

Peroxisome Proliferator-activated Receptor γ (PPAR γ) and Its Target Genes Are Downstream Effectors of FoxO1 Protein in Islet β -Cells

MECHANISM OF β -CELL COMPENSATION AND FAILURE*

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Background: The molecular mechanisms for islet β -cell compensation and failure are not fully known.

Results: FoxO1/PPAR γ signaling regulates key β -cell genes, with this network being up-regulated in nondiabetic insulin-resistant rats and impaired in rodents with diabetes.

Conclusion: We examine the potential for the FoxO1/PPAR γ network as a feature of β -cell compensation and failure.

Significance: We identify targets for prevention of type 2 diabetes.

The molecular mechanisms and signaling pathways that drive islet β -cell compensation and failure are not fully resolved. We have used *in vitro* and *in vivo* systems to show that FoxO1, an integrator of metabolic stimuli, inhibits PPAR γ expression in β -cells, thus transcription of its target genes (*Pdx1*, glucose-dependent insulinotropic polypeptide (GIP) receptor, and pyruvate carboxylase) that are important regulators of β -cell function, survival, and compensation. FoxO1 inhibition of target gene transcription is normally relieved when upstream activation induces its translocation from the nucleus to the cytoplasm. Attesting to the central importance of this pathway, islet expression of PPAR γ and its target genes was enhanced in nondiabetic insulin-resistant rats and markedly reduced with diabetes induction. Insight into the impaired PPAR γ signaling with hyperglycemia was obtained with confocal microscopy of pancreas sections that showed an intense nuclear FoxO1 immunostaining pattern in the β -cells of diabetic rats in contrast to the nuclear and cytoplasmic FoxO1 in nondiabetic rats. These findings suggest a FoxO1/PPAR γ -mediated network acting as a core component of β -cell adaptation to metabolic stress, with failure of this response from impaired FoxO1 activation causing or exacerbating diabetes.

Peroxisome proliferator-activated receptor γ (PPAR γ)² is a member of the nuclear hormone receptor family of ligand-inducible transcription factors and plays a pivotal role in diverse

biological processes such as glucose and lipid homeostasis, inflammation, and cellular proliferation and differentiation (1, 2). In addition to adipocytes and several other cell types, it is expressed in islet β -cells (3). We have shown that PPAR γ in β -cells regulates transcription of the prodifferentiation transcription factor *Pdx1* (4, 5) and the receptor for the incretin hormone glucose-dependent insulinotropic polypeptide (GIP) (6). Others have reported that PPAR γ regulates expression of the anaplerotic enzyme pyruvate carboxylase in adipose tissue (7), but whether this occurs in β -cells is unknown. Collectively, these genes are key controllers of β -cell mass, mitochondrial fuel metabolism, and insulin secretion, suggesting the potential for a central role of PPAR γ in β -cell compensation and/or failure. Consistent with this proposal, we have shown increased expression of PPAR γ and its target genes in islets from rodents with successful β -cell adaption to an experimental reduction of β -cell mass (4, 6) and to obesity and insulin resistance (6, 8) and decreased expression in a diabetic rodent model (6, 8). Also, clinical trials with thiazolidinedione PPAR γ agonists (9) have shown a high success rate at stabilizing β -cell function and preventing type 2 diabetes (10).

Understanding why β -cell PPAR γ expression is defective under hyperglycemic conditions requires characterizing its upstream regulation. In adipocytes, PPAR γ gene expression is under inhibitory control by FoxO1 (11, 12). FoxO1 is a member of the family of winged-helix/forkhead transcription factors that serve important roles in cellular differentiation, proliferation, apoptosis, and the response to cellular stress in many tissues. FoxO1 is highly expressed in β -cells and is a key regulator of β -cell development, mass, and function (13–16). The best described FoxO1 target gene in β -cells is *Pdx1* (17). The mechanism is reported to be competition for the FoxA2-binding element on the *Pdx1* promoter, with phosphorylation of FoxO1 resulting in nuclear export and release of its blockade of FoxA2-dependent *Pdx1* expression. However, the evidence is mostly correlative: EMSA and competition assays for FoxO1 and FoxA2 on the *Pdx1* promoter, and imaging studies showing

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² The abbreviations used are: PPAR γ , peroxisome proliferator-activated receptor γ ; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide 1; PANC PPAR $\gamma^{-/-}$, pancreas epithelium-specific PPAR γ knockout mice; PC, pyruvate carboxylase; PPRE, peroxisome proliferator-activated receptor response element; Px, partial pancreatectomy; ZF, Zucker fatty; ZL, Zucker lean.

cytoplasmic FoxO1 in Pdx1-positive β -cells and nuclear FoxO1 in Pdx1-negative β -cells (14, 17).

FoxO1 activity is modulated by post-translational modifications and protein-protein interactions that impact its intracellular trafficking and function (18). In the β -cell, insulin or growth factor PI3K/Akt-induced FoxO1 phosphorylation leads to its translocation from the nucleus to cytoplasm, relieving its inhibition of target gene transcription (14). The incretin hormones GIP and glucagon-like peptide 1 (GLP-1) also activate this sequence (19, 20). Another is the balance between oxidative stress-induced FoxO1 acetylation and deacetylation (21). Thus, FoxO1 orchestrates β -cell transcriptional regulatory pathways for adaptive responses to many hormones, growth factors, and metabolic stresses. Whereas much is known about FoxO1 activity in β -cells, much less is known regarding its downstream target genes.

In this study we provide evidence for a central role for PPAR γ in FoxO1-dependent regulation of *Pdx1* expression and other key β -cell genes. Also, we show strong nuclear localization of FoxO1 in β -cells along with reduced expression of PPAR γ and its target genes in diabetic rats, suggesting a novel mechanism for β -cell failure.

EXPERIMENTAL PROCEDURES

Cell Culture and in Vitro Assays—INS-1 (832/13) cells and β TC6 cells were maintained as described previously (5). PPAR γ and FoxO1 siRNA were carried out in INS-1 cells using pooled SMART selection-designed siRNA duplexes with UU 3'-overhang and 5'-phosphate on the antisense strand that targeted rat FoxO1 (NM_012560) and rat PPAR γ (NM_013124) (Dharmacon RNA Technology). Transfections were carried out with Dharmafect 4 transfection reagent for 24 h at final siRNA concentrations of 100 μ M. The protocol was two siRNA transfections at 0 h and 48 h; control cells were transfected with scrambled siRNA.

EMSA used nuclear extracts from rat-derived INS-1 cells and mouse-derived β TC6 cells with PAGE-purified oligonucleotides for the mouse PPAR γ promoter containing the putative FoxO1 binding site (forward, 5'-GTCAGATAGATAAACAAATTT-3' and reverse, 5'-CTATCTATTTGTTAAAATCG-3') labeled by end filling with [α - 32 P]dCTP (PerkinElmer Life Sciences) as described previously (5). PPAR γ specificity was determined with rabbit polyclonal FoxO1 antibody (Chemicon, AB4130) and competition studies that added increasing amounts of unlabeled dsDNA oligonucleotide sequences.

ChIP was performed with β TC6 cells using ChIP-IT kit (Active Motif) and a previously described method (5). Rabbit polyclonal FoxO1 antibody (Chemicon) was added to aliquots of precleared 300–500-bp chromatin fragments and incubated overnight, with parallel samples incubated with the negative-control IgG provided with the kit. Protein G-agarose beads were added to the mixture for 1.5 h at 4 $^{\circ}$ C. After reversing cross-links, DNA was isolated and PCR performed using primers for the mouse PPAR γ promoter. Primer sequences were: forward, 5'-AACATCAACCATTGGAATACTACTGC-3' and reverse, 5'-TGGTTGAAACTCACACATCTGAAG-3' with an expected 281-bp PCR product. PCR conditions were 1 cycle at

94 $^{\circ}$ C for 3 min, 40 cycles at 94 $^{\circ}$ C for 30 s, 62 $^{\circ}$ C for 1 min, 72 $^{\circ}$ C for 1 min. A mouse-specific positive control was performed using a kit based on the binding of the transcription factor EFl- α by anti-RNA polymerase II (Active Motif).

Luciferase reporter gene assays were carried out in 60–70% confluent INS-1 cells in 6-well plates incubated overnight in antibiotic-free medium. Co-transfections were performed with the FoxO1 overexpression cassette (pcDNA3-FLAG-FKHR from Dr. Kun-Liang Guan, University of Michigan) and the *Pdx1* promoter pTAL-PPRE-Pdx-1 luciferase plasmid (Dr. Roland Stein, Vanderbilt University) or 3 \times PPRE-luciferase reporter vector (Dr. Spiegelman laboratory, Addgene) with Lipofectamine 2000 transfection reagent (Invitrogen). *Renilla* luciferase reporter plasmid (pRL-TK, Promega) was included (0.05 μ g) in all transfections as internal control. Cells were lysed 48 h after transfection, and luciferase assay performed in a TD 20/20 luminometer (Turners Design) using a dual luciferase assay kit (Promega). Firefly luciferase activity was normalized to *Renilla* luciferase and expressed as relative luciferase activity of the reporter constructs with and without transfection of the FoxO1 overexpression cassette.

Animal Studies—All protocols were approved by the University of Vermont Institutional Animal Care and Use Committee. FoxO1-haplodeficient mice (17) and mice that overexpress a constitutively nuclear FoxO1 transgene (FoxO1^{S253A}) in β -cells and liver (13, 22) were obtained from D. Accili (Columbia University) on a mixed background and were backcrossed for >10 generations to the C57BL/6 background. Mice with PPAR γ deficiency restricted to pancreatic epithelium (PANC PPAR γ ^{-/-}) were generated by crossing Pdx1-Cre mice (original source, D. Melton, Harvard University) and mice with two floxed PPAR γ alleles as detailed previously (5). Some animals underwent glucose tolerance testing after an overnight fast that consisted of 2 g/kg intraperitoneal glucose with serum glucose measured at 0, 30, 60, 90, and 120 min (Freestyle glucose meter). Zucker lean (ZL, fa/+ or +/+) and Zucker fatty (ZF, fa/fa) male rats (Harlan) underwent 60% pancreatectomy (Px) or sham Px surgery at 6 weeks of age as described previously (8). Islets were isolated by pancreas duct perfusion with collagenase, Histopaque gradient separation, and hand picking.

Islet Expression Studies—Immunoblot and quantitative PCR analyses were performed as described previously (5). Immunoblot antibodies were rabbit polyclonal anti-Pdx1 (1:2000, Upstate/Millipore), mouse monoclonal anti-PPAR γ (1:1000, Chemicon/Millipore), and rabbit polyclonal anti-FoxO1 (1:1000, Chemicon/Millipore), followed by goat anti-mouse/rabbit-HRP-conjugated antibody (Bio-Rad). Pyruvate carboxylase affinity blotting was performed using HRP-conjugated streptavidin. Detection was by chemiluminescence using HyperFilm-ECL (Amersham Biosciences). Membranes were stripped and reprobed to establish equivalent loading using anti- β -actin (Sigma). Islet quantitative PCR was carried out in a PTC-200 Peltier Thermal Cycler (MJ Research) using cDNAs, *Taq* polymerase (Promega), and primer combinations (sequences available on request). The thermal cycle program was denaturing step at 95 $^{\circ}$ C for 2 min followed by 35 cycles for PPAR γ , 25 cycles for Pdx1, 30 cycles for FoxO1, at 94 $^{\circ}$ C for 15 s, 56 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C for 60 s, with an extension step of 5 min at 72 $^{\circ}$ C.

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Results are expressed relative to control gene expression (cyclophilin B).

GLP-1 Induction of Pdx1 Gene Expression—Isolated islets from PANC PPAR $\gamma^{-/-}$ and WT mice were cultured overnight in RPMI 1640 medium, 10% FBS, 11.2 mM glucose, 100 units/ml penicillin, 100 μ g/ml streptomycin, followed by overnight culture in serum-free medium with 3% BSA. Islets were incubated in serum-free medium with 3% BSA and 11.2 mM glucose plus freshly dissolved 100 nM mouse GLP-1 (Phoenix Pharmaceutical) or vehicle for 8 h followed by quantitative PCR analysis.

Pyruvate Carboxylase (PC) Activity—Freshly isolated islets from 3 week postsurgery sham and 60% Px ZF and ZL rats were frozen in liquid nitrogen and shipped to Dr. Ye Q. Liu at the University of Louisville. PC activity was measured as described previously (23).

Pancreas Immunofluorescence and Islet Morphometry—Excised pancreata were immersion-fixed overnight in 4.0% paraformaldehyde in 0.1 M phosphate buffer at 4 °C followed by embedding in paraffin. β -Cell mass was quantified using a previously detailed computer planimetric method (24). For multiple-labeling immunofluorescence studies of FoxO1 and Pdx1, sections were stained with guinea pig anti-insulin (Linco/Millipore) to mark β -cells, mouse anti-Pdx1 (Developmental Studies Hybridoma Bank, University of Iowa), and rabbit anti-FoxO1 (Cell Signaling, clone C29H4). Secondary antibodies were multiple-labeling grade anti-species-specific IgG conjugated to either CY2, CY3, or Alexa Fluor 647, respectively (Jackson ImmunoResearch or Molecular Probes/Invitrogen). Sections were imaged confocally using an LSM 510 META (University of Vermont Microscopy Imaging Center).

Statistical Analysis—Data are presented as mean \pm S.E. or S.D., as indicated. Each data point from the animal studies represents an individual animal. Statistical significance was determined by the unpaired Student's *t* test or two-way ANOVA.

RESULTS

Identification and Characterization of FoxO1 Binding Site on Mouse PPAR γ Promoter—The initial studies sought to identify a biological basis for FoxO1 regulation of PPAR γ expression. The consensus binding sequence for FoxO1 is GTAAACA (25). We searched GenBank and identified a putative FoxO1 binding site within the mouse PPAR γ promoter at positions -825 to -831 from the transcription start site (GenBank AY236530) with one nucleotide mismatch from the consensus sequence (Fig. 1A). MatInspector software identified homologous sequences in the rat and human PPAR γ promoters. We confirmed that this sequence binds FoxO1 by performing EMSA and FoxO1 antibody inhibition studies using α - 32 P-labeled complementary strand oligomers of the identified sequence and nuclear extracts from mouse-derived β TC6 and rat-derived INS-1 cells (Fig. 1B). Also, the specificity of binding was established with a competition assay using excess unlabeled probe.

ChIP assay was performed in β TC6 cells to confirm FoxO1 binds to this sequence in an intact insulin-containing cell line, using FoxO1 antibody-precipitated DNA fragments as the PCR template and a primer pair that spanned the region containing the FoxO1 binding site (Fig. 2A). Input DNA and the FoxO1

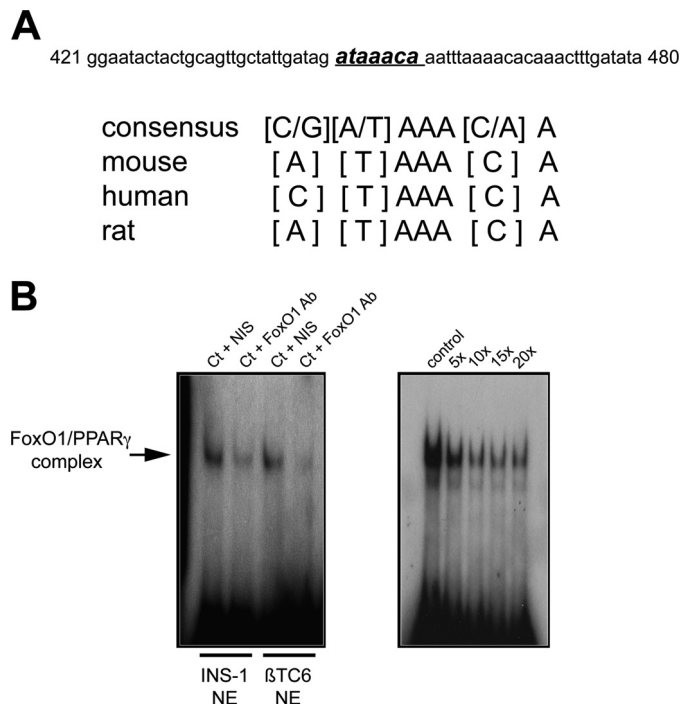


FIGURE 1. Identification of a FoxO1 binding site within the mouse PPAR γ promoter. A, identified sequence in the mouse PPAR γ promoter with one nucleotide mismatch from the FoxO1 consensus binding sequence. Homologous sequences within the rat and human PPAR γ promoters also are shown. B, left, EMSA showing inhibition with FoxO1 antibody, using nuclear extracts (NE) from rat-derived INS-1 cells and mouse-derived β TC6 cells incubated with α - 32 P-labeled complementary strand oligomers of the identified sequence. B, right, competition assay using increasing -fold amounts of unlabeled oligomers of the identified sequence. Ct, control; NIS, nonimmune serum.

antibody-precipitated chromatin fragments both contained the correct 281-bp product in contrast to the nonimmune IgG negative control (Fig. 2B). Also shown is the positive control based on the binding of RNA polymerase II to the transcription factor EFl- α (Fig. 2C).

Expression studies confirmed FoxO1 inhibits PPAR γ expression in an insulin-containing cell line. A FoxO1 siRNA protocol was established in INS-1 cells that resulted in a 60% knockdown of FoxO1 mRNA level and 47% reduction in FoxO1 protein by 96 h (Fig. 3A). PCR analysis showed a 2.0 ± 0.1 -fold increase in PPAR γ mRNA level compared with the scrambled siRNA control, and a similar increase in Pdx1 mRNA level: 2.2 ± 0.2 -fold (Fig. 3B). Also, FoxO1 was overexpressed by transient transfection in INS-1 cells and assayed for PPAR γ promoter activity using two PPRE luciferase reporters, Pdx1-PPRE and 3 \times PPRE. A 48-h overexpression of FoxO1 lowered Pdx1-PPRE driven luciferase activity by 41% compared with no FoxO1 overexpression (Fig. 3C). In addition, 3 \times PPAR γ -PPRE reporter activity was decreased 44% with the FoxO1 overexpression (Fig. 3D). Together, these *in vitro* findings confirmed that FoxO1 binds to the identified binding site sequence and inhibits PPAR γ promoter activity in insulin-containing cell lines.

In Vivo Studies—FoxO1 inhibition of PPAR γ expression in native β -cells was investigated using isolated islets from genetically modified mice. We first tested global FoxO1-haploinsufficient mice. Null mice are embryonically lethal, but the haploinsufficient mice are normoglycemic with relatively normal

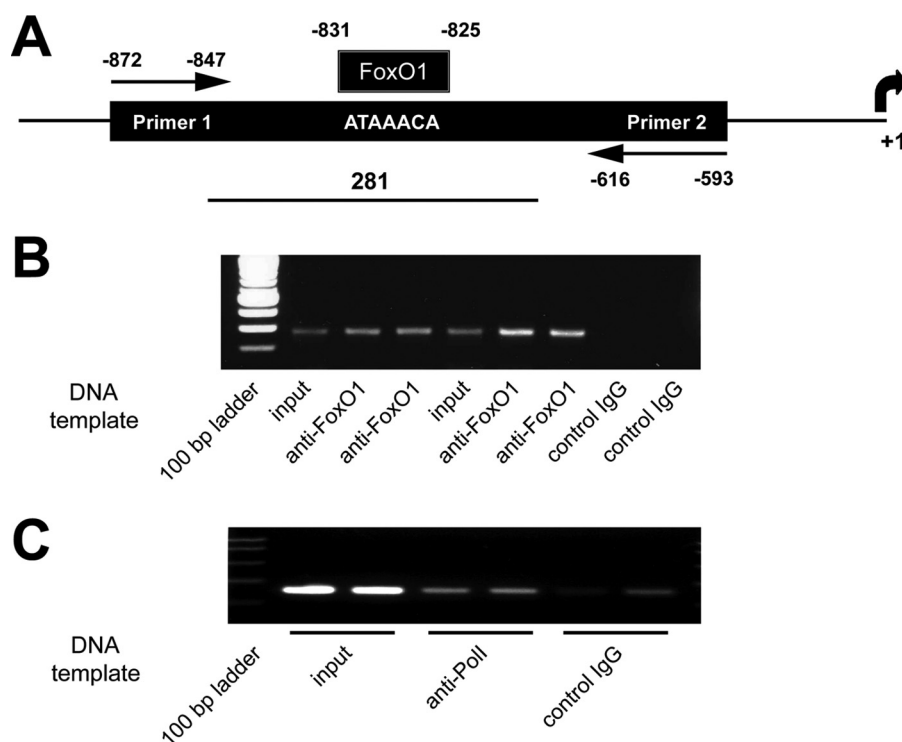


FIGURE 2. **ChIP assay confirming FoxO1 binds to the identified FoxO1 binding sequence in BTC6 cells.** *A*, diagram of the ChIP assay showing the primers spanning a 281-bp sequence that contains the FoxO1 binding sequence in the mouse PPAR γ promoter sequence. *B*, representative gel showing that input DNA and FoxO1 antibody-precipitated chromatin fragments contain the correct 281-bp product and the absence of a band with the control nonimmune IgG. *C*, positive control based on binding of RNA polymerase II to the transcription factor EFl- α (middle two lanes) and only a minimal product with the control IgG. Each lane represents an individual sample.

pancreas morphology and β -cell mass, insulin content, and insulin secretion (17, 22). Islet lysates from 8-week-old FoxO1 $^{+/-}$ mice had twice the PPAR γ mRNA and protein levels of WT mice (Fig. 4). Pdx1 mRNA and protein levels also were increased 2-fold in the FoxO1 $^{+/-}$ islets.

We next studied mice that hyperexpress a constitutively nuclear FoxO1 transgene in β -cells and liver; the transgene encodes an amino acid substitution at the Ser 253 phosphorylation site that prevents insulin-stimulated translocation of the mutant FoxO1 to the cytoplasm and is under control of the transthyretin promoter that directs expression to liver and β -cells (22). Others have shown that these mice on a mixed background have reductions in islet Pdx1 levels and early onset mild diabetes (13, 22). We backcrossed the transgene to the C57BL/6 background and confirmed glucose intolerance in 8–10-week-old mice, also noting a 25% reduction in β -cell mass compared with the littermate WT mice (Fig. 5A). As predicted, islet lysate immunoblots revealed a 45% reduction of PPAR γ and a similar loss of Pdx1 versus WT islets (Fig. 5B).

PPAR γ Is Required for GLP-1 Induction of Pdx1 Gene Expression in Vitro—We used GLP-1, a known mediator of FoxO1 nuclear translocation (20), to probe the FoxO1/PPAR γ /Pdx1 system. Normal mouse islets were incubated for 8 h with or without 100 nM GLP-1(7–36 amide) at 11.1 mM glucose followed by real time PCR analysis. PPAR γ and Pdx1 signals were increased 2–2.5-fold whereas MafA and FoxA2 were unchanged, showing the selectivity of the effect (Fig. 6A). We used this protocol with islets from PANC PPAR $\gamma^{-/-}$ mice. In agreement with the prior results, GLP-1 stimulation nearly

doubled PPAR γ and Pdx1 mRNA expression in the floxed control mice. In contrast, in the PANC PPAR $\gamma^{-/-}$ islets a GLP-1-induced increase in Pdx1 expression was absent (Fig. 6B), demonstrating that PPAR γ is essential for *in vitro* GLP-1 activation of Pdx1 expression.

PPAR γ Regulates PC Expression in Islet β -Cells—We have shown that PPAR γ transcriptionally regulates Pdx1 (4, 5) and GIP receptor genes (6) in β -cells. Others have shown PPAR γ regulation of PC expression in white and brown adipose tissue (7). We previously studied ZF rats that are obese, hyperlipidemic, and insulin-resistant secondary to a mutated leptin receptor, but are virtually normoglycemic because of compensatory increases in β -cell mass and function (26, 27). Those studies showed that PC plays a critical role in their β -cell adaptive response by preventing dysmetabolism-induced defects in β -cell mitochondrial fuel metabolism (27). Also, reduced islet PC expression is found in rodents (28, 29) and humans with type 2 diabetes (30).

We investigated whether β -cell PC expression is PPAR γ -regulated using an established siRNA for PPAR γ in INS-1 cells that lowers PPAR γ mRNA and protein 75% (4). Cells were treated 72 h with or without the PPAR γ agonist troglitazone. PPAR γ siRNA reduced PC mRNA band intensity >60% (Fig. 7A, first and fourth lanes) whereas troglitazone enhanced it 3-fold (second lane); that effect was lost when the PPAR γ siRNA and troglitazone were used together (third lane).

We previously developed and characterized a diabetic rat model by performing a 60% Px in ZF rats (8). Px ZF rats became hyperglycemic (\sim 15 mM) by 3 weeks after surgery with the

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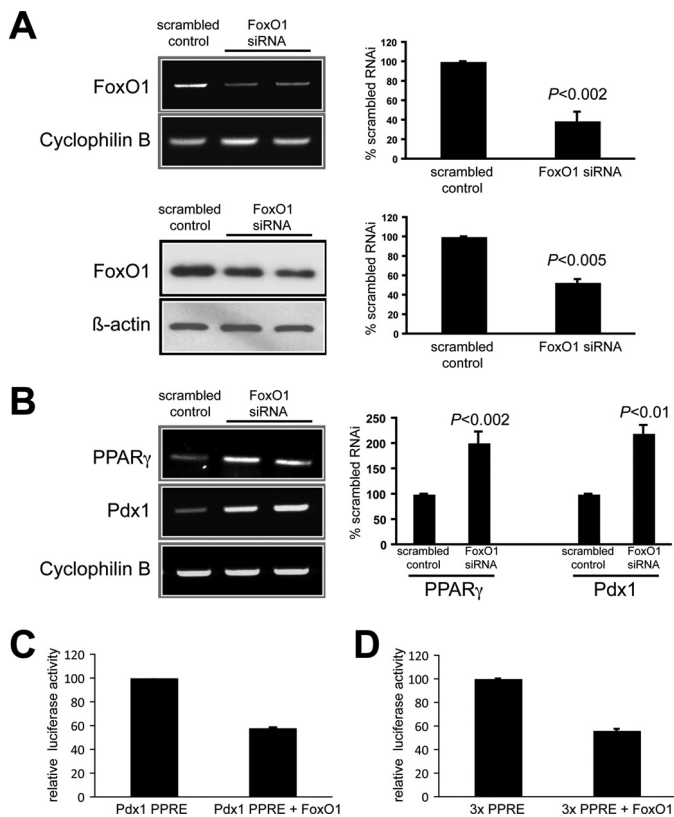


FIGURE 3. *In vitro* studies showing FoxO1 inhibits PPAR γ expression in INS-1 cells. A, 96-h FoxO1 siRNA showing marked reductions in FoxO1 mRNA (top row, gel on left and mean \pm S.D. (error bars) results of three experiments on right) and protein (bottom row, blot on left and mean \pm S.D. results of three experiments on right) compared with control scrambled siRNA transfections. B, siRNA-induced reduction in FoxO1 expression resulting in a doubling of PPAR γ and Pdx1 mRNA levels. Left, representative gel. Right, mean \pm S.D. results of three experiments. Each lane on the gels represents individual samples. C, transfection of pTAL-PPRE-Pdx1 promoter luciferase reporter system in INS-1 cells, showing a large reduction in gene expression activity when cotransfected with pcDNA3-FLAG-FKHR FoxO1 overexpression cassette. $n = 6$, $p < 0.001$. D, very similar results obtained with the 3 \times PPRE-luciferase vector in INS-1 cells, again showing a large reduction in gene expression activity with cotransfection of the pcDNA3-FLAG-FKHR FoxO1 cassette. $n = 6$, $p < 0.001$.

same free fatty acid levels and obesity as normoglycemic ZF shams. Additional controls were sham and 60% Px ZL rats that were euglycemic and metabolically indistinguishable. Islet immunoblots from 3 week postsurgery sham ZF rats had a doubling of PPAR γ protein and its transcriptional target GIP receptor compared with both groups of ZL rats, whereas the diabetic Px ZF rats displayed 20–30% reductions *versus* the ZL rats (6), supporting our hypothesis of importance for PPAR γ signaling in β -cell compensation and failure. We predicted a similar pattern for PC and have now assessed PC expression using streptavidin A affinity labeling of SDS-PAGE-resolved islet extracts from 3-week postsurgery Px and sham ZL and ZF rats. As expected, the band intensity in the sham ZF islets was nearly twice that of the ZL rats ($171 \pm 10\%$) whereas it was subnormal in the diabetic Px ZF rats ($67 \pm 12\%$, Fig. 7B). Comparable results were obtained for PC activity of islet extracts (Fig. 7C), with a 60% increase in sham ZF islets *versus* ZL rats ($p < 0.001$), but no increase in the Px ZF islets ($p < 0.002$ sham ZF *versus* Px ZF).

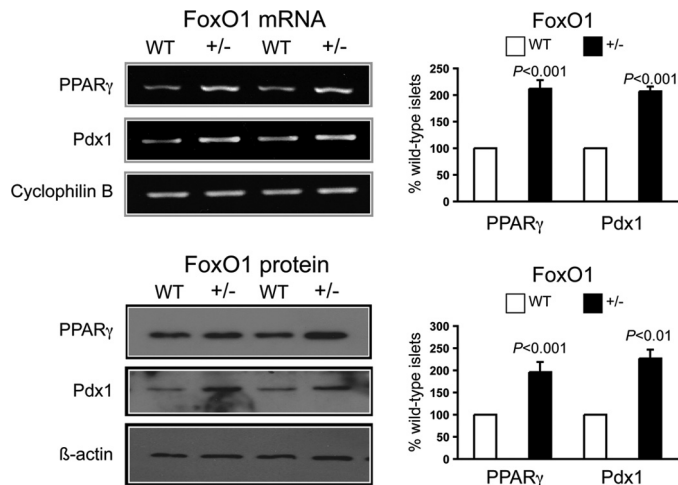


FIGURE 4. Increased islet PPAR γ and Pdx1 expression in 8-week-old FoxO1-haploinsufficient mice. In agreement with the siRNA results in INS-1 cells, PPAR γ and Pdx-1 mRNA (top panels) and protein (bottom panels) levels were doubled in isolated islets from global FoxO1-haploinsufficient mice. Left, representative gels showing results for two WT and two FoxO1-haploinsufficient mice. Right, mean \pm S.D. results of three mice in each group.

Nuclear Localization of FoxO1 in β -Cells of Hyperglycemic Rats—We sought an explanation for the markedly lowered expression of PPAR γ and its target genes in diabetic ZF rats by examining the subcellular distribution of β -cell FoxO1 in 3-week postsurgery Px and sham ZL and ZF rats using confocal microscopy (Fig. 8). In both ZL groups, FoxO1 immunoreactivity was chiefly cytoplasmic (the hyperintense staining areas are erythrocytes in capillaries). In sham ZF rats, the FoxO1 staining was both nuclear and cytoplasmic. In contrast, in the Px ZF rats a striking increase in nuclear FoxO1 immunoreactivity was observed. In addition, in both ZL groups and sham ZF rats there was strong nuclear Pdx1 staining, whereas in the Px ZF rats the nuclear Pdx1 signal was markedly reduced. We conclude from these findings that in the diabetic ZF rats a defect in FoxO1 activation and/or translocation causes loss of the adaptive increase in the FoxO1/PPAR γ pathway that is present in the nondiabetic insulin-resistant ZF rats.

DISCUSSION

The current results have established PPAR γ expression in β -cells is under transcriptional restraint by FoxO1. That conclusion is supported by our having identified a FoxO1 binding site in the mouse PPAR γ promoter with homologous sequences in rats and humans, and *in vitro* studies demonstrating FoxO1 binding and inhibition of PPAR γ expression. Also, transgenic mice with altered FoxO1 expression or defective islet FoxO1 nuclear translocation exhibited changes in islet PPAR γ expression and parallel findings for its target gene *Pdx1*. Of particular importance was our demonstrating that PPAR γ is necessary for GLP-1 mediated augmentation of *Pdx-1* expression *in vitro*, as GLP-1 is an established FoxO1 activator (20). We have also shown that PPAR γ regulates PC gene expression in β -cells, as has previously been characterized using molecular and *in vivo* techniques in white and brown adipose (7), making it the third PPAR γ target gene we have identified in β -cells along with the *Pdx1* (4, 5) and GIP receptor (6) genes.

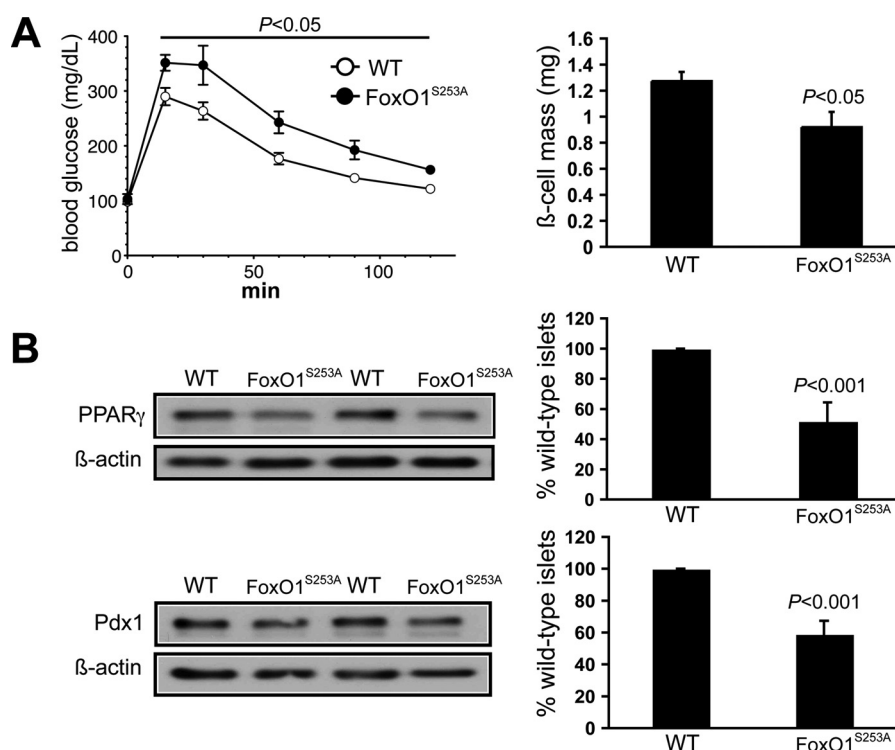


FIGURE 5. Reduced islet PPAR γ and Pdx1 expression in mice that hyperexpress a constitutively nuclear FoxO1 transgene with amino acid substitution at the Ser²⁵³ phosphorylation site preventing insulin-stimulated translocation of the mutant FoxO1 to the cytoplasm, under control of the transthyretin promoter directing expression to the liver and β -cells. A, results in 8–10-week-old FoxO1^{S253A} and WT mice showing glucose intolerance in the FoxO1^{S253A} mice assessed by intraperitoneal glucose tolerance test (left) and a 25% reduction in the β -cell mass in FoxO1^{S253A} mice (right). Values are mean \pm S.E. (error bars). B, islet immunoblotting results for PPAR γ and Pdx1 in WT and FoxO1^{S253A} mice. Left, representative gels showing results for two WT and two FoxO1^{S253A} mice. Right, mean \pm S.D. results of three mice in each group.

These results are especially notable because of the importance of the identified PPAR γ target genes for regulating β -cell development and postnatal β -cell mass and function. Pdx1 is an essential transcription factor for pancreas morphogenesis and postnatally is a key regulator of β -cell differentiation, maturation, and survival (31). It is required for the normal adaptive increase in β -cell mass and function to insulin resistance (32, 33). Distal from *Pdx1* is a network of genes that impact diverse aspects of β -cell signaling, mass, and function (34) including glucokinase with its well known regulatory effects on glycolysis, insulin secretion, and β -cell proliferation (35).

PC catalyzes mitochondrial pyruvate conversion to malate. In most cells, the normal pathway for pyruvate metabolism is through pyruvate dehydrogenase with entry into the citric acid cycle for ATP production. However in β -cells there is unusually high PC expression so that 50% of pyruvate undergoes carboxylation (36) with entry into the anaplerotic pathways of insulin secretion (37). Obese, insulin-resistant, hyperlipidemic ZF rats normally do not develop diabetes because of compensatory increases in β -cell mass and function (27). We previously looked for the Randle effect (inhibition of pyruvate dehydrogenase activation by elevated levels of free fatty acid) in islets from ZF rats and found 30% lowered pyruvate dehydrogenase activity, but, paradoxically, glucose oxidation and glucose-induced insulin secretion were increased. The explanation was increased PC expression with diversion of pyruvate metabolism to the anaplerotic pathways (this study and Ref. 27). We observed the same protective effect in high glucose-cultured rat

islets (38). Also, Liu and co-workers showed a role for this enzyme in the β -cell mass and functional adaptation to a 60% Px in rats (23, 39). As such, PC is believed to play a key role in the β -cell adaptation to insulin resistance and dysmetabolism by preserving mitochondrial fuel metabolism (37, 40).

The β -cell GIP receptor mediates that incretin hormone regulation of insulin secretion and glucose tolerance. GIP receptor gene knock-out mice are glucose-intolerant (41). Also, GIP receptor allelic variations correlate with the heterogeneity in serum glucose and insulin levels after oral glucose challenge in nondiabetic humans (42).

The second aspect of our results relates to evidence that this signaling pathway is enhanced with successful β -cell compensation to insulin resistance, whereas defective nuclear translocation of FoxO1 resulting in persistent inhibition of PPAR γ expression is a feature of failed β -cell compensation and hyperglycemia. We previously studied ZF rats made hyperglycemic by a 60% pancreatectomy and observed subnormal islet expression of PPAR γ and its target gene GIP receptor *versus* the hyperexpression in nondiabetic ZF rats (6). The current study has shown the same pattern for PC along with reduced nuclear Pdx1 immunostaining in Px ZF rats, which are in accord with our previous report showing subnormal PC and Pdx1 mRNA expression in Px ZF islets (8).

Insight into the impaired PPAR γ expression with hyperglycemia came with our finding a distinct change in the subcellular localization of FoxO1 in β -cells of ZF diabetic rats, specifically, instead of the nuclear and cytoplasmic pattern seen in the con-

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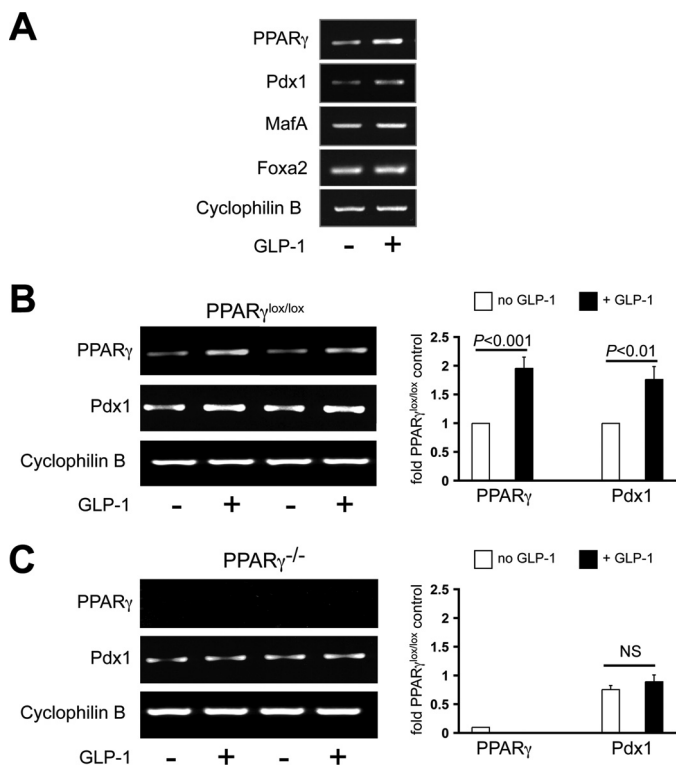


FIGURE 6. PPAR γ is required for GLP-1 induction of Pdx1 expression in mouse islets. *A*, normal mouse islets were incubated 8 h with or without 100 nM GLP-1(7–36 amide) followed by real time PCR analysis. Expression of PPAR γ and Pdx1 was doubled compared with no observable effect on MafA or Foxa2 expression. *B* and *C*, that protocol was used in isolated islets from 8-week-old floxed control mice (*B*) and PANC PPAR γ ^{-/-} mice (*C*) with the normal GLP-1 induction of Pdx1 expression absent in the PANC PPAR γ ^{-/-} islets. *Left*, representative gels with each lane representing islets from an individual mouse. *Right*, mean \pm S.D. (error bars) results of three mice in each group.

trol rats, there was an intense nuclear distribution. This finding is not unique to the Px ZF model, as nuclear FoxO1 and reduced overall islet FoxO1 expression were found in insulin-resistant diabetic mice created by deletion of insulin receptors in GLUT4-expressing tissues (43). Also, below normal islet expression of Pdx1, GIP receptor, and PC is well known to occur in animals (6, 28, 29, 44) and humans (30, 45, 46) with type 2 diabetes. Based on this collective evidence, we propose that nuclear retention of FoxO1 impairing PPAR γ -mediated defenses against metabolic stresses is an unrecognized feature of failed β -cell adaptation.

The mechanism of the defective nuclear to cytoplasmic translocation of FoxO1 with diabetes is not known, as the regulation of FoxO1 activity related to compartmentalization in β -cells is multifactorial related to several post-translational modifications and protein interactions (14, 18, 21). Best known for the β -cell is insulin or growth factor PI3K/Akt pathway-induced phosphorylation causing nuclear export that removes its inhibition of target gene transcription (14). Supporting a defect at this level of the signaling pathway is a study performed in New Zealand obese mice that became diabetic when fed a carbohydrate-rich diet; reductions in islet FoxO1 phosphorylation were noted (47). Furthermore, islets from these mice, and MIN 6 cells, incubated with high glucose and palmitate for 48 h underwent dephosphorylation of FoxO1 along with its

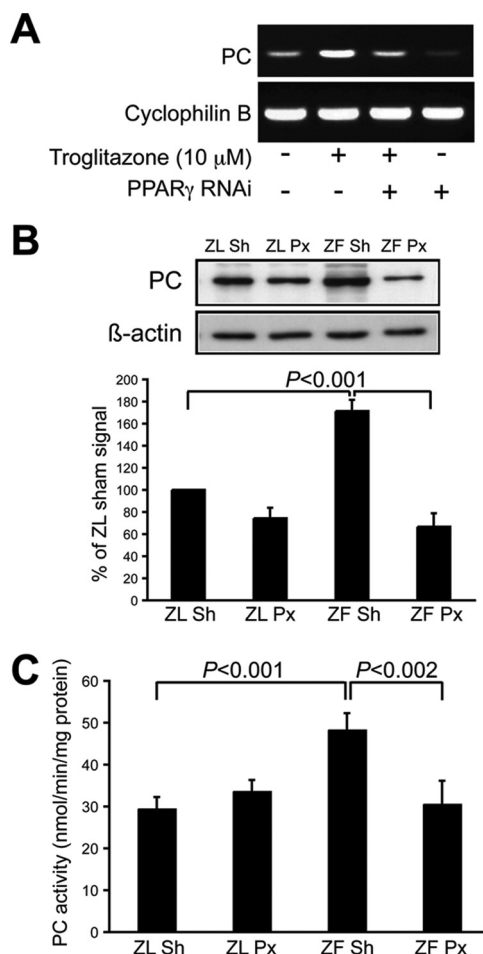


FIGURE 7. PPAR γ regulates PC gene expression in β -cells. *A*, representative results of PPAR γ siRNA in INS-1 cells with or without the PPAR γ agonist troglitazone, showing a 60% reduction in PC mRNA with the PPAR γ siRNA (*first* and *fourth* lanes) whereas troglitazone enhanced it 3-fold (*second* lane); the latter was lost when the PPAR γ siRNA and troglitazone were used together (*third* lane). *B*, PC expression assessed with streptavidin A affinity labeling of PAGE-resolved islet extracts from 3 weeks after surgery 60% Px and sham ZL and ZF rats. Sham (Sh) ZF rats are obese and insulin-resistant, but normoglycemic because of compensatory increases in β -cell mass and function. In contrast, Px ZF rats become hyperglycemic (\sim 15 mM) by 3 weeks after surgery with the same obesity and free fatty acid levels as the normoglycemic ZF shams. As we have seen with other PPAR γ -regulated genes in β -cells, the PC band intensity in the sham ZF islets was nearly twice that of the ZL rats and below normal in the diabetic Px ZF rats. *Top*, representative gel. *Bottom*, mean \pm S.D. (error bars) results of four rats in each group. *C*, PC enzyme activity measured in islet extracts from 3 weeks after surgery 60% Px and sham ZL and ZF rats. Results are mean \pm S.D. of three rats in each group.

upstream kinase Akt (47). Another study reported reduced insulin receptor and insulin receptor substrate 2 expression in islets from humans with type 2 diabetes, and it noted nuclear retention of FoxO1 in islets of mice with a β -cell-specific deletion of insulin receptors (48). Alternatively, FoxO1 activity and its subcellular compartmentation in β -cells is regulated by other serine/threonine kinases as well the balance between acetylation and deacetylation (21). Recently β -cell oxidative stress was linked to hyperexpression of a B55a-containing PP2A holoenzyme that dephosphorylates FoxO1 and promotes its nuclear location (49).

The importance of PPAR γ in β -cells has been controversial. A β -cell-specific knock-out mouse for PPAR γ based on a rat insulin promoter Cre/loxP recombinase system was reported to

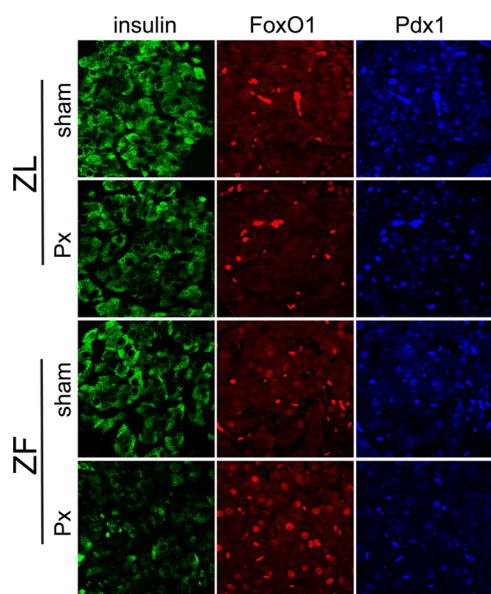


FIGURE 8. β -Cell FoxO1 and Pdx1 subcellular distribution in normoglycemic and diabetic rats. Representative islet confocal fields ($n = 3/\text{group}$) showing mainly cytoplasmic FoxO1 immunoreactivity and nuclear Pdx1 immunoreactivity in the β -cells of normoglycemic sham-operated and 60% Px ZL rats (top panels). In the β -cells of normoglycemic ZF-sham rats, there was both nuclear and cytoplasmic FoxO1 distribution along with strongly nuclear Pdx1. However, in the hyperglycemic ZF-Px rats (bottom panel), FoxO1 staining was principally nuclear along with decreased Pdx1 immunoreactivity. For reference, β -cells are stained green. Bright, autofluorescing bodies are erythrocytes in islet capillaries. Field widths are 138 μm .

be normoglycemic with a standard chow diet and also after fat feeding (50). Another publication from these authors (51) using a tamoxifen-inducible Pdx1 Cre/loxP system that lowered islet PPAR γ mRNA by 90% found no β -cell phenotype after 7 months of normal chow; glucose tolerance, insulin secretion, and β -cell mass were unchanged from WT mice as was expression of multiple islet genes including *Pdx1*, pyruvate carboxylase, and GIP receptor. In addition, 11 weeks of a high fat diet induced the same hyperglycemia, obesity, and insulin resistance in the PPAR γ knock-out and WT mice. Also, there was no difference in β -cell mass or islet gene expression profiles between the fat-fed WT and PPAR γ knock-out mice although the atypical finding of no effect of fat feeding on these parameters in WT mice makes these latter findings difficult to interpret. The authors concluded that PPAR γ in β -cells is inconsequential. In contrast, we studied noninducible Pdx-1 Cre/loxP PPAR γ -null mice (52) and found hyperglycemia at 8 weeks of age with standard chow along with near total absence of glucose-induced and GIP-induced insulin secretion in isolated islets, and a marked reduction in islet expression of Pdx-1 and GIP receptor (5, 6). There was no difference in β -cell mass or pancreas histology from control mice (5). As such, this mouse showed a phenotype of functional β -cell failure, with an expression profile that agreed exactly with the proposed signaling pathway in this study. Also, others have reported PPAR γ regulation of gene expression for GLUT2 (53), glucokinase (54), and GPR40 (55) in β -cells, plus key modulatory effects of PPAR γ in β -cell endoplasmic reticulum stress related to a cytokine-induced loss of SERCA2 expression have been shown (56, 57). In addition, numerous β -cell effects have been attributed to

PPAR γ from thiazolidinedione studies in islets, insulin-containing cell lines, and various animal models, although direct proof that the findings are PPAR γ -mediated is mostly lacking. Thus, although the basis for the different β -cell phenotypes in the mouse models is unknown, we conclude that there is extensive support for an important regulatory role for PPAR γ in β -cells, especially in the adaptation response to metabolic stresses.

In summary, our results are consistent with a novel mechanism for β -cell compensation and failure based on an integral relationship between FoxO1 and PPAR γ , whereby FoxO1 exerts hierarchal control over several key β -cell genes, and PPAR γ is a vital intermediate. FoxO1 is well poised to be a master transcriptional regulator over β -cell adaptive responses, as its expression and activity are influenced by numerous stimuli such as nutrient overload, growth factors, incretin hormones, and oxidative stress (13, 14). Downstream from PPAR γ is a network of genes that exert important regulatory control over prodifferentiation processes, incretin effects, glucose and mitochondrial fuel metabolism, and β -cell compensation to obesity and insulin resistance (13, 27, 32, 33). We have shown that islet PPAR γ and its target genes are hyperexpressed in nondiabetic insulin-resistant rats (present study and Refs. 6, 8) and rats during the β -cell adaptation after a partial pancreatectomy (4, 6), plus we and others have shown impaired expression of these same genes in diabetic animals (6, 29, 44) and humans with type 2 diabetes (30, 45, 46). Collectively, these findings support, but do not prove, a FoxO1/PPAR γ -mediated transcriptional network functioning as a core element of how β -cells adapt to metabolic stress, with failure of this response causing or exacerbating diabetes.

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