluorescence ratio at 28 mM glucose divided by the ratio at 3 mM glucose. (C) The insulin content of islets used for static release assays was measured by ELISA after acid extraction. (D) Islets were incubated in 3 and 28 mM glucose for 1 h, and insulin secretion into the supernatant was measured by ELISA. Data represent the means of the results of at least three independent experiments performed using 50 islets per group. *, significantly (P < 0.05) different compared to C57BLKS/J mice; #, significantly (P < 0.05) different compared to db/db mice.
pioglitazone-treated islets in the expression of genes controlling the G<sub>1</sub>-to-S transition (data not shown). These data suggested to us that the larger islet size in pioglitazone-treated animals was not secondary to enhanced islet replication but instead may have resulted from protection from islet cell dropout.

Next, we examined islet insulin production and secretory capacity. Immunofluorescence staining demonstrated that the islets of control db/db mice exhibited noticeably lower staining intensity for insulin than those of lean C57BLKS/J mice, suggestive of β-cell degranulation (Fig. 3D and E). Pioglitazone treatment, however, restored islet insulin-staining intensity (Fig. 3F). Of note, whereas glucagon-producing α cells occurred in a normal pattern at the islet periphery in lean mice, this architecture appeared disrupted in control-treated db/db mice, with α cells frequently occupying central portions of the islets; pioglitazone treatment did not appear to restore this normal architecture to islets (Fig. 3D to F). Total pancreatic insulin content was measured for three pancreata per group following acid extraction. Mean pancreatic insulin content was significantly higher in the C57BLKS/J group compared to the db/db group (11,335 ng ± 1,064 ng versus 4,854 ± 475 ng [P < 0.05]). Pioglitazone restored total pancreatic insulin content to the levels observed in the C57BLKS/J group. Mean pancreatic insulin content in pioglitazone-treated db/db mice was likewise significantly increased compared to that seen with untreated db/db mice (11,941 ng ± 1,506 ng versus 4,854 ± 475 ng [P < 0.05]). These data suggested that PPAR-γ activation enhances islet insulin production.

To directly assess islet function, we next isolated islets from control and pioglitazone-treated db/db mice and compared their function to that of islets isolated from lean C57BLKS/J mice. Figure 4 shows results from GSCa studies of isolated islets. GSCa is a measure of islet glucose sensitivity that captures the dynamics of the biphasic response, which is similar, but not identical, to those of glucose-stimulated insulin secretion (17). The GSCa, as measured by the change in the fura-2 AM fluorescence ratio after glucose stimulation, was reduced in control db/db islets such that the glucose stimulatory index was only 26% of that observed in islets from lean C57BLKS/J mice.
mice. Also, the initial reduction in [Ca\(^{2+}\)]\(_i\), following exposure to high glucose levels was blunted or absent in vehicle-treated db/db mice. This phenomenon has been attributed to the activity of SERCAs (47). Pioglitazone treatment of db/db mice restored both the initial fall in [Ca\(^{2+}\)]\(_i\) and, relative islet GSCa responsiveness, with the GSCa index increasing to about 80% of that seen with lean C57BLKS/J islets (Fig. 4A to B). As shown in Fig. 4C, average islet insulin content was significantly lower in vehicle-treated db/db mice compared to that seen with the background strain. Insulin content was restored after pioglitazone treatment. Glucose-stimulated insulin secretion paralleled the GSCa studies for all three groups, with islets from the background strain. Insulin content was restored after pioglitazone treatment, with the GSCa index increasing to about 80% of that seen with lean C57BLKS/J islets (Fig. 4A to B). As shown in Fig. 4C, average islet insulin content was significantly lower in vehicle-treated db/db mice compared to that seen with the background strain. Insulin content was restored after pioglitazone treatment. Glucose-stimulated insulin secretion paralleled the GSCa studies for all three groups, with islets from the background strain. Insulin content was restored after pioglitazone treatment, with the GSCa index increasing to about 80% of that seen with lean C57BLKS/J islets (Fig. 4A to B). As shown in Fig. 4C, average islet insulin content was significantly lower in vehicle-treated db/db mice compared to that seen with the background strain. Insulin content was restored after pioglitazone treatment. Glucose-stimulated insulin secretion paralleled the GSCa studies for all three groups, with islets from the background strain. Insulin content was restored after pioglitazone treatment, with the GSCa index increasing to about 80% of that seen with lean C57BLKS/J islets (Fig. 4A to B). As shown in Fig. 4C, average islet insulin content was significantly lower in vehicle-treated db/db mice compared to that seen with the background strain. Insulin content was restored after pioglitazone treatment.
Because previous data have demonstrated a positive link between IRS1 signaling and the expression of genes involved in intracellular calcium homeostasis (28), we also examined how PPAR-\(\gamma\)/

activation might affect the expression of the genes encoding the SERCAs. In this regard, db/db mice have also been shown to exhibit reduced SERCA activity and protein expression in islets, a finding that may explain the defects in GSCa and calcium oscillations that we observed (47). As shown in Fig. 7D, expression of genes encoding SERCA2a, SERCA2b, and SERCA3 was decreased 8- to 10-fold in db/db mice compared to the control background strain. Strikingly, pioglitazone treatment resulted in a dramatic improvement in the expression of each of these genes, restoring them to the levels seen in the control strain (Fig. 7D).

Pioglitazone treatment enhances markers of euchromatin at the \(\text{Ins1/2}\) and \(\text{Glut2}\) genes. In prior studies, we and others had demonstrated that \(\beta\)-cell genes, particularly \(\text{Ins1/2}\) and \(\text{Glut2}\), appear to be regulated through changes in covalent histone modifications that alter chromatin accessibility (6). Expression of \(\text{Ins1/2}\) and \(\text{Glut2}\) was increased 15- and 7-fold, respectively, in islets following 6 weeks of daily oral therapy with pioglitazone (Fig. 7). As shown in Fig. 7D, expression of genes encoding SERCA2a, SERCA2b, and SERCA3 was decreased 8- to 10-fold in db/db mice compared to the control background strain. Strikingly, pioglitazone treatment resulted in a dramatic improvement in the expression of each of these genes, restoring them to the levels seen in the control strain (Fig. 7D).

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PPAR-\(\gamma\) activation increases nuclear Set7/9 localization. To investigate the underlying mechanism for the observed enhancement of H3-dimethyl-Lys4 at the \(\text{Ins1/2}\) and \(\text{Glut2}\) genes, we examined the mRNA and protein levels of Set7/9. Set7/9 is a histone H3-Lys4-specific methyltransferase (37, 54) that is enriched in islets, physically interacts with Pdx1, and occupies known Pdx1 target genes such as \(\text{Ins1/2}\) and \(\text{Glut2}\) (4, 6, 11).

FIG. 7. Pioglitazone treatment improves the expression of key \(\beta\)-cell genes. Islets from age- and sex-matched male C57BLKS/J and db/db mice, treated with either vehicle (db/db) or pioglitazone (Pio-db/db) for 6 weeks, were harvested and subjected to real-time RT-PCR for analysis of islet growth and function genes (A), glucose-sensing genes (B), growth factor-signaling genes (C), and SERCA genes (D). Data represent the means \pm standard errors of the results of at least three different biologic replicate experiments. Data were analyzed by one-way ANOVA. *, statistically \((P < 0.05)\) different compared to C57BLKS/J mice; #, statistically \((P < 0.05)\) different compared to db/db mice.
RT-PCR and immunoblot analysis of islets revealed that Set7/9 protein levels were increased in response to pioglitazone treatment but with no effect seen on the transcript levels of the gene encoding Set7/9 (Setd7) (Fig. 9A and B). Consistent with the RT-PCR data (Fig. 7A), Pdx1 protein levels were also increased in islets from pioglitazone-treated mice (Fig. 9B). Interestingly, whereas Set7/9 exhibits a predominantly nuclear staining pattern in islets of normal mice (11), it displayed a strikingly more cytoplasmic pattern in islets from vehicle-treated db/db mice (Fig. 9C and G). Pioglitazone treatment of db/db mice, however, appeared to completely restore the nuclear staining pattern of Set7/9 (Fig. 9D and H), thereby suggesting a potential mechanism for the increased H3-dimethyl-Lys4 modifications observed at the Ins1/2 and Glut2 genes in treated animals. Importantly, Pdx1 staining, although reduced in control db/db mice, remained nuclear in both groups (Fig. 9E and H).

**PPAR-γ activation directly improves islet function in db/db mice.** To characterize the potential direct effect of pioglitazone on islet function, we incubated islets from 8- to 9-week-old diabetic db/db mice with pioglitazone at a concentration of 10 μM for 24 h, followed by an assessment of islet function and gene expression. Islet function was assessed via the measurement of GSCa and insulin secretion. Although GSCa results were not as robust in islets treated in vitro as in islets exposed to chronic pioglitazone treatment in vivo (compare Fig. 10A and Fig. 4A), we did observe significant improvements in treated islets compared to untreated islets. Figure 10A and B demonstrate that pioglitazone-treated islets exhibited a significantly reduced basal fura-2 ratio and an improved GSCa index, trends similar to those observed in islets exposed to pioglitazone in vivo. However, static glucose-stimulated insulin release assays failed to reveal differences in insulin secretory function between the two treatment groups, suggesting that the Ca²⁺ imaging data reflect a more sensitive early measure of islet functionality.

To characterize what factors might be contributing to the observed improvements in islet Ca²⁺ responses, we first measured the expression of the same key β-cell genes as examined in our studies in vivo (Fig. 7A and B). Surprisingly, we did not see changes in the expression of any of these genes following short-term treatment with pioglitazone (data not shown). Importantly, however, the genes encoding the SERCA proteins were all uniformly activated, in similarity to data observed with animals treated in vivo (Fig. 11A). We hypothesized that the improved GSCa and enhanced SERCA gene expression might have arisen from a reduction in ER stress. Others have previously described an induction of ER stress in islets from db/db mice as a mechanism underlying the pathogenesis of islet failure in these animals (9). Moreover, known inducers of ER...
FIG. 9. Expression patterns of Pdx-1 and the methyltransferase Set7/9. Islets and pancreata from male db/db mice, treated with either vehicle (db/db) or pioglitazone (Pio-db/db), were harvested and subjected to real-time RT-PCR, immunoblotting, or immunohistochemistry. (A) Results of real-time RT-PCR analysis for Setd7 mRNA by the use of RNA from isolated islets. Data represent the means ± standard errors of the results obtained from two independent islet isolation experiments using six animals in each experiment. (B) Results of immunoblot analysis for Set7/9 (upper panel), Pdx-1 (lower panel), or GAPDH (both panels) obtained using total protein from isolated islets. Data are from pooled-islet experiments performed using six different animals per group. Data from a second islet pool were similar. (C to H) Pancreata from db/db and Pio-db/db animals were peroxidase stained for Set7/9 (C and D) and Pdx-1 (E and F) and counterstained with hematoxylin or stained for both Set7/9 (green) and Pdx-1 (red) and visualized by immunofluorescence (G and H). Nuclei were counterstained using Hoechst dye in (G and H). Data in panels C to H show representative islets from among three pancreata analyzed per group of mice.
stress (e.g., thapsigargin) have been shown to produce defects in GSCa that are similar to those we observed in islets of untreated db/db mice (47). Thus, we considered the possibility that the direct effects of pioglitazone on db/db islet calcium homeostasis that we observed here may have been secondary to attenuation of ER stress. We measured the expression of several genes in the ER stress pathway, including Chop, Bip, and total and spliced Xbp1. We observed a significant reduction in the expression of Chop and spliced Xbp1, together with a nonsignificant decrease in total Xbp1 expression (Fig. 11B). There was no difference in the levels of Bip expression between treated and untreated islets. These data suggest that a direct effect of PPAR-γ activation in islets may be to reduce ER stress.

PPAR-γ activation reduces ER stress and maintains Set7/9 nuclear occupancy. To investigate further the relationship between PPAR-γ activation, ER stress, and Set7/9 nuclear occupancy, we studied a model of ER stress in vitro through the use of INS-1 (832/13) cells, a well-characterized glucose-responsive β-cell line (19). INS-1 cells were treated with 1 μM thapsigargin for 6 h to induce a state of ER stress similar to that observed in db/db mice (47). As shown in Fig. 12, treatment with thapsigargin led to a reduction in Pdx1 protein levels and increases in CHOP and cleaved caspase 3 levels, whereas coincubation with pioglitazone led to recovery of Pdx1 levels and decreases in CHOP and cleaved caspase 3 levels. These data demonstrate a direct effect of PPAR-γ activation in the reduction of ER stress and preservation of Pdx1 levels in β cells.

Next, we assessed the nuclear occupancy of Set7/9 in INS-1 cells treated with thapsigargin and/or pioglitazone. Panels A and B of Fig. 13 show that Set7/9 occupies a slightly more predominant nuclear distribution in INS-1 cells (see quantitation in Fig. 13G), whereas treatment with thapsigargin led to strongly cytoplasmic redistribution (see Fig. 13C, D, and G) similar to that observed in db/db mice. Concurrent treatment with thapsigargin and pioglitazone caused restoration of Set7/9 nuclear occupancy, a finding again similar to that observed

FIG. 10. PPAR-γ activation in vitro improves glucose-stimulated calcium response in db/db islets. Islets from 8-week-old male db/db mice were incubated for 24 h with 10 μM pioglitazone (Pio-db/db) or vehicle control (db/db). (A) Results of GSCa studies of isolated islets. The panel shows the continuous fura-2 AM fluorescence ratio (340/380 nm) changes as glucose in the incubation chamber was increased from 3 mM to 28 mM. Data represent the means ± standard errors of the results for at least 10 islets per group. (B) Data from panel A were used to calculate a GSCa index, which represents the fura-2 AM fluorescence ratio under conditions of 28 mM glucose divided by the ratio under conditions of 3 mM glucose. (C) Islets were incubated in 3 and 28 mM glucose for 1 h, and insulin secretion into the supernatant was measured by ELISA. Data represent the means of the results of at least three independent experiments performed using 50 islets per group. Results were analyzed using a t test. *, statistically different (P < 0.05) compared to db/db islets treated with vehicle control.

FIG. 11. PPAR-γ activation in vitro upregulates SERCA genes and reduces ER stress in db/db islets. Islets from 9- to 10-week-old db/db mice, treated with either vehicle (db/db) or 10 μM pioglitazone (db/db + Pio) for 24 h, were subjected to real-time RT-PCR for analysis of SERCA genes (A) and ER stress markers (B). Data represent the means ± standard errors of the results of five biologic replicate experiments. Results were analyzed by one-way ANOVA. *, significantly (P < 0.05) different compared to db/db mouse group results.
with pioglitazone-treated db/db mice (Fig. 13E, F, and G). Thus, the results in Fig. 13 suggest that the islet chromatin and functional phenotype we observed in pioglitazone-treated db/db mice may have resulted from a direct action of PPAR-γ in reducing ER stress in the islet.

Finally, we asked how PPAR-γ activation could lead to alterations in Set7/9 localization. In prior studies, we showed that Set7/9 is recruited to target genes such as Ins1/2 and Glut2 via physical interaction with Pdx1 (6, 11). We hypothesized therefore that this interaction may lead to the “chaperoning” of Set7/9 into the nucleus and that states that lead to reduced Pdx1 levels (such as ER stress) may reduce Set7/9 nuclear transport. To test this hypothesis directly, we utilized Tet-off mice (see reference 20). As shown in Fig. 14, control mice exhibited normal nuclear localization of both Pdx1 and Set7/9 in pancreatic islets. However, administration of doxycycline for 7 days led to nearly complete elimination of Pdx1 and to both reduction and redistribution of Set7/9 into the cytoplasm. These data therefore suggest strongly that recovery of Pdx1 levels upon PPAR-γ activation (either by direct activation of the Pdx1 gene or indirectly through the reduction of ER stress) may therefore allow recovery of Set7/9 levels in the nucleus.

**DISCUSSION**

Prior reports from studies using islet-derived cell lines, rodent models of type 2 diabetes, and humans with alterations in glucose tolerance suggest that administration of PPAR-γ agonists leads to preservation of islet mass and function and may prevent the onset of diabetes (10, 13, 18, 22, 25, 33, 57, 58). To date, however, few studies have extended these observations to investigation of the molecular basis for the observed improvements in islet function. In this study, we used a mouse model of progressive type 2 diabetes and another of glucose intolerance and insulin resistance to provide novel molecular evidence linking PPAR-γ-mediated improvements in whole-body glucose homeostasis to specific improvements in islet gene transcription and ER stress.

The nuclear receptor PPAR-γ is expressed in a variety of tissues, including liver, muscle, blood cells, and fat, and regulates a host of genes that favorably affect insulin signaling, lipid metabolism, and adipocyte differentiation (29). Consistent with this mechanism of action, db/db mice displayed substantial improvements in glucose homeostasis and significant reductions in plasma lipids (triglycerides and free fatty acids)
following treatment with the PPAR-γ agonist pioglitazone. These animals also gained more weight than controls, presumably secondary to increased adipocyte differentiation (29).

Although PPAR-γ is expressed in the islet β cell (8, 48), its role in β-cell physiology is less well characterized. Interestingly, targeted elimination of PPAR-γ in β cells resulted in mice with increased β-cell mass and replication but with normal glucose homeostasis (48). However, islets from these mice were not capable of increasing insulin secretion in response to PPAR-γ agonists, thereby suggesting that PPAR-γ may play a role in β-cell insulin secretion. Recent studies of the β-cell-specific ABCA1 knockout mouse suggested that the beneficial effect of PPAR-γ agonists on insulin secretion may be partially secondary to inhibition of cholesterol accumulation in β cells (2). Studies of cell line models in vitro also suggested a potential direct effect of PPAR-γ agonists in β cells (33).

Our results obtained with db/db and HFD-fed mice confirm a positive effect of PPAR-γ agonists on islet function, as assessed by increases in random insulin levels of treated mice as well as by analysis of islets isolated from treated mice. Not only did we observe improvements in GSCa and glucose-stimulated insulin secretion from these islets, but we also observed significant improvements in islet Ca²⁺ oscillations, all approaching values similar to those observed with the lean background strain. Defects in Ca²⁺ oscillatory activity have previously been observed in islets from db/db mice (47), but to our knowledge this is the first report that this dysfunction can be corrected following treatment of diabetes. In this regard, loss of insulin pulsatility in nondiabetic relatives of diabetic patients has been previously shown (43), suggesting that loss of oscillatory activity may be an early warning sign of β-cell dysfunction. A particularly novel finding in our studies is that Ca²⁺ homeostasis in treated animals may well be secondary to recovery of the genes encoding the SERCAs. SERCA genes have been shown to be activated downstream of the IRS1 arm of the insulin/IGF-1 receptor-signaling pathway, and IRS1 is a known direct target of PPAR-γ (28). Incubation of db/db islets in vitro with pioglitazone also confirmed a stimulatory effect of pioglitazone on the SERCAs and therefore raised the additional attractive possibility that PPAR-γ may directly activate genes encoding the SERCAs. In support of this possibility, SERCA genes contain conserved PPAR-responsive elements within their regulatory regions (61).

Improvements in islet function following pioglitazone treatment appeared secondary to increases in the expression of genes that encode proteins involved in glucose sensing and β-cell differentiation, including Glut2, Kcnj11, Pdx1, Nkx6.1, NeuroD1, Ins1/2, and Irs1. Importantly, Glut2 has been shown to contain PPAR-γ recognition sequences and is positively regulated by PPAR-γ agonists (21, 26). However, from our studies we cannot know whether increases in the activation of these genes are a direct result of the presence of PPAR-γ agonists, as these genes are also direct targets of Pdx1 (5, 53, 55). To gain further insight into the mechanism underlying activation of these genes following PPAR-γ activation, we looked more closely at the chromatin structure of the two most highly upregulated genes, Glut2 and Ins1/2. Both genes exhibited significant increases in the level of the euchromatin marker histone H3-dimethyl-Lys4. Interestingly, we also found that with progressive diabetes, levels of H3-dimethyl-Lys4 decreased at the Ins1/2 promoter, implying that a loss of favorable chromatin architecture is yet another explanation for the progressive islet dysfunction seen with type 2 diabetes. With respect to H3-dimethyl-Lys4, this particular marker appears to serve as a recognition site for the docking of chromodomains-containing chromatin remodeling complexes (e.g., SNF2H and Chd1) (45), which subsequently catalyze a more open, euchromatic, structure at the gene. In a recent study, our group demonstrated that the islet-enriched histone methyltransferase Set7/9 may be responsible for H3-Lys4 dimethylation at the Ins1/2 and Glut2 genes (6). Consistent with the increases in H3-Lys4 dimethylation in islets of pioglitazone-treated mice,
we observed significant increases in Set7/9 protein levels and increased nuclear localization. Although Set7/9 harbors no known nuclear localization signals, we have previously proposed that it may be chaperoned into the nucleus by virtue of its direct interaction with Pdx1 (11) and demonstrate here that loss of Pdx1 directly leads to a loss in Set7/9 nuclear occupancy. Thus, the greater nuclear occupancy of Set7/9 in pioglitazone-treated versus vehicle-treated animals in our study may be secondary to higher Pdx1 levels in treated animals.

An important but unresolved issue in the literature has been whether the improved islet function and gene expression patterns following PPAR-γ agonist therapy are direct effects of the presence of these agonists in islets or indirect effects of improved glycemic and/or lipid control. In this regard, in vitro studies have yielded conflicting results with regard to insulin release after short-term incubation of rodent cell lines and cultured islets. In an attempt to address this question, we incubated islets from db/db mice with pioglitazone for 24 h. We observed increases in SERCA gene expression and favorable changes in Ca²⁺ responsiveness in isolated islets and evidence for diminished ER stress responses. ER stress has been shown to be an important mechanism leading to islet dysfunction in various mouse models of diabetes, including HFD-fed and db/db mice (51). The direct effects of PPAR-γ activation in mitigating ER stress were also observed in thapsigargin-treated INS-1 cells. Thus, we propose that PPAR-γ may reduce ER stress in islets by a mechanism involving at least SERCA gene activity, but further studies to clarify the mechanism are still needed.

FIG. 14. Inducible deletion of Pdx1 in pancreatic islets causes a nuclear-to-cytoplasmic shift in Set7/9. Pdx1<sup>TetOn</sup> Tg<sup>Pdx1</sup> mice (20) were treated with vehicle for 7 days as described in Materials and Methods, and pancreata were harvested and fixed for immunostaining for Pdx1, Set7/9, and DAPI, as indicated. (A to C) Pancreatic section from a representative control animal treated with vehicle (−DOXY); (D to F) pancreatic section from a representative animal treated with doxycycline (+DOXY).

FIG. 15. Schematic diagram proposing a model for PPAR-γ action in the setting of diabetes or insulin resistance. The figure suggests two arms in the pathway through which PPAR-γ might improve glycemic control in the setting of diabetes and insulin resistance. The left arm proposes PPAR-γ action with respect to insulin-sensitive tissues such as muscle and adipose tissue, and the right arm proposes PPAR-γ action in the islet directly. See the text for details.
Finally, we would like to point out that our studies were conducted using pioglitazone, whereas many of the other studies in the literature used the related PPAR-γ agonists troglitazone or rosiglitazone. Although all three agonists are believed to act as agonists of PPAR-γ, outcome reports from clinical trials suggest differential effects of the three agents with respect to cardiovascular complications and mortality outcomes (30, 39, 50). In addition, a recent microarray analysis suggested that each agent regulates both common and distinct subsets of genes in non-β cells (49). It is possible that the differences in gene regulation profiles may be caused by different binding affinities of each agent for PPAR-γ (49), and it therefore remains possible that pioglitazone may have unique effects on the β-cell compared to other agonists. In a clinical context, it is noteworthy that pioglitazone appears to have effects on overall cardiovascular mortality in type 2 diabetic patients that are different from those observed with other PPAR-γ agonists studied (30, 38, 56).

Taken together, our results suggest a model (Fig. 15) whereby PPAR-γ may have direct effects on islet function in diabetes and insulin resistance through the activation of IRS1 signaling, SERCA gene activation, and attenuation of ER stress. Another and related arm of PPAR-γ activation may arise from effects on Pdx1 activation, which in turn leads to increased Set7/9 nuclear occupancy and islet gene euchromatin. These findings therefore provide new evidence for pathways of PPAR-γ that may be specific to islets.

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