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PPARG Regulates Gonadotropin-Releasing Hormone Signaling in LbetaT2 Cells In Vitro and Pituitary Gonadotroph Function In Vivo in Mice[1]

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ABSTRACT

Peroxisome proliferators-activated receptor gamma (PPARG) ligands improve insulin sensitivity in type 2 diabetes and polycystic ovarian syndrome (PCOS). Despite clinical studies showing normalization of pituitary responsiveness to gonadotropin-releasing hormone (GnRH) in patients with PCOS, the precise role of PPARG in regulating the hypothalamic-pituitary-gonadal axis remains unclear. In the present study, we tested the hypothesis that the PPARG agonist rosiglitazone has a direct effect on the pituitary. In mouse LbetaT2 immortalized gonadotrophs, rosiglitazone treatment inhibited GnRH stimulation of the stress kinases p38MAPK and MAPKs/JNKs, but did not alter activation of ERKs, both in the presence and absence of activin. Furthermore, p38MAPK signaling was critical for both *Lhb* and *Fshb* promoter activity, and rosiglitazone suppressed the GnRH-mediated induction of *Lhb* and *Fshb* mRNA. Depletion of PPARG using a lentivirally encoded short hairpin RNA abolishes the effect of rosiglitazone to suppress activation of JNKs and induction of the transcription factors EGR1 and FOS as well as the gonadotropin genes *Lhb* and *Fshb*. Lastly, we show conditional knockout of *Pparg* in pituitary gonadotrophs caused an increase in luteinizing hormone levels in female mice, a decrease in follicle-stimulating hormone in male mice, and a fertility defect characterized by reduced litter size. Taken together, our data support a direct role for PPARG in modulating pituitary function in vitro and in vivo.

*female fertility, follicle-stimulating hormone, gonadotropin, gonadotropin-releasing hormone, luteinizing hormone, NR4A1 (*Nur77*), PPARG, PCOS, pituitary, pituitary hormones, rosiglitazone*

INTRODUCTION

Thiazolidinediones (TZDs) are synthetic ligands for the peroxisome proliferators-activated receptor γ (PPARG) and are used clinically to treat insulin resistance in type 2 diabetes [1–4] and polycystic ovarian syndrome (PCOS). The beneficial clinical effects of TZDs include increased insulin sensitivity, decreased blood pressure, correction of dyslipidemia, and improvement in inflammation [5, 6]. Although TZDs generally are thought to improve insulin sensitivity through effects on skeletal muscle and adipose tissue, TZDs are now also known to have a number of biological effects in other systems. For example, PPARG is expressed in macrophage cells of the immune system, and TZDs exert anti-inflammatory effects by suppressing macrophage gene expression. Indeed, recent studies suggest that a major portion of the beneficial clinical effects of TZDs may result from their anti-inflammatory properties.

Polycystic ovarian syndrome is a common endocrine disorder affecting 5–10% of women of reproductive age and is the major cause of anovulation and infertility [7]. In PCOS, inappropriate pituitary gonadotropin secretion leads to increased circulating luteinizing hormone (LH) and normal or decreased follicle-stimulating hormone (FSH) levels [8, 9]. Basal LH secretion is elevated, and pituitary LH responsiveness to gonadotropin-releasing hormone (GnRH) is exaggerated. However, it is not known whether this reflects a hypothalamic or a pituitary defect. Genetic studies have also linked polymorphisms in PPARG to PCOS, which provides additional support for a connection between PPARG activation and hypothalamic-pituitary-gonadal (HPG) axis function [10, 11]. Insulin-sensitizer therapy with TZDs in PCOS corrects the underlying insulin resistance and metabolic defects, decreases serum LH and androgen levels, and increases ovulation rate [12, 13]. Although the insulin-sensitizing effects of these drugs are well documented [12, 14], neither the mechanism underlying this amelioration nor the site of action on the HPG axis is understood. Clinical studies by Mehta et al. [15] showed that pioglitazone therapy reduces peak serum LH responses to multidose GnRH, which indicates that absolute pituitary responsiveness, but not sensitivity, is altered in vivo. This could be a direct effect of TZDs on the pituitary or an indirect effect resulting from changes in circulating androgens that might in turn alter feedback to the hypothalamus.

In view of the multiple potential actions of TZDs, we were interested in examining the mechanism by which PPARG agonists regulate reproductive hormone responses. We hypothesized that TZDs might have direct effects on the pituitary to alter gonadotropin synthesis and secretion. The LβT2 immortalized gonadotroph cells express the *Lhb* and *Fshb* subunit genes and respond to GnRH and activin. They also express PPARG and respond to rosiglitazone and so are a useful model to study the interaction of PPARG and GnRH signaling [16]. In the present study, we show that treatment of LβT2 cells with rosiglitazone suppresses the GnRH activation

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of the stress kinases p38 mitogen-activated protein kinases (MAPKs) and JNKs and the expression of the gonadotropin *Lhb* and *Fshb* subunit genes. We also show that pituitary-specific deletion of PPARG leads to elevated LH levels in female mice and a fertility defect characterized by reduced litter size, supporting the idea that PPARG can modulate pituitary function and alter the HPG axis.

MATERIALS AND METHODS

Materials

The GnRH was from Dr. A.F. Parlow (National Hormone and Pituitary Program, Harbor UCLA Medical Center, Torrance, CA). The inhibitors PD98059, SP600125, and PD169316 were purchased from Calbiochem. Activin A was purchased from R&D Systems. Antibodies to phosphorylated and total p38MAPKs, JNKs, ERKs, ATF2, PPARG, phosphorylated c-jun (JUN), CREB, and SRF were from Cell Signaling Technology. β-Tubulin and horseradish peroxidase (HRP)-linked secondary antibodies were purchased from Santa Cruz Biotechnology. All other reagents were purchased from either Sigma or Fisher Scientific. Vehicle for all experiments was dimethyl sulfoxide (DMSO), unless stated otherwise.

Cell Culture and Agonist Treatment

The LβT2 cells were maintained in monolayer cultures in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics in a humidified 10% CO_2 atmosphere at 37°C. Cells were treated with 10 μM rosiglitazone for 24, 48, or 72 h. Twenty-four hours before GnRH stimulation, the cells were starved in media containing 0.1% bovine serum albumin (BSA) and simultaneously treated with activin A (25 ng/ml) in the starvation media for 24 h wherever indicated. GnRH was added at the indicated concentrations for 30 min. Inhibitors of MEKs (PD98059: 5, 10, and 20 μM), JNKs (SP600125: 2.5, 5, and 10 μM), and p38MAPKs (PD169316: 1.2, 2.5, and 5 μM) were added 30 min before GnRH stimulation.

Western Blot Analysis

After treatment, cells were washed once with chilled PBS and solubilized in radioimmunoprecipitation lysis buffer containing a Protease inhibitor cocktail (Roche), 2% SDS, and 4% β-mercaptoethanol. Lysates were then subjected to SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (Immobilon-P; Millipore). Following blocking with 5% milk in Tris-buffered saline containing 0.1% Tween 20, membranes were probed with primary antibodies at the dilutions recommended by the manufacturer and subsequently with HRP-linked secondary antibodies for chemiluminescent detection (Pierce). Following phospho-protein analysis, blots were stripped in 62.5 mM Tris-HCl (pH 6.7), 100 mM β-mercaptoethanol, and 2% SDS in a shaking water bath at 50°C for 30 min and reprobed for respective total proteins.

Plasmids and Transfections

The LβT2 cells were treated with rosiglitazone (10 μM) or DMSO as vehicle for a total of 72 h. After 24 h of rosiglitazone treatment, the cells were transiently transfected with 500 ng of 1.8-kb rat *Lhb-luc* reporter gene [17] or 1-kb mouse *Fshb-luc* reporter gene [18]. As an internal control for transfection efficiency, cells were cotransfected with 50 ng of the TK-LacZ plasmid containing β-galactosidase driven by the herpesvirus thymidine kinase promoter. After overnight incubation, the cells were washed and starved in DMEM medium containing 0.1% BSA and treated with hormones, inhibitors, and agonists as indicated. At the end of 8 h, the cells were washed once with PBS and then lysed. After lysing, the cells were assayed for luciferase activity using a buffer containing 100 mM Tris-HCl (pH 7.8), 15 mM $MgSO_4$, 10 mM ATP, and 65 μM luciferin. β-Galactosidase activity was measured by chemiluminescence (Galacto-Light; Tropix). Both luciferase and β-galactosidase activity were measured using a Veritas microplate luminometer (Turner Design).

Lentivirus Production and Infection of LβT2 Cells

The lentivirus expressing a short hairpin (sh) RNA against *Pparg* or the control gene luciferase has been described previously [19]. Briefly, the infection of cells with shLuciferase (shLuc) and shPPARG lentivirus was carried out by addition of lentivirus along with polybrene to the LβT2 cell culture. Following overnight incubation, the medium was changed to fresh serum containing medium. After 3–4 days, the confluent cells were trypsinized and centrifuged at 4000 rpm for 5 min. The cell pellet was first washed in PBS and then in PBS containing 5% BSA. Approximately 8×10^6 cells were used for sorting by fluorescence-activated cell sorting. The green fluorescent protein positive cells were collected and cultured until they were confluent.

Real-Time PCR

Total RNA was extracted from the cells using TRIzol (Invitrogen) following the manufacturer's instructions. First-strand cDNA was synthesized using a High Capacity cDNA Synthesis kit (Roche). Samples were run in 20-μl triplicate reactions on an MJ Research Chromo4 instrument using SYBR Green Master Mix (Bio-Rad) and the sequence-specific primers for *Lhb* (forward, GTGCCGGCTACTGTCCTAGCATGGTC; reverse, CAGCTGAGGGCTA CAGGAAAGGAGAC), *Fshb* (forward, CCACTTGGTGTGCGGGCTACTG; reverse, GTGTAGAGGGAGTCTGAGTGGCGGGC), *Egr1* (forward, CCCTATGAGCACCTGACCAC; reverse, TCGTTTGGCTGGGATAACTC), *Fos* (forward, AACCTGGTGCTGGATTGTAT; reverse, CGAAAGACCT CAGGGTAGAA), *Nr4a1* (forward, CTGCACAGCTTGGGTGTT GATGTTCC; reverse, TCCTGGAGCCCGTGTCGATCAGTG), *Pparg* (forward, CTGTCGGTT TCAGAAGTGCCT; reverse, CCCAAACCTGATGG CATTGTGAGACA), and M36B (forward, ACCCTGAAGTGCTCGACAT CACAG; reverse, GCAGGGGCAGCAGCCGCAAATGC) under the following conditions: initial activation of DNA polymerase at 95°C for 3 min, followed by 45 cycles of denaturing at 94°C for 15 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec. Gene expression levels were calculated after normalization to the housekeeping gene, M36B, using the ΔΔCt method and expressed as relative mRNA levels compared with the control.

Generation of Pituitary Pparg Knockout Mice

Pituitary *Pparg* knockout (PKO) mice were created using the cre/loxP strategy by crossing pituitary-specific *Lhb-cre* mice [20] with $Pparg^{flox/flox}$ mice (official symbol, $Pparg^{tm2Rev}$) [21]. Heterozygous mice ($Pparg^{flox/+}$:*Lhb-cre*) from the F_1 generation were back-crossed with $Pparg^{flox/flox}$ mice or with heterozygous cre-ve littermates to generate PKO mice. Genotypes were determined by PCR of tail DNA as previously described [22]. All mice were housed under controlled light (12L:12D) and temperature (22°C) conditions and had free access to food and water. Fertility was assessed by mating four pairs of PKO or control littermate mice over 6 mo. The number of litters and each litter size were recorded. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of the University of California, San Diego.

Hormone Measurements

Animals used for hormone measurements were age matched at 10–12 wk. Blood was collected from PKO and cre-ve control mice from the tail vein. Estrous cycles were determined by vaginal lavage. Vaginal smears were taken daily between 0800 and 0900 h for 3 wk in normal saline. Based on the cytology, the smears were classified into various stages of the estrous cycle: A diestrous smear had almost exclusively leukocytes; a proestrous smear showed both leukocytes and nucleated epithelial cells, but predominantly nucleated epithelial cells; estrous smears had large, irregularly shaped, cornified, and clumped cells; and metestrous smears showed approximately equal numbers of leukocytes and epithelial cells. Blood was collected during diestrus. Plasma obtained after centrifugation using Adams MHCT II Microhematocrit centrifuge at $13\,700 \times g$ for 5 min was stored at −80°C until hormone analysis. Twenty-five microliters of plasma were used to measure LH and FSH levels by Luminex assay kit (Millipore Corporation) following the manufacturer's instructions.

Statistical Analysis

All the assays and SDS-PAGE data presented here are representative examples of at least three separate experiments. Differences between groups are shown as the mean ± SEM of at least three separate experiments and were analyzed for statistical significance using ANOVA followed by Tukey post-test (JMP IN Version 5.1; SAS Institute, Inc.). Sigmoidal dose-response curves were fit using the program Prism (Version 4.0; GraphPad Software) and tested both against the null hypothesis (i.e., no difference in the curves) and for significant differences in curve parameters. Hormone measurements were founds to be nonnormally distributed by Kolmogorov-Smirnov,

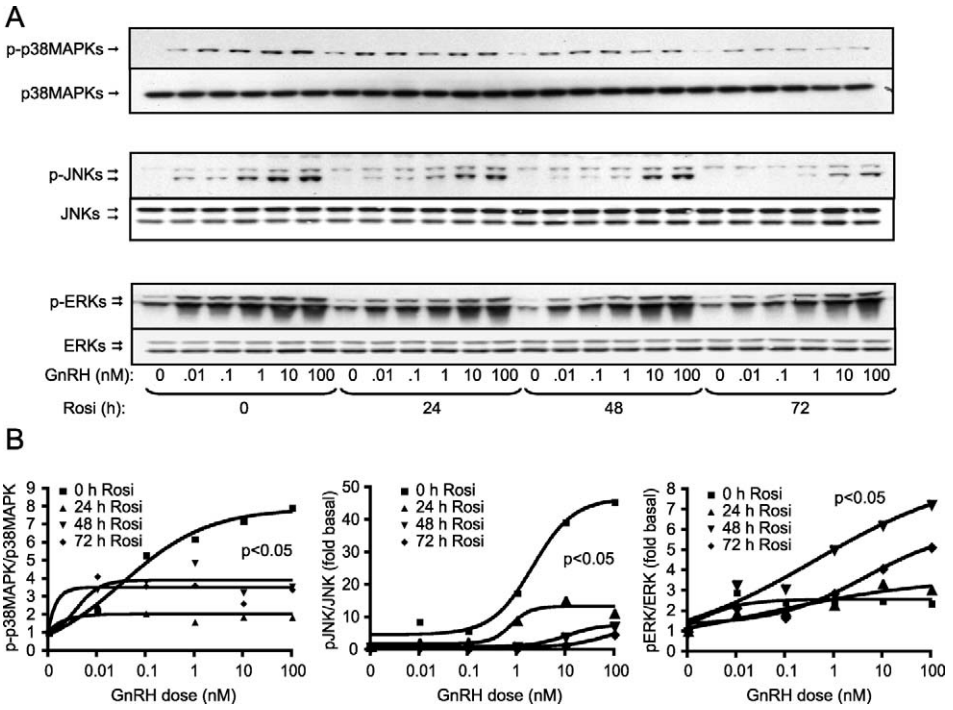


FIG. 1. Effect of rosiglitazone (Rosi) on GnRH-induced activation of p38MAPKs, JNKs, and ERKs. LβT2 cells (1.5×10^6 cells/well) were treated with rosiglitazone (10 μM) or DMSO as vehicle for the indicated time periods. After 0, 24, or 48 h of pretreatment with rosiglitazone, the cells were incubated in serum-free DMEM containing 0.1% BSA for 24 h before treatment with varied concentrations of GnRH (0, 0.01, 0.1, 1, 10, or 100 nM) for 30 min. Whole-cell lysates were separated on SDS-PAGE and immunoblotted with an antibody for phospho-p38MAPKs (p-p38), phospho-JNKs (p-JNK), and phospho-ERKs (p-ERK; **A**). The blots were stripped and reblotted for p38MAPKs, JNKs, or ERKs, respectively, to determine the total protein loading. The blots are representative experiments, each repeated three times. Quantification of the GnRH dose response in the presence of rosiglitazone is also shown (**B**). Sigmoidal dose-response curves were modeled and tested for significance. Rosiglitazone caused a statistically significant decrease in the dose-response curve for p38MAPK and JNK activation by GnRH ($P < 0.05$) and a significant increase in the dose-response curve for ERK activation ($P < 0.05$).

D'Agostino-Pearson, and Shapiro-Wilk tests ($P < 0.001$), so data were analyzed by nonparametric Mann-Whitney and Wilcoxon signed-rank tests (Prism). A P value of 0.05 or less was considered to be statistically significant.

RESULTS

Rosiglitazone Suppresses GnRH- and Activin-Induced Activation of JNKs and p38MAPKs

We and others have reported that the three MAPK families—ERKs, p38MAPKs, and JNKs—are activated by GnRH [23]. To investigate the effects of TZDs on pituitary LβT2 gonadotroph cells, we pretreated cells with rosiglitazone for increasing times (24, 48, and 72 h) and measured the dose-dependent activation of the MAPKs by GnRH. At 10 μM rosiglitazone, we observed a dose-dependent increase in the phosphorylation levels of p38MAPKs, JNKs, and ERKs upon GnRH treatment, with maximum induction observed at 10–100 nM GnRH (Fig. 1A). Treatment with rosiglitazone for 24–72 h caused a significant decrease in phosphorylation of the stress kinases p38MAPKs and JNKs in response to GnRH (Fig. 1A). We saw a slight but significant increase in ERK activation at 48 h, but not at 24 or 72 h, of rosiglitazone treatment. These changes were verified by quantification of multiple blots (Fig. 1B). None of these treatments caused any visible effects on cell viability. Inhibition, albeit somewhat weaker, was also seen at 1 μM rosiglitazone; therefore, all further experiments used the maximal dose of 10 μM.

Previously, we have shown that activin enhances the GnRH activation of the JNKs and p38MAPKs [23]. Therefore, we tested the effect of rosiglitazone on activin-enhanced activation of the stress kinases. After pretreatment for 48 h with 10 μM rosiglitazone, the cells were incubated in serum-free DMEM containing 25 ng/ml of activin and 10 μM rosiglitazone for another 24 h before treatment with GnRH (10 or 100 nM) for 30 min. Rosiglitazone reduced the effect of activin in enhancing the GnRH-mediated activation of JNKs and p38MAPKs but had no effect on ERKs (Fig. 2).

Rosiglitazone Inhibits Phosphorylation of Downstream Targets of p38MAPKs and JNKs

To further examine the inhibitory effects of rosiglitazone in the presence of activin and GnRH, we measured the phosphorylation of known downstream targets of the p38MAPKs. Treatment with rosiglitazone inhibited the phosphorylation of ATF2 and JUN in the presence of GnRH and activin (Fig. 3). In contrast, rosiglitazone had no effect on the phosphorylation of CREB, which is a downstream target of the phosphokinase A pathway. To verify that these transcription factors are indeed targets for the stress kinases, we used specific inhibitors of p38MAPKs, MEKs, and JNKs. Phosphorylation of ATF2 was blocked only by the p38 inhibitor (Fig. 4). ATF2 appears to be a relatively specific target, because the MEK and JNK inhibitors had no effect, or even slightly increased, the phosphorylation of ATF2. Inhibition of either JNKs or p38MAPKs reduced JUN phosphorylation, indicating that both kinases target this transcription factor. As a control, the MEK inhibitor reduced phosphorylation of SRF, a known target for ERKs. Interestingly, inhibition of p38MAPKs also reduced phosphorylation of SRF in response to GnRH. The inhibitors alone had no effect on basal phosphorylation of ATF2, JUN, or SRF (data not shown). These data indicate that rosiglitazone reduces GnRH activation of JNKs and p38MAPKs and downstream transcription factor targets.

p38MAPKs Are Critical for Induction of Lhb *and* Fshb *Promoter Activity*

Inhibition of the p38MAPK pathway impairs GnRH and activin induction of the *Fshb* promoter [24]. Therefore, we tested whether any of these kinases is responsible for the GnRH activation of *Lhb* promoter activity. Cells were transfected with a 1.8-kb rat *Lhb* promoter luciferase reporter [17]. Twenty-four hours after transfection, cells were serum-starved overnight, pretreated with inhibitors for MEKs (20 μM PD98059), JNKs (10 μM SP600125), or p38MAPKs (5 μM PD169316) for 30 min, then stimulated with 100 nM GnRH for

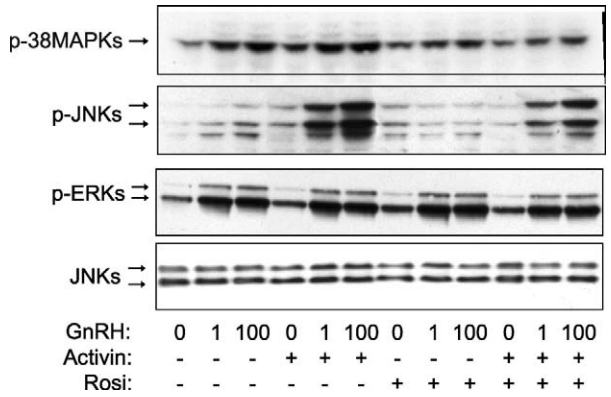


FIG. 2. Effect of rosiglitazone (Rosi) on activin-enhanced GnRH activation of p38MAPKs and JNKs. LβT2 cells (1.5×10^6 cells/well) were treated with rosiglitazone (10 μM) or DMSO as vehicle for a total of 72 h. After 48 h of rosiglitazone pretreatment, the cells were incubated in serum-free DMEM containing activin A (25 ng/ml) for 24 h before treatment with GnRH (1 or 100 nM) for 30 min. Whole-cell lysates were separated on SDS-PAGE and immunoblotted with an antibody for phospho-p38MAPKs (p-p38), phospho-JNKs (p-JNK), and phospho-ERKs (p-ERK). The blots were stripped and reblotted for JNKs to demonstrate equal protein loading. The blots are representative experiments, each repeated three times. Rosiglitazone caused a statistically significant decrease in levels of phosphorylation of p38MAPKs and JNKs, but not of ERKs, induced by 1 and 100 nM of GnRH in the presence of activin compared to no rosiglitazone treatment ($P < 0.05$).

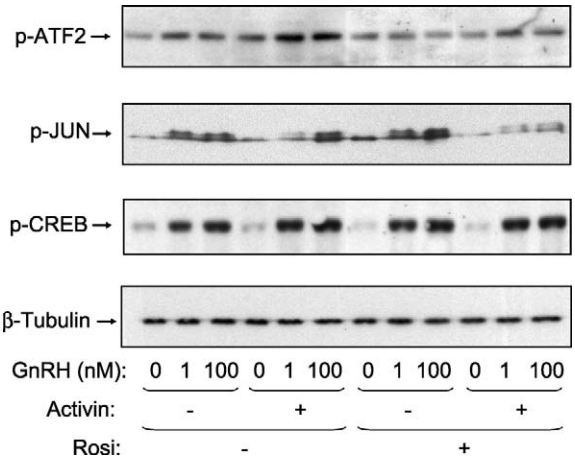


FIG. 3. Effect of rosiglitazone (Rosi) on GnRH-stimulated phosphorylation of ATF2, CREB, and JUN. LβT2 cells (1.5×10^6 cells/well) were treated with rosiglitazone (10 μM) or DMSO as vehicle for a total of 72 h. After 48 h of rosiglitazone pretreatment, the cells were incubated in serum-free DMEM containing activin A (25 ng/ml) for 24 h before treatment with GnRH (1 or 100 nM) for 30 min. Whole-cell lysates were separated on SDS-PAGE and immunoblotted with an antibody for phospho-ATF2 (p-ATF2), phospho-JUN (p-JUN), or phospho-CREB (p-CREB). The blots were stripped and reblotted for β-tubulin to demonstrate equal protein loading. The blots are representative experiments, each repeated three times. Rosiglitazone caused a statistically significant decrease in the level of ATF2 phosphorylation induced by 1 and 100 nM GnRH in the presence of activin and a significant decrease in the level of JUN phosphorylation in response to GnRH (100 nM) and activin ($P < 0.05$).

8 h. We observed a statistically significant decrease in GnRH-stimulated *Lhb* promoter activity in the presence of the p38MAPK inhibitor (Fig. 5A). We then tested whether inhibition of p38MAPKs altered sensitivity of the *Lhb* promoter to GnRH stimulation. Cells were transfected with the 1.8-kb rat *Lhb* promoter, serum-starved in the absence or presence of 25 ng/ml of activin for 16 h, treated with p38MAPK inhibitor or vehicle for 30 min, then stimulated with increasing doses of GnRH (0.01–100 nM) for 8 h. Inhibition of p38MAPK caused a decrease in responsiveness of the *Lhb* promoter but did not change the sensitivity to GnRH (Fig. 5, B and C). Similar experiments were performed with a 1-kb mouse *Fshb* promoter-luciferase reporter [18]. Pretreatment with the p38MAPK inhibitor, but not with the JNK or MEK inhibitors, caused a significant decrease in *Fshb* promoter activity (Fig. 5D) and decreased responsiveness of the promoter without changing sensitivity to GnRH (Fig. 5E). In the presence of activin, the basal promoter activity and the maximal response were increased (Fig. 5F), as expected. Interestingly, inhibition of p38MAPKs not only caused a decreased basal activity and maximal response to GnRH but also significantly altered the sensitivity to GnRH (median effective dose, 0.2 nM vs. 0.009 nM in the presence of PD169316; $P < 0.05$) (Fig. 5F).

Rosiglitazone Inhibits GnRH-Stimulated Lhb *and* Fshb *mRNA Expression*

Because GnRH-mediated activation of p38MAPKs was suppressed by rosiglitazone and p38MAPK inhibition impaired transcription of the *Lhb* (Fig. 6A) and *Fshb* (Fig. 6B) promoters, we examined the effect of rosiglitazone on *Lhb* and *Fshb* gene expression. Cells were treated with 10 μM rosiglitazone or vehicle for 48 h, then serum-starved in the presence of 25 ng/ml of activin plus rosiglitazone overnight and finally stimulated with 100 nM GnRH for 8 h. GnRH tended to increase, though not significantly, *Lhb* mRNA (Fig. 6A) but to up-regulate the expression of the *Fshb* mRNA by

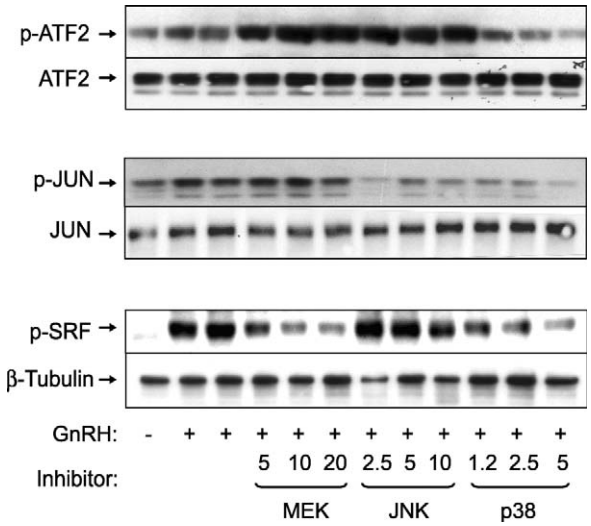


FIG. 4. Effect of p38MAPK, JNK, and MEK inhibitors on the GnRH-induced phosphorylation of ATF2, SRF, and JUN. LβT2 cells (1.5×10^6 cells/well) were seeded in DMEM containing 10% serum and incubated overnight. This was followed by serum starving the cells through incubation in serum-free DMEM containing 0.1% BSA. After 24 h, the cells were treated with the inhibitors of MEKs (PD98059: 5, 10, and 20 μM), JNKs (SP600125: 2.5, 5, and 10 μM), or p38MAPKs (PD169316: 1.2, 2.5, and 5 μM) for 30 min, followed by treatment with 100 nM GnRH for 30 min. Whole-cell lysates were separated on SDS-PAGE and immunoblotted with an antibody for phospho-ATF2 (p-ATF2), phospho-JUN (p-JUN), or phospho-SRF (p-SRF). The blots were stripped and reblotted for ATF2, JUN, or β-tubulin to demonstrate equal protein loading. The blots are representative experiments, each repeated three times. PD169316 at a concentration of 2.5 and 5 μM caused a significant decrease in the GnRH-induced ATF2, JUN, and SRF phosphorylation ($P < 0.05$). SP600125 caused a significant decrease in GnRH-induced JUN phosphorylation, and PD98059 at 5, 10, and 20 μM significantly suppressed the GnRH-induced phosphorylation of SRF ($P < 0.05$).

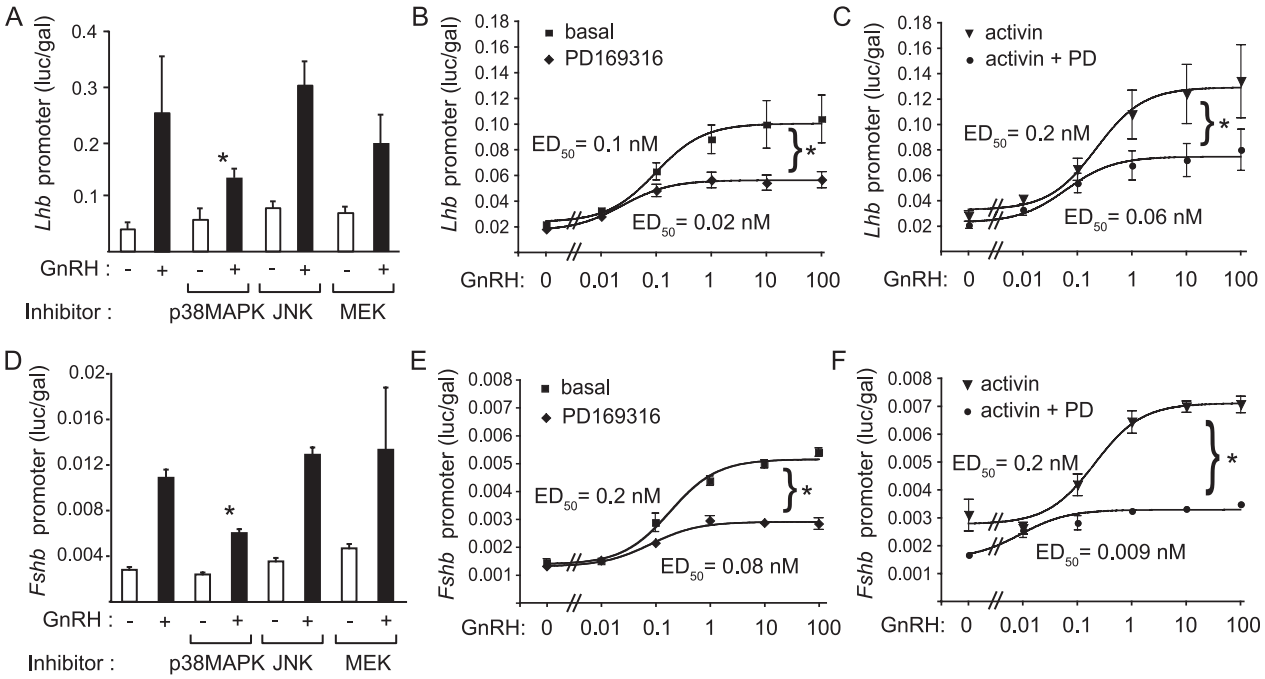


FIG. 5. GnRH signals to the *Lhb* and *Fshb* promoters via p38MAPKs. Induction of the *Lhb* and *Fshb* promoters requires p38MAPK, but not ERK or JNK, signaling. LβT2 cells (5×10^5 cells/well) were transiently transfected with 500 ng of the 1.8-kb *Lhb luc* reporter gene along with 50 ng of TK-LacZ plasmid containing β-galactosidase. After overnight starvation in serum-free DMEM, the cells were treated with the inhibitors of MEKs (PD98059: 20 μM), JNKs (SP600125: 10 μM), or p38MAPKs (PD169316: 5 μM) or with DMSO for 30 min followed by treatment with 100 nM GnRH for 30 min (**A**). Cells were lysed and assayed for *Lhb* promoter activity and β-galactosidase activity. The graph shows promoter activity as luciferase activity (relative light units) normalized to the β-galactosidase activity (relative light units) from the control plasmid. Cells were also transfected with the *Lhb* promoter as described above. After overnight starvation in serum-free media with or without 25 ng/ml of activin, cells were treated with 5 μM PD169316 or DMSO for 30 min, then with increasing doses of GnRH (0, 0.01, 0.1, 1, 10, or 100 nM). Graphs show GnRH dose-response curves for *Lhb* promoter activity in the absence (**B**) or presence (**C**) of activin pretreatment. Experiments were then repeated using the 1-kb mouse *Fshb* promoter luciferase reporter gene. Treatment with PD169316 inhibited FSHB induction by GnRH (**D**). Dose-response curves for *Fshb* promoter activity showed a decreased maximal response in the presence of the p38MAPK inhibitor (**E**). GnRH dose-response curves showed increased basal and maximal promoter activity in the presence of activin (**F**). PD169316 reduced basal promoter activity and maximal response but increased sensitivity. Asterisks indicate statistical significance ($P < 0.05$) compared to the corresponding sample without inhibitor. Dose-response curves were compared by testing the best-fit sigmoidal curves against the global null hypothesis curve. ED_{50}, median effective dose.

10-fold (Fig. 6B). Rosiglitazone treatment significantly repressed *Lhb* expression down to basal levels and also repressed *Fshb* induction by 80%. Activin pretreatment enhanced the basal expression levels of the *Lhb* gene by 3-fold and levels of the *Fshb* gene by 80-fold, with no significant change upon rosiglitazone treatment. Furthermore, cotreatment with GnRH and activin caused a synergistic increase in both *Lhb* (5-fold) and *Fshb* (160-fold), as published previously [24]. Treatment with rosiglitazone eliminated this synergistic induction and decreased *Lhb* and *Fshb* mRNA levels to those induced by activin alone. Elimination of the synergistic effect on *Fshb* expression was consistent with published data showing that inhibition of p38MAPKs abolishes synergy on the *Fshb* promoter [24].

PPARG Mediates Rosiglitazone Suppression of GnRH Induction of JNKs and Lhb *and* Fshb *mRNA Expression*

Although rosiglitazone is a well-accepted ligand for PPARG, PPARG-independent effects have also been documented in the pituitary [25] and other tissues [26, 27]. Therefore, to determine whether the observed suppressive effects of rosiglitazone on the stress kinases and gonadotropin mRNA expression are mediated via PPARG, we knocked down *Pparg* by infecting LβT2 cells with a lentivirus encoding an shRNA against *Pparg* or *Luciferase* as a control. A 70% decrease in PPARG protein and mRNA (Fig. 7A) was obtained. *Pparg* knockdown abolished the effect of rosiglitazone on GnRH-induced phosphorylation of JNKs and p38MAPKs (Fig. 7B). At the level of gonadotropin gene expression, rosiglitazone suppressed the GnRH induction of *Lhb* (Fig. 7C) and *Fshb* (Fig. 7D) mRNA expression in shLuc cells, but the suppression was abolished in shPPARG cells.

PPARG Activation Suppresses GnRH Induction of Transcription Factors EGR1 and FOS But Not NR4A1

To define the mechanism leading to the PPARG-mediated suppression of gonadotropin gene expression, we analyzed the effects of rosiglitazone and GnRH on the expression of *Egr1* and *Fos* (transcription factors for LHB and FSHB, respectively) in shLuc and shPPARG cells. GnRH induced *Egr1* by 24-fold and *Fos* by 4-fold at the mRNA level. Rosiglitazone suppressed the GnRH induction of both *Egr1* and *Fos* only in shLuc cells (Fig. 8A), not in shPPARG cells (Fig. 8B). A similar repression of EGR1 and FOS protein expression was observed. Rosiglitazone treatment caused a 20% decrease in both EGR1 and FOS protein (both $P < 0.05$) in shLuc cells but no decrease in shPPARG cells (data not shown). To verify the specificity of the PPARG effect, we analyzed the induction of NR4A1, an orphan nuclear receptor known to be induced by GnRH [27]. *Nr4a1* mRNA expression was increased by 15-fold upon GnRH stimulation; however, the induction was unchanged by rosiglitazone in either shLuc or shPPARG cells

(Fig. 8C). These data reinforce the conclusion that the effect of rosiglitazone is mediated by PPARG, and they suggest that suppression of *Egr1* and *Fos* may underlie the suppression of *Lhb* and *Fshb* mRNA expression, respectively.

Pituitary-Specific Deletion of PPARG Elevates LH Levels In Vivo

To study the role of PPARG in vivo in the pituitary, we generated PKO mice following the cre-loxP strategy by crossing *Pparg-floxed* mice with *Lhb-cre* mice that express the cre recombinase in LH-positive pituitary gonadotroph cells with little or no expression in other organs [20]. The PKO mice had the genotype $Pparg^{flox/flox}$:*Lhb-cre,* and the control group was made up of littermates with the genotype $Pparg^{flox/flox}$ but lacking the *Lhb-cre* allele. As expected, recombination of PPARG alleles occurred in the pituitaries of PKO mice and not in the control cre-ve littermates as determined by PCR (Fig. 9A). The PKO females had a 3-fold elevated plasma diestrous LH (286 ng/ml) compared to the control female mice (85 ng/ml) (Fig. 9B). This alteration was specific for the females; the LH levels in PKO male mice were unchanged compared to controls. The difference was not related to a hypomorphic $Pparg^{flox/flox}$ allele, because the LH levels in $Pparg^{flox/flox}$ mice ($n = 17$) were not significantly different from those in $Pparg^{flox/+}$ mice ($n = 18$). No difference was observed in the plasma FSH levels in control and PKO female mice, but the male PKO mice had slightly, but significantly, lower FSH (Fig. 9B). The female PKO mice showed normal patterns of estrous cycles by vaginal lavage over a 2-wk period before hormone measurements. To analyze the PKO mice for reproductive phenotype, we bred four independent pairs of PKO or control mice over the course of 6 mo. The PKO mice were fertile and produced litters at the same rate as control mice. The total number of mice produced by the PKO breeding pairs was lower than that in the wild-type mice, and the mean litter size was significantly smaller (Fig. 9B).

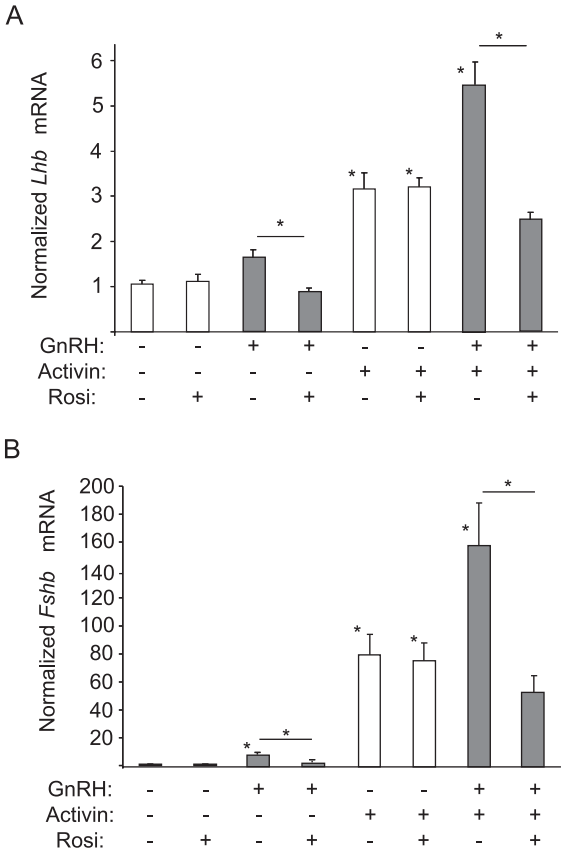


FIG. 6. Rosiglitazone (Rosi) represses gonadotropin gene expression. LβT2 cells were treated with rosiglitazone (10 μM) or DMSO as vehicle for a total of 72 h. After 48 h of rosiglitazone treatment, the cells were starved in serum-free DMEM with or without activin A (25 ng/ml). After a further 24 h, the cells were treated with GnRH (100 nM) as indicated and incubated for 8 h. RNA was harvested and analyzed by quantitative PCR for *Lhb* expression (**A**) or *Fshb* expression (**B**). Data for *Lhb* or *Fshb* mRNA are normalized to the housekeeping gene M36B4 and presented as fold-induction compared to untreated samples. Asterisks indicate a statistically significant ($P < 0.05$) decrease in *Lhb* or *Fshb* mRNA levels compared to the untreated control or to the matched, non-rosiglitazone-treated sample.

DISCUSSION

In the present study, we show that rosiglitazone down-regulates GnRH-mediated phosphorylation of the JNKs and p38MAPKs in LβT2 cells. These kinases are activated by many cytokines and inflammatory signals, so this TZD effect is consistent with its known anti-inflammatory actions. This inhibition is also seen in the presence of activin, which we showed previously to enhance phosphorylation of the stress kinases by GnRH [23]. The elevated p38MAPK signaling in the presence of activin and GnRH likely causes a synergistic increase in the expression of *Lhb* and *Fshb*, which could contribute to the preovulatory LH and FSH surge when inhibin levels are low. Our results are consistent with a previous report [28] that both PPARA and PPARG agonists reduced the basal activity of the gonadotropin gene promoters and increased inhibin-α and follistatin levels, which would inhibit activin signaling. Therefore, it is possible that rosiglitazone suppresses the activin-enhanced stress kinase activation by inducing the antagonistic hormones follistatin and inhibin.

The GnRH receptor is a classical G protein-coupled receptor that couples to Gq/11, leading to activation of phosphokinase C and CaMK [29, 30], and to Gs, leading to activation of phosphokinase A [31, 32]. Both the Gq/11 and Gs pathways are upstream of ERK activation and the Gq/11 pathway is upstream of JNKs, but the signals leading to p38MAPKs are not known [32–34]. All these kinases phosphorylate specific nuclear targets to mediate transcriptional activation. GnRH up-regulates phosphorylation of both JNK2 and p38MAPKs in primary rat pituitary cultures [35] and in pituitary gonadotroph cell lines [32, 33]. Our results suggest that p38MAPKs are the main mediator for GnRH-stimulated *Lhb* and *Fshb* promoter activity, which disagrees with a report that JNKs are the critical regulator of the *Lhb* gene in primary rat pituitary cultures [35]. It is possible that GnRH uses different stress kinases to induce the *Lhb* gene in mouse and rat pituitary cells, because many of the transcription factor targets are redundant. Another study utilized a dominant-negative JUN expression vector to demonstrate that JNK signaling is critical in LβT2 cells [34], but in the present study, we show that JUN is a target for both JNKs and p38MAPKs. Therefore, the data in the previous study could be consistent with p38MAPK signaling to *Lhb*.

Conflicting reports also suggest the ERKs play a role in the transcriptional activation of *Lhb* in response to GnRH [36]. Blockade of ERK and p38MAPK signaling has an additive effect to reduce transcription of the *Lhb* promoter, both the minimal proximal promoter and one containing the upstream response element [37]. Blocking ERK, but not p38MAPK, signaling reduced EGR1 expression, yet blocking p38MAPK signaling eliminated EGR1 DNA binding but not expression, so the two signals may regulate different aspects of *Lhb* induction [37]. The role of ERKs has been supported by a

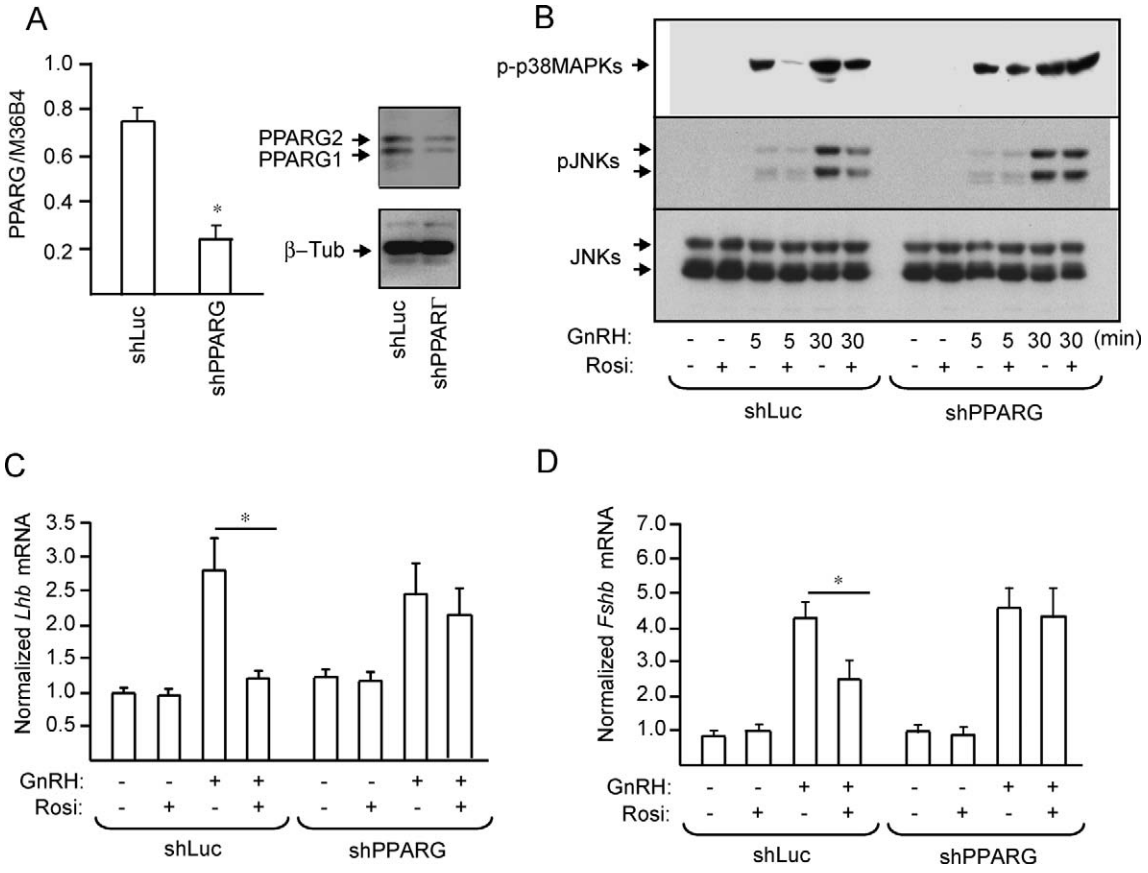


FIG. 7. PPARG depletion abolishes the effect of rosiglitazone (Rosi) on GnRH-induced JNK phosphorylation and gonadotropin gene expression. Quantitative PCR analysis of *Pparg* mRNA expression and immunoblot for protein expression in shLuc and shPPARG cells were performed. The blot was stripped and reprobed for β-tubulin to demonstrate equal protein loading (**A**). To assess stress kinase activation, shLuc and shPPARG cells were treated with rosiglitazone (10 μM) or DMSO vehicle for 72 h. After 48 h of rosiglitazone treatment, the cells were starved for 24 h in serum-free DMEM with or without rosiglitazone, then treated with GnRH (100 nM) for 5 or 30 min. Whole-cell extracts were immunoblotted for phospho-p38MAPKs (p-p38) and phospho-JNKs (p-JNK), then stripped and reblotted for β-tubulin or JNKs, respectively (**B**). The blots are representative experiments, each repeated three times. JNK and p38MAPK phosphorylation was significantly decreased by rosiglitazone treatment only in the shLuc cells ($P < 0.05$). To assess gonadotropin subunit gene expression, shLuc and shPPARG cells were treated with rosiglitazone (10 μM) or DMSO vehicle for 72 h. After 48 h of rosiglitazone treatment, the cells were starved for 24 h in serum-free DMEM with or without rosiglitazone, then treated with GnRH (100 nM) for 8 h. RNA was analyzed by quantitative PCR for *Lhb* expression as before (**C**), and the same RNA was analyzed for *Fshb* expression as before (**D**). Data for *Lhb, Fshb,* or *Pparg* mRNA are presented as fold-induction compared to the control or untreated samples. Asterisks indicate statistical significance ($P < 0.05$) compared to the untreated controls or to the matched, non-rosiglitazone-treated samples.

pituitary double knockout of MAPK3/1 (ERK1/2) that led to decreased expression of *Egr1* and *Lhb* [38], but that study is complicated by the observed lack of specificity of the GSU-cre mouse, which also expresses cre recombinase in the ovary and testis [39]. Our observed suppression of both JNK and p38MAPK activation and *Lhb* and *Fshb* gene expression by rosiglitazone is supported by the finding that rosiglitazone impairs induction of EGR1 and FOS. Female *Egr1* knockout mouse are infertile because of low LH levels [40], and *Egr1* regulates the *Lhb* promoter in vitro. Similar studies have shown the importance of FOS for induction of *Fshb* and that female *Fos* knockout mice are infertile, although it has not been shown whether this is the result of decreased FSH [24, 41]. The suppressive effect of rosiglitazone on *Egr1* expression is confirmed by a report that rosiglitazone attenuates postoperative ileus in the mouse colon through PPARG-dependent down-regulation of EGR1 [26]. Whereas the effect of rosiglitazone on *Egr1* and *Fos* expression is modest compared to the suppression of *Lhb* and *Fshb*, PPARG and p38MAPKs may also be involved in posttranscriptional regulation of DNA binding [37], which could further contribute to the repression of gonadotropin expression.

In agreement with the in vitro data, we find that PPARG regulates fertility in vivo, because female PKO mice have elevated plasma LH and reduced litter size compared to the control cre-negative littermates. PPARG may provide a tonic repression of inflammatory signaling in female gonadotrophs that is lost in the PKO mice, resulting in elevated LH. Although our in vitro data also point to repression of *Fshb* expression, the female PKO mice have normal FSH, but male PKO mice have reduced FSH, as expected. Activin is a physiologically more important stimulus of FSH levels than GnRH in vivo, and rosiglitazone does not repress activin-induced *Fshb*. Therefore, the normal FSH levels in female PKO mice may be a reflection of normal activin signaling, but we do not yet have an explanation for the reduced FSH in male PKO mice. Activin expression is responsive to inflammatory stimuli, so it is possible that activin tone is reduced in the male PKO mice [42]. Would the elevated LH alone cause the subfertility in the female mice? Transgenic mice with a 10- to 12-fold overexpression of a stabilized form of LH causes chronic anovulation with enlarged ovaries, ovarian cysts, or ovarian tumors, so the 3-fold overexpression seen in the PKO mice might be sufficient to reduce fertility. A similar phenotype of

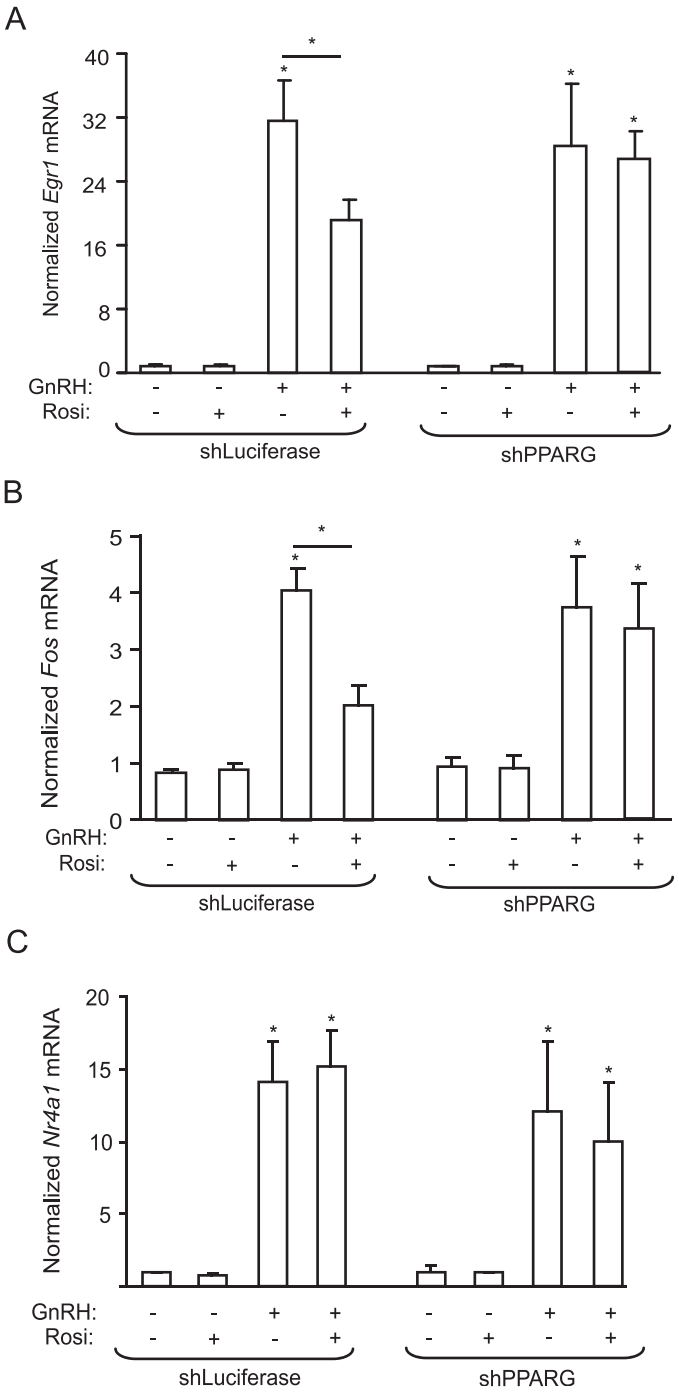
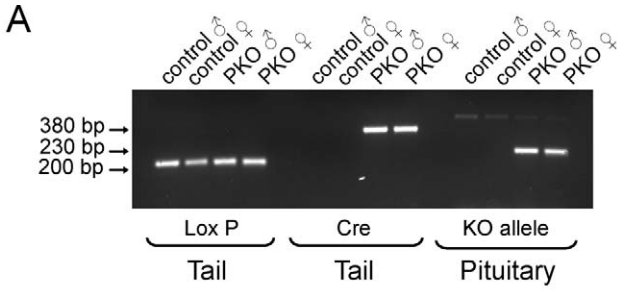


FIG. 8. Rosiglitazone (Rosi) suppresses GnRH-induced *Egr1* and *Fos*, but not of *N4a1*, mRNA expression. To assess *Egr1* and *Fos* gene expression, shLuc and shPPARG cells were treated with rosiglitazone (10 μM) or DMSO vehicle for 72 h. After 48 h of rosiglitazone treatment, the cells were starved for 24 h in serum-free DMEM with or without rosiglitazone, then treated with GnRH (100 nM) for 8 h. RNA was analyzed by quantitative PCR for *Egr1* (**A**), *Fos* (**B**), or *Nr4a1* (*Nur77;* **C**) mRNA expression. Data for *Egr1, Fos,* or *Nr4a1* mRNA are normalized to the housekeeping gene M36B4 and presented as fold-induction compared to the untreated samples. Asterisks indicate statistical significance ($P < 0.05$) compared to the untreated controls or to the matched, non-rosiglitazone-treated samples.



B

	Control	PKO
Number of female mice	19	20
Female LH (pg/ml)[a]	85	286*
Female FSH (ng/ml)[a]	14	19
Number of male mice	29	21
Male LH (pg/ml)[a]	113	87
Male FSH (ng/ml)[a]	175	136*
Number of litters	16	14
Number of pups	103	64
Number of pups/litter	6.4±0.5	4.6±0.5*

FIG. 9. Pituitary-specific deletion of *Pparg* causes reduction in the litter size and alters plasma gonadotropin profile in female mice. Genotyping of floxed *Pparg* allele and cre transgene in tail DNA shows that all mice contained the floxed *Pparg* allele but that only the PKO mice carried the *Lhb-cre* allele (**A**). PKO mice had the genotype $Pparg^{flox/flox}$:*Lhb-cre,* and the control mice had the genotype $Pparg^{flox/flox}$, but without the *Lhb-cre*. Analysis of DNA extracted from the pituitaries after mice were euthanized demonstrates the presence of the deleted knockout (KO) *Pparg* allele only in PKO mice. Reproductive hormones were measured during diestrus in females and randomly in males (**B**). Median hormone levels are given, because data are not normally distributed. Significance was assessed using nonparametric Mann-Whitney and Wilcoxon signed-rank tests. Fertility was assessed by breeding four pairs of PKO or control mice over 6 mo. Total number of litters and pups are shown along with the litter size (mean ± SEM). Significance was assessed using Student *t*-test. Asterisks indicate statistical significantly ($P < 0.05$) compared to the untreated controls.

increased LH levels and reduced litter size is reported for a subset of subfertile female mice lacking estrogen receptor alpha (ESR1) in pituitary gonadotrophs [43]. Estrogen has anti-inflammatory effects similar to those of PPARG agonists and prevents metabolic complications of diet-induced obesity. It is conceivable that ESR1 and PPARG have similar effects in the gonadotroph to suppress inflammatory signaling that would otherwise elevate LH levels. This overlapping role is seen in other tissues as well: Muscle-specific knockout of *Pparg* causes insulin resistance and glucose intolerance, and knock out of *Esr1* causes a similar phenotype, with muscle insulin resistance and increased tissue inflammation [21, 44]. The ability of ESR1 to repress inflammation is only seen in estrogenized females. Males and postmenopausal females are not protected because of low estrogen levels [45]. The PPARG effects also appear to be sex-specific: LH is not elevated in males compared to control littermates. It is possible that PPARG expression is low in male mice, that they do not produce endogenous PPARG ligands, or that other pathways, such as androgen receptor repression of *Lhb*, may compensate for lack of PPARG signaling in male PKO mice.

If GnRH uses the same signaling pathways as inflammation, then one would predict that states of inflammation would alter the reproductive axis. Indeed, this has been seen in both humans and animals. Chronic filarial infection lowers LH levels in women [46], and obese PCOS is associated with

elevated inflammatory markers [47, 48]. Men with untreated systemic lupus erythematosus, rheumatoid arthritis, type 2 diabetes, metabolic syndrome, or chronic obstructive pulmonary disease—all conditions that are associated with increased inflammation—also have alterations in the HPG axis [49–52]. In animals, endotoxin (lipopolysaccharide) administration decreases serum LH in female and male rats [53, 54] and decreases GnRH pulsatility and pituitary responsiveness in sheep [53, 55]. Some of these effects can be replicated by cytokine treatment alone. For example, interleukin 1β suppresses LH in female rats [56, 57] and suppresses kisspeptin expression but increases gamma-aminobutyric acid in the hypothalamus [58, 59], so the effect can be central as well as on the pituitary.

In conclusion, the ability of PPARG to repress *Lhb* and the increased LH in PKO female mice provide an interesting model of reproductive dysfunction. A more complete analysis of the reproductive and inflammatory phenotype of the PKO mice is underway to fully understand how lack of PPARG signaling in the gonadotroph influences the HPG axis.

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