Peroxisome Proliferator-activated Receptor-γ Activation Augments the β-Cell Unfolded Protein Response and Rescues Early Glycemic Deterioration and β Cell Death in Non-obese Diabetic Mice*

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Type 1 diabetes is an autoimmune disorder that is characterized by a failure of the unfolded protein response in islet β cells with subsequent endoplasmic reticulum stress and cellular death. Thiazolidinediones are insulin sensitizers that activate the nuclear receptor PPAR-γ and have been shown to partially ameliorate autoimmune type 1 diabetes in humans and non-obese diabetic (NOD) mice. We hypothesized that thiazolidinediones reduce β cell stress and death independently of insulin sensitivity. To test this hypothesis, female NOD mice were administered pioglitazone during the pre-diabetic phase and assessed for insulin sensitivity and β cell function relative to controls. Pioglitazone-treated mice showed identical weight gain, body fat distribution, and insulin sensitivity compared with controls. However, treated mice showed significantly improved glucose tolerance with enhanced serum insulin levels, reduced β cell death, and increased β cell mass. The effect of pioglitazone was independent of actions on T cells, as pancreatic lymph node T cell populations were unaltered and T cell proliferation was unaffected by pioglitazone. Isolated islets of treated mice showed a more robust unfolded protein response, with increases in Bip and ATF4 and reductions in spliced Xbp1 mRNA. The effect of pioglitazone appears to be a direct action on β cells, as islets from mice treated with pioglitazone showed reductions in PPAR-γ (Ser-273) phosphorylation. Our results demonstrate that PPAR-γ activation directly improves β cell function and survival in NOD mice by enhancing the unfolded protein response and suggest that blockade of PPAR-γ (Ser-273) phosphorylation may prevent type 1 diabetes.

Type 1 diabetes (T1D)2 is characterized by the loss of insulin production, as β cells succumb to targeted autoimmunity. The non-obese diabetic (NOD) mouse spontaneously develops T1D and is used as a model that closely mimics human disease (1). In this animal model insulitis (infiltration of the islet by cells of the immune system) is observed as early as 4 weeks of age, with the subsequent development of diabetes in many animals by 12–20 weeks of age (2). The concept that the only defect in T1D lies in the immune system has been revisited in recent years. Our group and others have demonstrated that endoplasmic reticulum (ER) stress and resultant insulin secretory defects in the β cell precede the onset of T1D in mice (3, 4). These findings raise the intriguing possibility that ER stress in the β cell might contribute to the aberrant production of “neoantigens” that subsequently invites autoimmunity and insulitis (5–7). Dysfunction of the β cell in pre-T1D has been observed in other studies in NOD mice (8), and both β cell ER stress and dysfunction have been observed in humans with early T1D or destined to develop T1D (9, 10). Therefore, therapies that directly enhance the ER stress-remediating response known as the unfolded protein response (UPR) may prove useful as adjunctive therapies to delay/prevent T1D.

Thiazolidinediones (TZDs) are activators of the nuclear transcription factor peroxisome proliferator-activated receptor-γ (PPAR-γ) and have been traditionally viewed as insulin sensitizers, due to their effects on adipose tissue. The anti-diabetic action of TZDs in adipose tissue occurs, in part, by blocking the phosphorylation of PPAR-γ at Ser-273 (11). Additionally, studies in humans with type 2 diabetes (T2D) and in rodent models of T2D suggest that TZDs may also directly enhance β cell function (12, 13). In addition to their potential effects on β cells, TZDs have also been implicated in the reduction of inflammation and autoimmunity due to the effects on dendritic cells, macrophages, and T cells of the immune system (14–16).

2 The abbreviations used are: T1D, type 1 diabetes; T2D, type 2 diabetes; NOD, non-obese diabetic; ER, endoplasmic reticulum; UPR, unfolded protein response; TZD, thiazolidinedione; PPAR, peroxisome proliferator-activated receptor; CFSE, carboxyfluorescein succinimidyl ester; AUC, area under the curve; CC3, cleaved caspase 3; GTT, glucose tolerance test; 4-HNE, 4-hydroxynonenal; PCNA, proliferation marker proliferating cell nuclear antigen.

* This work was supported by National Institutes of Health Grants R01 DK60581; R01 DK105588; UC4 DK104166 (all to RGM); and F32 DK094489 (to S. C. C.). This work was also supported by a Junior Faculty Award grant from the American Diabetes Association (to S. A. T.) and by grants from the George and Frances Ball Foundation and the Ball Brothers Foundation (to R. G. M.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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The potential utility of TZDs in T1D has been limited to only a few studies. In humans, treatment of established T1D with the TZD pioglitazone resulted in modest improvements in glycemic control (17–19), raising the prospect that intervention with the drug in the pre-diabetic phase might have greater impact. In accordance with this possibility, studies in NOD mice have shown that administration of the TZDs rosiglitazone and troglitazone at the time of weaning significantly reduced the incidence of T1D (20, 21). These studies in NOD mice did not address the possibility that direct effects of TZDs on the β cell may have led to reduced β cell stress early in the pathogenesis of T1D. To address this possibility, we studied the effects of pioglitazone in the pre-diabetic phase in NOD mice. Our results were consistent with a β cell sparing effect of pioglitazone independent of effects on insulin sensitivity or body fat distribution and suggest that reductions in early β cell stress in NOD mice may underlie the reduction in T1D incidence caused by TZDs.

Results

TZD Therapy Improves Glycemic Control in Pre-diabetic Female NOD Mice—Pre-diabetic female NOD mice show progressively worsening β cell function, failing UPR, and increasing ER stress in pancreatic islets as they age from 6 weeks onward (3, 4, 8). To determine if TZD therapy can restore the UPR and ameliorate β cell stress in the pre-diabetic phase, 6-week-old female NOD mice were fed a standard chow diet containing pioglitazone (0.01 wt%) until 10 weeks of age. Control mice were fed an identical diet not containing pioglitazone. By design, none of the animals developed overt diabetes (defined as blood glucose on two consecutive measurements >250 mg/dl) during the course of the study. Whereas pioglitazone is known to increase body weight and fat mass in mouse models of T2D (22), during this study pioglitazone-treated NOD mice gained weight no differently than controls (Fig. 1B), consistent with a direct effect of pioglitazone on islet β cells, we pretreated the mouse β cell-derived cell line MIN6 with 10 μM pioglitazone or 10 μM roscovitine (an inhibitor of cyclin-dependent kinases, which are known to promote Ser-273 phosphorylation) (24) then performed an immunoblot for phospho-PPAR-γ (Ser-273). As shown in Fig. 4A, levels of phospho-PPAR-γ (S273) was detectable in non-treated (control) cells. By contrast, phospho-PPAR-γ (S273) levels were blunted in cells pretreated with 10 μM pioglitazone and 10 μM roscovitine (Fig. 4A). Islets of pioglitazone-treated NOD mice also showed reductions in phospho-PPAR-γ (S273) (Fig. 4B), consistent with a direct effect of pioglitazone on islets.

TZD Therapy Augments Insulin Secretion and Reduces β Cell ER Stress and Death in Pre-diabetic Female NOD Mice—To determine if the improved glycemic control in pioglitazone-treated NOD mice was a consequence of enhanced β cell function, serum insulin levels were measured after an intraperitoneal glucose load. As shown in Fig. 5A, pioglitazone-treated mice displayed significantly higher levels of serum insulin compared with the control group at 10 min after the intraperitoneal glucose load. Isolated islets from treated mice also showed a lower basal insulin secretion at low glucose (2.5 mm) in vitro and a significantly more robust response to glucose stimulation (25 mm) compared with control islets (Fig. 5B). To assess if pioglitazone treatment affected β cell stress and prohormone

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processing, we next measured random-fed insulin and proinsulin and the proinsulin:insulin ratio. Higher proinsulin:insulin ratios have been shown to correlate to cell ER stress in both humans and NOD mice (4, 25, 26). As shown in Fig. 5C, whereas the random-fed insulin was a significant 2-fold greater in the treatment group compared with controls, the proinsulin levels were not significantly different (Fig. 5D), resulting in a proinsulin:insulin ratio that trended lower (p < 0.07) in the treatment group (Fig. 5E).

To assess more directly the possibility that pioglitazone enhanced the UPR and reduced ER stress, we next isolated islets from treated and control mice at the end of the study and measured both mRNA and protein markers of the UPR. The UPR is characterized by variable activation of three distinct pathways, IRE1α, PERK, and ATF6. In islet β cells, the IRE1α and PERK pathways predominate and are evident by increases in spliced Xbp1 mRNA levels and ATF4 protein levels (27). As shown in Fig. 6A, there was significant reduction in spliced Xbp1 (sXbp1) mRNA levels in islets from pioglitazone-treated mice compared with controls, demonstrating attenuation of the IRE1α pathway by pioglitazone treatment. By contrast, an increase in ATF4 protein levels was observed by immunoblot analysis (Fig. 6B). The elevation of ATF4 protein levels was confirmed by an increase in the mRNA encoding DNA damage-inducible protein 34 (Gadd34), a known ATF4 target gene (Fig. 6C) (28). GADD34 is a phosphatase that dephosphorylates eIF2α to restore protein synthesis in the remediation phase of the UPR (28, 29). As shown in Fig. 4B, phosphorylated eIF2α is reduced in islets of pioglutazone-treated mice. Consistent with the recovery of protein synthesis, a slight, but significant, increase in the levels of the protein folding chaperone BIP was observed in the islets of treated mice (Fig. 6C).
Failure of the UPR to adapt to the underlying stress leads to frank ER stress and to activation of the proapoptotic pathway mediated by CHOP (30). Concordant with the adaptive UPR in pioglitazone-treated mice, there was a reduction in Chop mRNA in islets (Fig. 6E) and a corresponding reduction in CHOP protein in β cells by immunohistochemistry (Fig. 7A). Likewise, cleaved caspase 3 (CC3), a marker of apoptotic cells, was reduced in insulin+ cells of pioglitazone-treated mice compared with control mice (Fig. 7B).

To clarify the underlying mechanism promoting an adaptive UPR, we evaluated pancreas tissue sections for evidence of oxidative stress, which is known to drive the development of ER stress (27). Fig. 7C shows that control NOD mice exhibited evidence of oxidative stress in islets, as assessed by immunostaining for 4-hydroxynonenal (4-HNE). By contrast, minimal to no 4-HNE staining was observed in islets of pioglitazone-treated mice. As a likely consequence of reduced oxidative stress and more robust UPR, β cell area (as a percentage of total pancreatic area) was increased 2-fold upon pioglitazone treatment (Fig. 7D). This greater β cell area percent in pioglitazone-treated mice resulted primarily from reduced β cell death and not an increase in β cell proliferation, as β cell death parameters CC3 (Fig. 7C) and unmethylated preproinsulin DNA (Fig. 7E) (31) were reduced, but no changes in the proliferation marker proliferating cell nuclear antigen (PCNA) were observed (Fig. 7F).

Discussion

T1D develops as a disorder of the immune system in which β cell autoantigens, released as a result of β cell death, trigger the eventual selection and proliferation of autoreactive T cell clones (32). It has been speculated that the physiologic early turnover of β cells seen in neonatal mice (and possibly humans, as well) might contribute sufficient antigen exposure to trigger autoimmunity (33), but this possibility has been challenged in studies of NOD mice (34). A second hypothesis suggests that stress responses intrinsic to the β cell result in the pathologic formation of epitopes (as misfolded or posttranslationally modified proteins) that are immunogenic (5, 6, 35). In this regard, β cell ER stress has been shown to occur in the early pre-diabetic phase in NOD mice and in humans with new-onset T1D (4, 10, 25). The UPR is a coordinated response that is activated under a variety of stress conditions that put increased burden on the ER (ER stress). Two phases of the UPR have been recognized: an adaptive, stress-remediating phase, and a subsequent maladaptive, pro-apoptotic phase that is initiated when remediation is not possible (for a review, see Ref. 29).
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The adaptation to ER stress by enhancing the UPR has been proposed as viable, β cell-protective therapy for T1D (3). Because administration of TZDs has been shown to ameliorate ER stress (22), we asked if TZDs have the potential to directly impact β cell function and survival in NOD mice.

TZDs are traditionally viewed as insulin sensitizers, but evidence in recent years suggests that they have direct effects on the β cell. These effects include the stimulation of Pdx1 gene activity and increases in Pdx1 protein levels in isolated β cells (36, 37), stimulation of Serca2 gene activity and protein levels (22, 38), and reductions in β cell oxidative stress (39). Moreover, TZD administration was shown to delay the incidence of T1D in NOD mice (20, 21) and to improve T1D glycemic control in humans (17–19), although it is unclear if these findings were a result of β cell effects of TZDs. Notably, our studies showed that pioglitazone administration resulted in a reduction in phospho-PPAR-γ (Ser-273) in islets and an improvement in the islet UPR, as evidenced by enhanced ATF4 and BIP protein levels and a trend to reduced proinsulin:insulin ratio. Although our study points to a PPAR-γ-dependent effect of pioglitazone, we should point out that pioglitazone was also shown to have an acute effect of reducing metabolic flux and insulin secretion in β cells in a non-PPAR-γ-dependent fashion (40, 41). This effect may have allowed for reduced ER load early in disease pathogenesis and subsequent improvements in ER function. Nevertheless, because our findings were not accompanied by changes in body weight, body fat distribution, or insulin sensitivity, we believe they suggest a direct effect of pioglitazone on islet β cells.

It is noteworthy that we observed increased ATF4 levels despite a reduction in CHOP protein and mRNA in pioglitzone-treated mice. Whereas prior studies have linked ATF4 to activation of the gene encoding CHOP (42), it has become increasingly appreciated that other pathways and transcription factors may activate CHOP independently of ATF4 (43, 44) and that of GADD34 may dissociate ATF4 from Chop activation (45). We, therefore, propose that the elevation of ATF4 in our study likely represents an adaptive, ameliorative effect on ER stress.

A significant outcome in our studies was the increased area percent of β cells in pioglitzone-treated animals compared with controls. Importantly, β cell area was also higher in these animals when compared with 6-week-old NOD mice at the start of the study, suggesting that pioglitzone treatment either enhanced the rate of β cell replication or reduced β cell death or some combination of the two. This result is reminiscent of studies in type 2 diabetic db/db mice in which pioglitzone treatment resulted in increased β cell area that was coincident with improved glycemic control (22). In our case, pioglitzone-treated mice showed a reduction in the serum unmethylation index, a sensitive biomarker of β cell death (31), as well as CC3 staining in insulin+ cells. In light of the reduction in β cell death, it is noteworthy that we observed reductions in both oxidative stress (by 4-HNE immunostaining) and IRE1 activity (as judged by reduced spliced Xbp1 mRNA). IRE1α is an endoribonuclease that operates in a bifunctional manner, initially promoting the adaptive response of the UPR by leading to Xbp1 splicing and later, during prolonged ER stress, promoting the cleavage of other mRNAs and microRNAs and leading to cellular apoptosis (46–48). In prior studies, our group demonstrated that NOD mice show significant elevations in spliced Xbp1 mRNA (and, by inference, IRE1α activity) compared with non-diabetes-prone controls at 10–12 weeks of age (4). The studies of Kaufman et al. (27) suggest that oxidative stress is closely linked to ER stress. We suggest here that the reductions in IRE1α activity as a result of attenuated oxidative stress by pioglitzone likely reflect an augmented and more successful UPR.

Finally, we should comment on the significant reduction in insulitis we observed upon pioglitzone treatment. Although the reduction in insulitis, we did not observe differences in relative proportions of CD4+ T cell subtypes in the draining pancreatic lymph node. These findings raise the possibility that pioglitzone may have had a primary effect on reducing proliferation of T cells. We tested this possibility by performing T cell activation assays in vitro wherein no changes in CD4+ cell proliferation or differentiation were observed in response to pioglitzone. Studies have shown stimulatory and inhibitory effects of...
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PPAR-γ on Treg and Th17 cells, respectively (14, 49), so we cannot rule out the possibility that differences in CD4+ T cell subpopulations occurred in the immediate vicinity of the islets themselves. In the absence of a direct effect of pioglitazone on T cell proliferation, it nevertheless remains possible that the augmentation of the β cell UPR with pioglitazone may have diminished the stimulus for autoimmunity. In this regard recent studies have shown that chemical restoration of β cell UPR in type 1 diabetic mice results in a reduction in insulitis (3).

Certain key limitations to our study must be recognized. First, notwithstanding some evidence to the contrary, we cannot rule out non–β cell effects of pioglitazone in our study, and it remains possible that β cell-protective effects may still indirectly emanate from the effects on adipose tissue, muscle, or immune cells. Second, the implications of our findings to human T1D remain speculative, but recent observations suggesting elevations in β cell ER stress parameters in human T1D (3, 10, 25) suggest promise for the use interventions that enhance the UPR. Although pioglitazone and similar TZDs have fallen out of favor in clinical use, our findings linking pioglitazone treatment to reductions in phospho-PPAR-γ (Ser-273) open the exciting possibility that non-TZD approaches to blocking Ser-273 phosphorylation may be an alternative approach to enhancing the β cell UPR. Recently, imatinib (Gleevec) was shown to block PPAR-γ (Ser-273) phosphorylation (50) and as such represents a non-TZD with diabetes-ameliorating effects (50, 51). In this respect, an ongoing clinical trial (NCT01781975) is testing the potential for imatinib as therapy for recent-onset T1D. Taken together, our data support the conclusion that TZDs and similar-acting agents may have protective effects to reduce β cell stress and death in T1D and also support the notion that an aggressive approach to β cell function represents an important adjunctive means to control progression or severity of T1D.
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Female NOD/ShiLtJ (NOD) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained at the Indiana University Laboratory Animal Resource Center under pathogen-free conditions according to protocols approved by the institutional animal care and use committee. Mice were allowed to acclimate for 1 week before experimentation. Mice were fed with either a standard chow diet or a standard chow diet containing 0.01 wt% pioglitazone (Harlan-Teklad Global). This concentration of pioglitazone was designed to deliver ~20 mg/kg body weight pioglitazone based on average daily food intake (22, 39). Body weights and blood glucose levels were measured weekly. At 10 weeks of age, mice underwent an intraperitoneal GTT at 2 g/kg body weight of glucose as described previously (4). Serum during the GTT was collected for insulin measurements at 0 and 10 min post glucose injection. Lean body mass and fat mass was determined by dual-energy x-ray absorptiometry (DEXA) using a Lunar PIXImus2 densitometer (GE Medical Systems). Insulin tolerance tests were performed with 1.5 units/kg body weight of insulin injected intraperitoneally as described (22). At the end of the study, mice were euthanized, serum was collected, and pancreata harvested or islets isolated as described (53). Serum insulin was measured using an Ultra Sensitive Mouse Insulin ELISA kit (Crystal Chem), and serum proinsulin was measured using a Mouse Proinsulin ELISA kit (ALPCO). Some mice were stimulated with 10 units/kg insulin (Humulin®; Lilly) 5 min before euthanasia, and liver and muscle were harvested.

Quantitative Real-time RT-PCR and Immunoblot Analysis—Total RNA from islets or MIN6 cells was isolated, reverse-transcribed, and subjected to quantitative real-time RT-PCR as described previously (54). Results of RT-PCR analysis were normalized to Actb levels. Primers for mouse spliced Chop, GADD34, Chop, and Actb were described previously (30, 55–58). Measurement of methylated and unmethylated pre-insulin DNA in the serum by multiplex PCR was performed as we recently detailed (31).

Immunoblots were performed using 4–20% polyacrylamide gel electrophoresis as previously described (59). Antibodies were used as follows: anti-phospho-AKT (Ser-473) (1:1000 dilution, Cell Signaling Technology #9271), anti-total AKT (Ser-273) (1:1000 dilution, Bioss #bs-4888R), and anti-acetyl histone H3 (Lys14) (1:2000, Millipore #06-911).

Immunohistochemistry, Immunofluorescence, β Cell Area, and Insulitis Scoring—Pancreata were fixed, sectioned, and stained for insulin as described (22). Pancreata were stained for immunofluorescence for glucagon, CHOP, PCNA, cleaved caspase 3, or 4-HNE with insulin and counterstained with DAPI and quantified as described (4, 60). Images were captured using an AxioObserver Z1 equipped with a high resolution color concentration and applied directly to cell culture medium at concentrations of 1 μM or 10 μM as indicated in the figures. Cells were preincubated with pioglitazone or roscovitine overnight before experimentation.

Experimental Procedures

Cells, Animals, and Procedures—Mouse-derived MIN6 β cells were cultured and maintained as previously described (52). Pioglitazone and roscovitine were dissolved in DMSO at 1000× concentration and applied directly to cell culture medium at concentrations of 1 μM or 10 μM as indicated in the figures. Cells were preincubated with pioglitazone or roscovitine overnight before experimentation.
camera or LSM 700 confocal (Carl Zeiss). β cell area was calculated as described previously (61). For insulitis scoring, 3 pancreas sections at least 70 μm apart from 5 animals per group were scored using the following grading scheme (62): grade 1, no islet-associated mononuclear cell infiltrates; grade 2, peri-insulitis affecting <50% of the circumference of the islet without evidence of islet invasion; grade 3, peri-insulitis affecting >50% of the circumference of the islet with evidence of islet invasion; grade 4, islet invasion.

Flow Cytometric Analysis of T Cells—Single cell suspensions were prepared from harvested pancreatic lymph nodes from NOD mice (62, 63). For Treg cell analyses, equal volumes of the single cell suspensions were stained using anti-CD4-FITC (eBioscience RM4-5) and anti-CD25-APC (eBioscience PC61.5) antibodies and fixed overnight before being permeabilized and stained with anti-Foxp3-PE (eBioscience FJK-16s) antibody. For the Th1 and Th17 cell analyses, equal volumes of the single cell suspensions were first incubated with Cell Stimulation Mixture (eBioscience) for 4 h before staining with anti-CD4 antibody; cells were fixed overnight then permeabilized and stained for IL-17A (eBioscience eBioIL17B7) and IFNγ (eBioscience XMG1.2) according to the manufacturer’s instructions (eBioscience). Cells were analyzed using a BD FACSCalibur flow cytometer (BD Biosciences) and FlowJo software (TreeStar). For determination of cellular proliferation, single cell suspensions of unfractionated splenocytes from NOD mice were incubated in 5 μM membrane dye carboxyfluorescein diacetate succinimidyl ester (CFSE) for 10 min at 37 °C, diluted with 5 volumes of ice-cold media, and incubated for 5 min on ice. Samples were then washed with PBS three times and subjected to stimulation in vitro and subjected to flow cytometry as described above.

Statistical Analysis—All data are presented as the mean ± S.E. One-way analysis of variance (with Dunnett’s post-test) was used for comparisons involving more than two conditions, and a two-tailed Student’s t test was used for comparisons involving two conditions. Prism 7 software (GraphPad) was used for all statistical analyses. Statistical significance was assumed at p < 0.05.

Author Contributions—A. V. M. and R. G. M. designed the experiments, performed the research, and wrote the paper. S. A. T. performed the research and wrote the paper. F. S., J. B. N. and S. C. C. performed the research. B. M. designed the experiments.

Acknowledgments—We thank N. Stull and K. Benninger of the Center for Diabetes and Metabolic Diseases Islet and Physiology Core laboratory for expert assistance in the isolation of islets and in the metabolic characterization of mice. This work utilized core services supported by National Institutes of Health Grant P30 DK097512 to the Indiana University School of Medicine.

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