Rosiglitazone Promotes PPARγ-Dependent and -Independent Alterations in Gene Expression in Mouse Islets

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The glitazone class of insulin-sensitizing agents act, in part, by the activation of peroxisome proliferator-activated receptor (PPAR)-γ in adipocytes. However, it is unclear whether the expression of PPARγ in the islets is essential for their potential β-cell-sparing properties. To investigate the *in vivo* effects of rosiglitazone on β-cell biology, we used an inducible, pancreatic and duodenal homeobox-1 enhancer element-driven, Cre recombinase to knockout PPARγ expression specifically in adult β-cells (PPARgKO). Subjecting the PPARgKO mice to a chow diet led to virtually undetectable changes in glucose or insulin sensitivity, which was paralleled by minimal changes in islet gene expression. Similarly, challenging the mutant mice with a high-fat diet and treatment with rosiglitazone did not alter insulin sensitivity, glucose-stimulated insulin secretion, islet size, or proliferation in the knockout mice despite PPARγ-dependent and -independent changes in islet gene expression. These data suggest that PPARγ expression in the β-cells is unlikely to be directly essential for normal β-cell function or the insulin-sensitizing actions of rosiglitazone. (***Endocrinology*** **153: 4593–4599, 2012)**

Type 2 diabetes is a growing health care problem worldwide and continues to create a large economic burden. The pathophysiology of type 2 diabetes is characterized by insufficient insulin secretion to maintain normal glycemia, with a background of insulin resistance. The thiazoldinedione (TZD) class of drugs, which acts as ligands of the nuclear transcription factor, peroxisome proliferator-activated receptor (PPAR)-γ (1), have been widely used to effectively treat diabetes. TZD act mainly by increasing peripheral insulin sensitivity, predominantly by activating PPARγ in adipocytes promoting changes in lipid storage and circulation (2, 3), thus preventing lipid accumulation in nonadipose tissues, such as liver and skeletal muscle. However, there is also evidence that these drugs can directly improve β-cell function (4, 5).

In rodent models of diabetes, treatment with TZD has been shown to be effective at protecting islets against the destruction that occurs during the diabetes process and to improve β-cell function and insulin secretion (6–10) with similar observations made in some human studies (11). It has been proposed that the antidiabetic actions of TZD are due to direct activation of PPARγ in pancreatic β-cells, although *in vivo* evidence to support this action is lacking. PPARγ has been suggested to protect against fatty acid induced toxicity in β-cells (12) and to directly promote transcription of islet specific genes, including pancreatic and duodenal homeobox-1 (PDX1) (5, 13), gastric inhibitory polypeptide receptor (GIPR) (14), glucokinase (4), and glucose transporter 2 (Glut2) (15). However, the functional role of PPARγ in β-cells is unclear as there is also

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Abbreviations: ABCA1, ATP-binding cassette transporter A1; BrdU, 5-bromo-2′-deoxyuridine, GIPR, gastric inhibitory polypeptide receptor; GK, glucokinase; Glut2, glucose transporter 2; HSL, hormone-sensitive lipase; KO, knockout; LXR, liver X receptor; PDX1, pancreatic and duodenal homeobox-1; PPAR, peroxisome proliferator-activated receptor; SCD1, steroyl CoA desaturase; TZD, thiazoldinedione; WT, wild type.

evidence to suggest that PPARγ has no effect on, or is detrimental to, β-cell function and survival (16–20).

To directly assess the function of PPARγ in β-cells, we created an inducible β-cell-specific knockout (KO) mouse model. The data from these animals suggest that PPARγ is not essential for normal β-cell function and survival *in vivo* and that the antidiabetic actions of rosiglitazone are not directly dependent on PPARγ activation in the islets.

Materials and Methods

Mice

Study populations of mice were generated by breeding PPARγfl/fl mice (from Bruce Spiegelman, Dana-Farber Cancer Institute and Department of Cell Biology, Harvard Medical School, Boston, MA) (21) with mice expressing the tamoxifen-inducible cre driven by the PDX-1 enhancer element, pdx1^{PB}CreER^{TM} [from Maureen Gannon. Ph.D., Department of Medicine, Vanderbilt University, Nash-

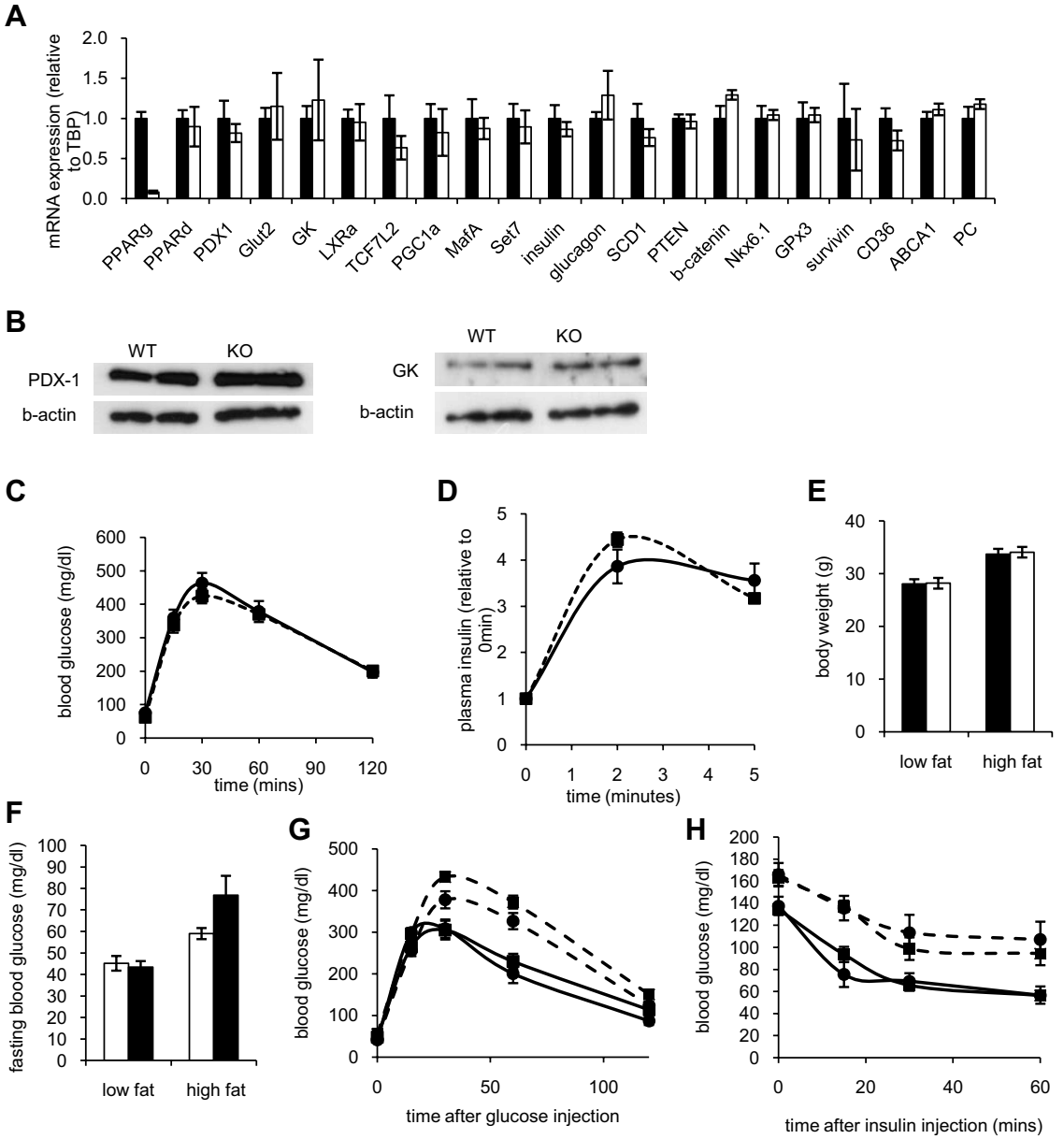

FIG. 1. Characterization of Pdx1^{PB}CreER^{TM} PPARγ KO mice on a normal or high-fat diet. A and B, Twenty-five weeks after tamoxifen injection, mRNA expression levels were measured in islets (A). *Black bars*, PPARγfl/fl (WT); *white bars*, PPARγfl/fl/pdx1^{PB}CreER^{TM} (KO). Results are shown as mean ± SEM (n = 5/6). B, Protein levels of PDX-1 and GK. C, Glucose tolerance tests 30 wk after tamoxifen injection. Glucose levels were measured from tail blood after ip glucose injection of 2 g/kg glucose body weight. D, Thirty-two weeks after tamoxifen injection, a glucose-stimulated insulin secretion test was performed with an ip glucose injection (3 g/kg body weight). *Solid line with circles*, PPARγfl/fl (WT); *dashed lines with squares*, PPARγfl/fl/pdx1^{PB}CreER^{TM} (KO). Results are shown as mean ± SEM (n = 10/11). E–H, Tamoxifen-injected mice were placed on a low-fat (10% calories from fat) or high-fat diet (60% calories from fat) for 10 wk, after which body weight (E) and fasting blood glucose levels (F) were measured and glucose tolerance (G) and insulin sensitivity (H) tests carried out. In the insulin sensitivity test, blood glucose levels were determined after injection of insulin (1 U/kg body weight). *Black bars*, PPARγfl/fl (WT); *white bars*, PPARγfl/fl/pdx1^{PB}CreER^{TM} (KO); *solid lines*, Low-fat diet; *dashed lines*, high-fat diet; *circles*, WT; *squares*, KO. Results are shown as mean ± SEM (n = 7).

ville, TN (22)]. Two populations of mice were used, namely, PPARγfl/fl/pdx1^{PB}CreER^{TM} (referred to as KO) and PPARγfl/fl [wild type (WT)]. Because mice were maintained on a mixed FVB/129/C57 background, littermates served as controls for all experiments. Genotyping was performed as described previously (21, 22). To activate the CreER^{TM} and induce recombination of the PPARγ flox gene, 6-wk old mice were injected sc with 400 μl of tamoxifen (8 mg/ml in corn oil), on three occasions at 48-h intervals. Detection of recombination was performed on islet genomic DNA (21).

All animals were housed in specific pathogen-free facilities and maintained on a 12-h light, 12-h dark cycle and fed standard rodent chow (except where otherwise stated) at the Foster Animal Laboratory, Brandeis University (Waltham, MA). All protocols for animal use were approved by the Institutional Animal Care and Use Committee of the Joslin Diabetes Center and Brandeis University and were in accordance with National Institutes of Health guidelines.

Physiological measurements

Blood glucose values from whole venous blood were determined using an automated glucose monitor (Contour; Bayer, Indianapolis, IN). Serum insulin levels and C-peptide levels were measured using ELISA (Crystal Chem Inc., Chicago, IL), and plasma glucagon was measured by RIA (Linco Research Inc., St. Charles, MO). Glucose tolerance tests and acute insulin secretion tests were performed on animals that had been fasted overnight for 16 h (23). Insulin tolerance tests were performed after a 3-h morning fast (23).

The high-fat diet (60% calories from fat; D12492) and the corresponding low-fat diet (10% calories from fat; D12450B) were purchased from Research Diets (New Brunswick, NJ). The high-fat diet containing 20 mg/kg rosiglitazone (Cayman Chemicals, Ann Arbor, MI) was custom made by Research Diets. Mice fed with rosiglitazone showed an average plasma level of rosiglitazone of 398 ± 38 nM.

β-Cell mass, size, and immunohistochemistry

Mice were injected with 100 mg/ml 5-bromo-2′-deoxyuridine (BrdU) 5 h before being anesthetized, and the pancreas was rapidly dissected, weighed, fixed in Z-fix solution, and sectioned for immunostaining (21).

Real-time quantitative RT-PCR

Islets were isolated from mice by collagenase digestion as described previously (24), and RNA was isolated using an RNeasy minikit (QIAGEN, Valencia, CA), and 500 ng of RNA was applied to a reverse transcriptase (RT) One-Step system (Applied Biosystems, Foster City, CA) to produce cDNA. Quantitative real-time RT-PCR was performed; cDNA samples were amplified by SYBR Green PCR master mix (Applied Biosystems) and analyzed on an ABI PRISM 7900 sequence detection system (Applied Biosystems). Primer sequences are available on request.

Statistics

Data are presented as means ± SEM and were analyzed using an unpaired two-tailed Student's *t* test or ANOVA as appropriate. A $P < 0.05$ was considered significant.

TABLE 1. Gene expression in islets of Pdx1^{PB}CreER^{TM} PPARγ KO mice treated with a high-fat diet and rosiglitazone

	Low fat		High fat		High fat + Rosi	
Gene	WT	KO	WT	KO	WT	KO
PPARγ	0.65 ± 0.13	0.11 ± 0.03[a]	0.58 ± 0.09	0.11 ± 0.01[a]	0.72 ± 0.12	0.17 ± 0.03[a]
PDX-1	8.04 ± 1.6	7.17 ± 0.9	7.03 ± 0.84	8.03 ± 0.97	7.09 ± 1.19	7.02 ± 1.02
Glut2	2.02 ± 0.77	2.63 ± 0.88	2.34 ± 0.38	2.5 ± 0.36	2.73 ± 0.47	4.39 ± 0.92
GK	3.38 ± 1.02	2.55 ± 0.6	2.03 ± 0.3	2.94 ± 0.5	2.83 ± 0.39	2.61 ± 0.17
GIPR	0.69 ± 0.06	0.87 ± 0.03[a]	0.86 ± 0.17	0.91 ± 0.2	1.15 ± 0.14[b]	0.92 ± 0.07
PC	0.53 ± 0.03	0.74 ± 0.11[a]	0.75 ± 0.06[b]	0.68 ± 0.1	0.79 ± 0.07[b]	0.83 ± 0.08
LXRα	0.78 ± 0.09	1.02 ± 0.07	0.91 ± 0.1	0.97 ± 0.09	1.12 ± 0.11[b]	1.04 ± 0.04
ABCA1	4.98 ± 0.96	6.22 ± 0.84	4.79 ± 0.94	5.55 ± 0.94	9.53 ± 1.05[b, c]	6.93 ± 0.42
Survivin	0.15 ± 0.02	0.12 ± 0.02	0.12 ± 0.03	0.09 ± 0.02	0.04 ± 0.01[b, c]	0.04 ± 0.004[b,c]
GPx3	20.91 ± 3.7	23.88 ± 2.7	19.54 ± 2.8	19.52 ± 2.5	22.97 ± 4.2	21.02 ± 2.0
MafA	20.42 ± 1.69	19.29 ± 5.09	34.84 ± 8.36	36.68 ± 5.15[b]	22.42 ± 2.99	24.21 ± 3.56
PGC1α	0.54 ± 0.09	0.52 ± 0.11	0.44 ± 0.11	0.69 ± 0.08	0.54 ± 0.1	0.51 ± 0.08
PPARδ	1.34 ± 0.12	2.01 ± 0.48	1.53 ± 0.21	1.67 ± 0.1	2.06 ± 0.29	1.62 ± 0.07
PTEN	8.51 ± 0.73	10.06 ± 0.44	10.13 ± 0.77	8.81 ± 2.03	11.47 ± 2.04	10.13 ± 0.74
SCD1	0.1 ± 0.01	0.12 ± 0.03	0.13 ± 0.02	0.11 ± 0.03	0.16 ± 0.02[b]	0.1 ± 0.01[a]
Nkx6.1	17.71 ± 7.4	15.03 ± 2.2	14.86 ± 2.64	19.11 ± 2.5	16.50 ± 1.23	16.57 ± 1.95
HSL	0.01 ± 0.001	0.01 ± 0.002	0.02 ± 0.004	0.02 ± 0.002	0.03 ± 0.003[b]	0.02 ± 0.003[b]
FAS	3.04 ± 0.38	3.51 ± 0.19	3.35 ± 0.68	4.38 ± 0.74	4.18 ± 0.57	4.18 ± 0.66
β-catenin	4.09 ± 0.6	5.03 ± 1.1	5.08 ± 1.1	6.15 ± 1.83	6.13 ± 0.71	6.46 ± 1.35
Insulin	81.9 ± 22.9	90.4 ± 22.4	71.9 ± 13.0	133.5 ± 59.5	101.0 ± 24.0	225.7 ± 115.9
Glucagon	39.74 ± 2.4	43.14 ± 5.6	37.18 ± 11.2	59.19 ± 19.3	45.65 ± 6.7	64.09 ± 17.6

After 10 wk of high fat diet, followed by 1 wk of a high fat diet ± rosiglitazone, mRNA was extracted from isolated islets and used in RT-PCR reactions to determine gene expression levels. Values represent the expression levels of the genes expressed in arbitrary units after normalization to the TATA box binding protein housekeeping gene. Results are expressed as mean ±SEM (n = 5/6). Rosi, Rosiglitazone.

[a] $P < 0.05$ compared with WT on corresponding diet.

[b] $P < 0.05$ compared with corresponding genotype on a low-fat diet.

[c] $P < 0.05$ compared with corresponding genotype on a high-fat diet.

Results and Discussion

β-Cell specific PPARγ knockouts

To investigate the *in vivo* effects of PPARγ in the β-cell, we created a β-cell-specific, PPARγ KO mouse model by crossing PPARγfl/fl mice with a tamoxifen-inducible, β-cell-specific cre expressing strain of mice. Injection of tamoxifen induced genomic recombination of the PPARγ flox gene, resulting in a 90% decrease in PPARγ mRNA levels (Fig. 1A). The islet specificity of the PPARγ gene knockout was confirmed by a lack of changes in DNA recombination and the PPARγ mRNA levels in other tissues in the KO mice (Supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org).

Effect of changes in PPARγ expression and rosiglitazone treatment on islet gene expression

Islets isolated from PPARγ KO mice fed a normal chow diet exhibited minimal changes in gene expression of important β-cell genes including insulin, PDX1, GIPR, glucokinase (GK), pyruvate carboxylase and Glut2 compared with controls (Fig. 1A). In parallel, we did not detect significant differences in protein levels of PDX-1 or GK between groups (Fig. 1B).

To assess the potential effects of *in vivo* rosiglitazone treatment on islet gene expression, we fed mice a high-fat diet for 11 wk and added rosiglitazone to the diet during the final week. We observed changes in gene expression that were both PPARγ dependent and independent (Table 1). For example, steroyl CoA desaturase (*SCD1*), ATP-binding cassette transporter A1 (*ABCA1*) and liver X receptor (*LXR*)α genes were increased by rosiglitazone treatment only in WT but not in PPARγ KO islets. In contrast, we observed an increase in hormone-sensitive lipase (HSL) and a decrease in survivin in both groups, suggesting that these changes were PPARγ independent. In contrast, MafA, a transcription factor that regulates insulin gene transcription (25, 26), showed a trend toward being increased by a high-fat diet but was decreased by rosiglitazone treatment in both WT and KO islets. How-

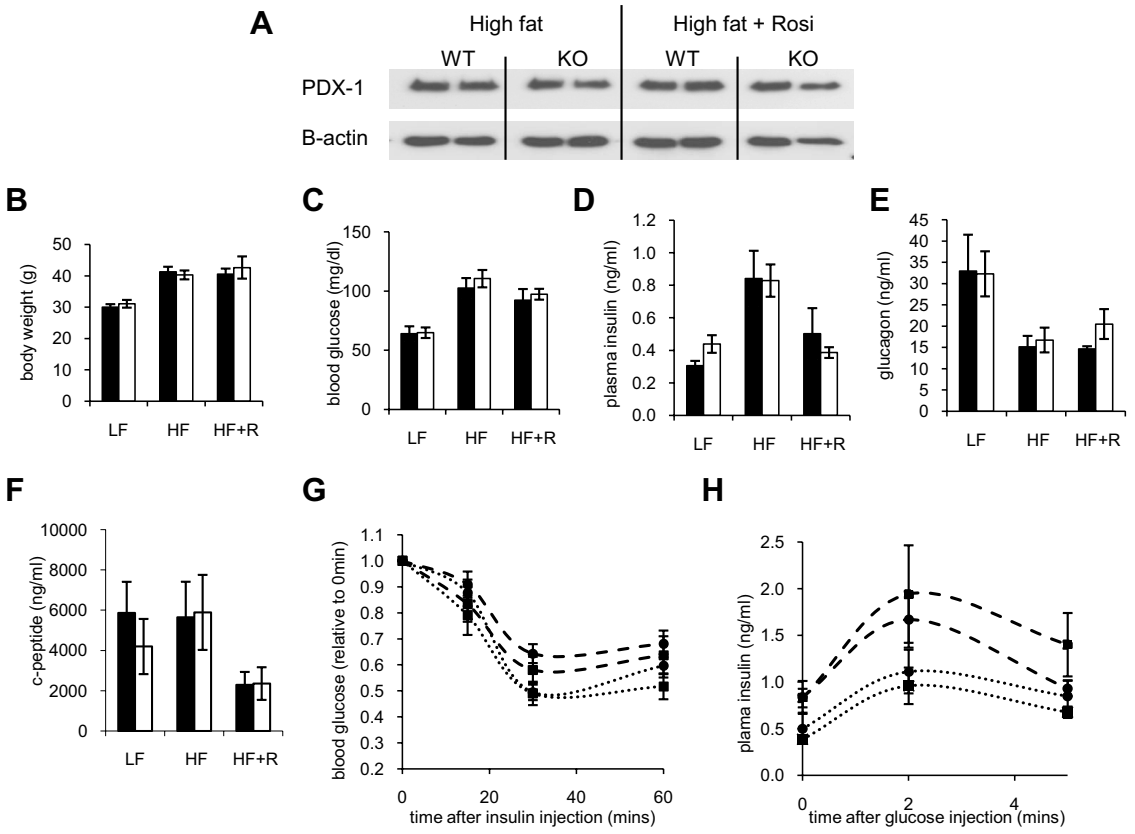

FIG. 2. Physiological characteristics of Pdx1^{PB}CreER^{TM} PPARγ KO mice on a high-fat diet treated with rosiglitazone. A, Tamoxifen-treated mice were fed a high-fat diet for 10 wk, followed by 1 wk of a high-fat diet ± rosiglitazone (20 mg/kg chow). Protein was extracted from isolated islets to determine PDX-1 protein levels. LF, Low-fat diet; HF, high-fat diet; R, rosiglitazone. B–H, Tamoxifen-injected mice were placed on a high-fat diet for 10 wk followed by an additional 6 wk of a high-fat diet ± rosiglitazone. At the end of the study, body weights (B), blood glucose (C), plasma insulin (D), glucagon (E), and c-peptide (F) levels were measured and glucose tolerance (G) and glucose-stimulated insulin secretion (H) tests were carried out. Results are shown as mean ± SEM (n = 6/7). *Black bars*, PPARγfl/fl (WT); *white bars*, PPARγfl/fl/pdx1^{PB}CreER^{TM} (KO); *dashed lines*, high-fat diet; *dotted lines*, high-fat diet + rosiglitazone; *circles*, WT; *squares*, KO.

ever, other important β-cell genes, which have been previously suggested to be regulated by PPARγ, including insulin, glucagon, *PDX1*, *GK*, and *Glut2*, were not significantly altered by either a high-fat diet or rosiglitazone treatment (Table 1). The levels of PDX1 protein levels were also unchanged (Fig. 2A).

The alterations in gene expression after rosiglitazone treatment could potentially have functional consequences for β-cell function. For example, ABCA1, the cholesterol transporter, has been previously suggested to be regulated by PPARγ in β-cells and to influence insulin secretion in both rodents and humans (27, 28). LXR-α, which has been shown to regulate ABCA1 levels (29), was increased by rosiglitazone treatment in a PPARγ-dependent manner in our studies. However, the functional effects of increasing LXRα expression in β-cells are still unclear. In human and rodent islets, activation of LXRα has been shown to increase insulin secretion (30, 31), whereas chronic activation of LXRα in pancreatic β-cells causes growth arrest and promotes lipotoxic apoptosis (32). In contrast, an increase in the expression of SCD1, which converts saturated fatty acids to unsaturated fatty acids in islets, has been reported to protect against lipoapoptosis (33), whereas HSL, which mobilizes fatty acids from stored triglycerides, is implicated in maintaining glucose stimulated insulin secretion after an overnight fast (34). Likewise, survivin has been implicated in the expansion of β-cell mass and prevention of apoptosis (35). Taken together, the changes in gene expression observed in our study suggest that rosiglitazone has both beneficial and detrimental effects, and its overall impact on β-cell function and survival may be context dependent.

Physiological characteristics of islet-specific PPARγ KO mice

On a normal chow diet, we observed no significant changes in the physiological characteristics of PPARγKO mice compared with controls, which correlates with the lack of changes seen in islet gene expression. When studied 7 months after the induction of recombination of the *PPARγ* gene, we observed no significant differences in body weight, plasma insulin, plasma glucagon, plasma glucose (Supplemental Fig. 2), or glucose tolerance (Fig. 1C) between groups. Furthermore, *in vivo* insulin secretion in response to acute ip glucose injection was similar between groups (Fig. 1D).

When challenged with a high-fat diet, both groups of mice exhibited increased body weight, accompanied by fasting hyperglycemia, impaired glucose tolerance, and increased insulin resistance (Fig. 1, F–H). However, we

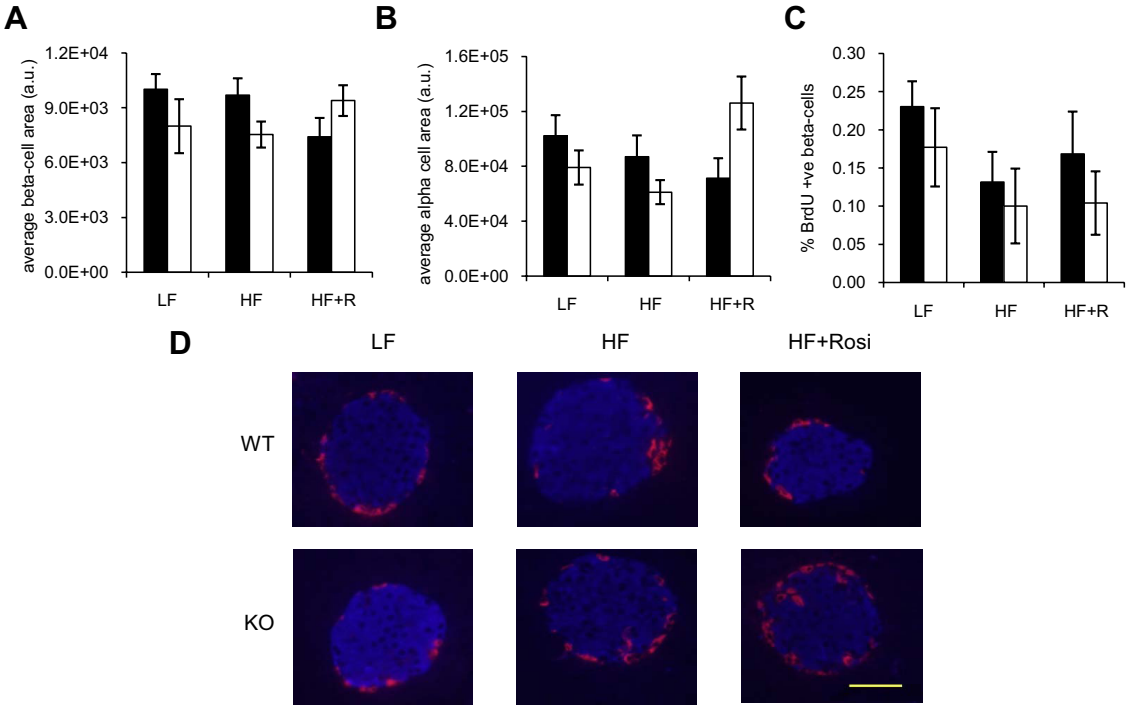

FIG. 3. Islet size and proliferation of Pdx1^{PB}CreER^{TM} PPARγ KO mice. Mice fed a high-fat diet for 10 wk followed by 6 wk of a high-fat diet ± rosiglitazone were injected with 100 mg/ml BrdU for 5 h and the pancreas fixed and stained with insulin and glucagon to allow determination of β-cell (A) and α-cell (B) areas, respectively. Other pancreas sections were stained with insulin, BrdU, and 4′,6′-diamino-2-phenylindole to assess the number of BrdU-positive β-cells (C). Results are shown as mean ± SEM (n = 6/7). *Black bars*, PPARγfl/fl (WT); *white bars*, PPARγfl/fl/pdx1^{PB}CreER^{TM} (KO). D, Immunhistochemistry of pancreas sections from PPARγfl/fl (WT) and PPARγfl/fl/pdx1^{PB}CreER^{TM} (KO) mice on a low-fat (LF), high-fat (HF), or high-fat + rosiglitazone (HR+Rosi) diet for 6 wk. Insulin is stained in *blue* and glucagon in *red*. *Bar*, 50 μM.

could not detect differences between PPARγKO mice and controls. In addition, increased expression of leptin and adipocyte protein 2 was detected in adipose tissue, suggesting that the high-fat diet was effective at increasing PPARγ activation in this tissue (Supplemental Fig. 3). To investigate whether changes in islet gene expression caused by rosiglitazone would lead to physiological alterations, we examined the effects of rosiglitazone treatment in mice fed a high-fat diet. Body weights in both groups were similar over the course of the experiment (Fig. 2B). Interestingly, rosiglitazone treatment improved the insulin sensitivity and reduced fasting insulin levels in both WT and KO mice (Fig. 2, G and D). Moreover, rosiglitazone treatment led to reduced levels of insulin secretion in response to an acute ip glucose stimulus compared with untreated mice on a high-fat diet in both groups (Fig. 2H). Furthermore, C-peptide levels were decreased after the rosiglitazone treatment (Fig. 2F), whereas randomly fed glucagon and fasting blood glucose levels were unchanged (Fig. 2, E and C).

Previous studies (21) have reported an increase in islet size in β-cell-specific PPARγKO mice. To study the effects on islet mass in our model, we measured islet size and β-cell replication (by measuring BrdU positive β-cells). At 2, 4, and 30 wk after tamoxifen injection, we did not detect alterations either in islet size or BrdU incorporation in β-cells. As expected, however, we observed an age-dependent increase in islet size that was associated with a decrease in BrdU incorporation into β-cells, although no differences were evident between genotypes (Supplemental Fig. 4, A and B).

In mice fed a high-fat diet, despite increasing insulin resistance, decreasing glucose tolerance and increasing fasting insulin secretion (Fig. 1, F–H), there was no significant change in islet area, β-cell number, or BrdU incorporation compared with mice on a low-fat diet. In addition, there was no difference between WT and KO islets on a high-fat diet (Fig. 3, A, B, and D). Treatment of mice on a high-fat diet with rosiglitazone had no effect on the islet size or BrdU incorporation in either WT or KO mice (Fig. 3, A and B). Although the α-cell area was increased in KO mice treated with rosiglitazone (Fig. 3C), this was not associated with a corresponding change in plasma glucagon levels (Fig. 2E).

In the present study, we found no significant changes in β-cell mass in islets from PDX-1 enhancer-driven inducible Cre PPARγKO mice, differing from the results using the rat insulin promoter Cre PPARγKO mouse, which has an increased β-cell mass (21). This may point to a role for PPARγ in regulating neonatal rather than adult β-cell biology. On the other hand, our data correlate with whole-pancreas PPARγKO mice, which have normal β-cell mass and islet architecture (5). Furthermore, mice that are haploinsufficient for PPARγ exhibit virtually no alteration in β-cell area on a high-fat diet compared with controls (36). Whether the duration of feeding and content of the diet or the timing and specificity of the cre expression or differences in genetic background contributed to the variable responses reported by different groups requires further investigation.

In summary, rosiglitazone treatment altered the expression of specific genes in β-cells in a PPARγ-specific manner, although the observed effects were not functionally significant. This may be related, in part, to the low levels of PPARγ expressed in islets compared with other tissues (results not shown). Indeed Matsui *et al.* (36) found that the level of PPARγ expression in islets was only 0.18% of that reported in adipose and 10–20% of that in muscle and liver tissues. It is possible that the minor effects of activating PPARγ in the islets are masked by the increased insulin sensitivity *in vivo*, which may be a dominant clinical response to TZD.

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