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Thiazolidinediones and Rexinoids Induce Peroxisome Proliferator-Activated Receptor-Coactivator (PGC)-1α Gene Transcription: An Autoregulatory Loop Controls PGC-1α Expression in Adipocytes via Peroxisome Proliferator-Activated Receptor-γ Coactivation

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Thiazolidinediones (TZDs) are insulin-sensitizing drugs currently used to treat type 2 diabetes. They are activators of peroxisome proliferator-activated receptor (PPAR)-γ, and adipose tissue constitutes a major site for their biological effects. PPAR coactivator (PGC)-1α is a transcriptional coactivator of PPARγ and other transcription factors. It is involved in the control of mitochondrial biogenesis, and its activity has been linked to insulin sensitization. Here we report that PGC-1α gene expression in brown and white adipocytes is a direct target of TZDs via PPARγ activation. Activators of the retinoid X receptor also induce PGC-1α gene expression. This is due to the presence of a PPARγ-responsive element in the distal region of the PGC-1α gene promoter that binds PPARγ/retinoid X receptor heterodimers. Moreover, there is a positive autoregulatory loop of control of the PGC-1α gene through coactivation of PPARγ responsiveness to TZDs by PGC-1α itself. These data indicate that some of the effects of TZDs, especially promotion of mitochondrial biogenesis and oxidative pathways in adipose depots, entail PGC-1α up-regulation via enhanced transcription of the PGC-1α gene. (*Endocrinology* 147: 2829–2838, 2006)

THIAZOLIDINEDIONES (TZDS) ARE insulin-sensitizing drugs of current use in type 2 diabetes. They are strong agonists of peroxisome proliferator-activated receptor (PPAR)-γ, and most of their effects are believed to involve the activation of this receptor (1). The mechanisms of insulin sensitization elicited by TZDs are not fully understood. Although TZDs can act on skeletal muscle, adipose tissue is their major target. PPARγ is highly expressed in adipose tissue and it acts as a master transcription factor of adipogenesis. Thus, it has been proposed that the main mechanisms of action of TZDs include stimulation of adipogenesis and subsequent fatty acid storage in adipose tissue, sparing other tissues such as liver, muscle, or β-cells from excessive exposure to free fatty acids (2). However, the effects of TZDs are complex, and several other mechanisms have been implicated in insulin sensitization, including the promotion of mitochondrial oxidative activity in adipose tissue (3).

PPAR-coactivator (PGC)-1α is a transcriptional coactivator involved in the control of energy metabolism. It coactivates members of the type II class of nuclear hormone receptors, including PPARγ as well as nuclear respiratory factors-1 and -2, responsible for concerted activation of genes determining mitochondrial respiratory capacity (4). PGC-1α is implicated in the control of multiple biological pathways related to energy metabolism in liver, muscle, and adipose tissues. It is required for efficient activation of gluconeogenesis in the liver (5), and it promotes mitochondrial biogenesis in skeletal muscle (6). PGC-1α is strongly expressed in brown adipose tissue from rodents and, to a lesser extent, white adipose tissue of humans and rodents (7, 8). When overexpressed in either rodent or human adipocytes, PGC-1α drives differentiation toward a brown adipocyte phenotype, characterized by the expression of the mitochondrial uncoupling protein (UCP)-1 and enhancement of overall mitochondrial oxidative activity (7, 9). It has been proposed that PGC-1α mediates the conversion of white adipocytes from storage to fat-oxidizing cells in response to such signals as leptin (10).

A deficient mitochondrial oxidative capacity associated with reduced expression of PGC-1α target genes has recently been identified as a primary event associated with insulin resistance in skeletal muscle (11, 12), and recent studies indicate that reduced expression of PGC-1α accounts for a reduction in the oxidative capacity in subjects prone to insulin resistance (13). Whereas this impairment of oxidative capacity has been reported mainly in skeletal muscle, recent data indicate that it may also be relevant in adipose tissue. Corvera and collaborators (14, 15) identified mitochondrial biogenesis as a key process in the differentiation of white

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Abbreviations: aP2, Adipocyte P2; C/EBP, CCAAT/enhancer-binding protein; ChIP, chromatin immunoprecipitation; 9-cis RA, 9-cis retinoic acid; COII, cytochrome oxidase subunit II; COIV, cytochrome oxidase subunit IV; CREB, cAMP response element-binding protein; FABP, fatty acid binding protein; GFP, green fluorescent protein; PGC, PPAR coactivator; PKA, protein kinase A; PPAR, peroxisome proliferator-activated receptor; PPRE, PPAR-responsive element; RXR, retinoid X receptor; TZD, thiazolidinedione; UCP, uncoupling protein.

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adipose tissue, similarly to brown fat differentiation. They recently reported that TZDs favor this process, including the induction of PGC-1α, in white adipose tissue depots in rodents. An overall reduction in marker genes characteristic of brown adipose phenotype, including PGC-1α, in sc adipose tissue of human adults has been associated with insulin resistance (16). This has led to the proposal that up-regulation of PGC-1α could constitute a strategy for preventing and reversing insulin resistance and obesity (17). PGC-1α gene transcription is induced by cAMP-dependent pathways. In response to adrenergic stimulus in brown adipose tissue, transcriptional activation is mediated by phosphorylation of activating transcription factor (ATF)-2 via p38 MAPK, which is activated by protein kinase A (PKA) in response to cAMP (18). A similar mechanism involving direct phosphorylation of cAMP response element-binding protein (CREB) by PKA takes place in liver in response to glucagon (19). ATF-2 and CREB action on the PGC-1α gene occurs through a cAMP-responsive site in the proximal promoter region. Myocyte enhancer factor-2, a specific myogenic transcription factor, is another transcription factor implicated in PGC-1α gene expression in skeletal muscle and its responsiveness to calcium (20).

Considering that TZDs exert most of their effects on adipose tissue and recent data indicating that they may up-regulate oxidative activity in adipose tissue of diabetic patients (3), we examined their effect on the transcription of the PGC-1α gene in brown and white adipose cells. We identified PGC-1α as a direct target of TZDs acting via a PPARγ-responsive element in the PGC-1α gene promoter as well as a positive autoregulatory loop of control of the PGC-1α gene through coactivation of PPARγ responsiveness by PGC-1α itself.

Materials and Methods

Materials

Rosiglitazone and ciglitazone were from Cayman Chemicals (Ann Arbor, MI). Troglitazone was provided by Glaxo Wellcome (Greenford, UK). Norepinephrine, 9-cis retinoic acid (9-cis RA), T_3, insulin, propanolol, prazosin, and cycloheximide were from Sigma (St. Louis, MO). AGN194204 was a gift from Dr. R. A. Chandraratna (Allergan Pharmaceuticals, Irvine, CA). TTNPB, GW9662, and H89 were from EMD Biosciences (La Jolla, CA).

Cell culture

The care and use of mice were in accordance with European Community Council Directive 86/609/EEC and were approved by the Comitè Ètic d'Experimentació Animal of University of Barcelona. Primary cultures of brown adipocytes from mice were performed as described previously (21), and experiments were done on d 9 of culture when 80–90% of the cells were differentiated. HIB-1B cells were cultured and differentiated as described elsewhere (22). 3T3-L1 cells were maintained and differentiated following standard procedures (23) and were studied 1 wk after induction of differentiation when more than 90% of the cells were differentiated on the basis of lipid accumulation. Human preplated adipocytes, corresponding to a mix of cells from six healthy individuals with normal body mass index, were obtained from Zen-Bio (Research Triangle Park, NC), cultured according to supplier's instructions, and studied 2 wk after induction of differentiation. Cells were exposed to 10 μM rosiglitazone, 10 μM troglitazone, 30 μM ciglitazone, or 1 μM 9-cis RA for 24 h, unless otherwise indicated. Treatment with norepinephrine was at 0.5 μM for 6 h. The effects of PPARγ inhibition were determined by using 30 μM GW9662 and those of retinoid X receptor (RXR) activation with 1 μM AGN194204. Propanolol plus prazosin were used at 10 μM for 24 h. H89 was used at 10 μM and cycloheximide was used at 5 μg/ml, as reported elsewhere (21).

RNA isolation, Northern blot, and quantitative real-time PCR analyses

RNA was extracted using the RNeasy minikit (QIAGEN, Hilden, Germany). Northern blot analysis and hybridization were carried out as described (24), using as probes the cDNA for mouse PGC-1α (7), rat UCP1 (25), adipocyte P2 (aP2)/fatty acid binding protein (FABP) (26), mouse cytochrome oxidase subunit II (COII) (27) and subunit IV (COIV) (American Type Culture Collection, Manassas, VA), and mouse β-actin (28). Hybridization signals were quantified using Molecular Image System GS-525 (Bio-Rad Laboratories, Foster City, CA). After individual densitometric analysis of the 6.5- and 5.0-kb transcript signals for PGC-1α, no differential changes in their expression were detected in any of the experimental treatments studied, except for the positive control norepinephrine (see Fig. 1), and both transcripts were modified in parallel in response to the TZDs or rexinoids. Therefore, data are expressed by integrating densitometry signals for both transcripts. For quantitative real-time PCR analysis of mRNA expression, TaqMan RT-PCR was performed on the ABI PRISM 7700HT sequence detection system and reagents (Applied Biosystems, Foster City, CA). One microgram of RNA was transcribed into cDNA using MultiScribe reverse transcriptase and random-hexamer primers. The reaction was performed in a final volume of 25 μl using TaqMan universal PCR master mix, No-AmpErase UNG reagent, and the specific gene expression primer pair probes. The Assay-on-Demand probes used were Mm00447183 for mouse PGC-1α, Hs00173304 for human PGC-1α, Hs00222453 for human UCP1, Mm00437762 for mouse β_2-microglobulin, and Hs99999904 for human cyclophilin A (housekeeping reference controls). Human COII mRNA was analyzed (Assay-by-Design, Applied Biosystems) using as primers CAAACCACTTTCACCGCTACAC (forward) and GGACGATGGGCATGAAACTGT (reverse), and the FAM-labeled probe was AAATCTGTGGAGCAAACC. The relative amount of PGC-1α mRNA in each sample was normalized to that of the reference control using the comparative ($2^{-\Delta CT}$) method according to the manufacturer's instructions.

Immunoblot assays

Immunoblot analysis of PGC-1α was performed as reported elsewhere (24). Protein extracts from brown adipocytes were prepared by homogenization in a buffer containing 100 mM Tris (pH 8.5), 250 mM NaCl, 1% Igepal CA-630 (Sigma), 1 mM EDTA, a cocktail of protease inhibitors (Complete-Mini; Roche Diagnostic, Sant Cugat del Valles, Spain), and 0.1% phenylmethylsulfonyl fluoride. Proteins (40 μg/lane) were separated by 8% SDS-PAGE, transferred to Immobilon-P membranes (Millipore, Bedford, MA), and probed with an antibody against murine PGC-1α (SC-5816; Santa Cruz Biotechnology, Santa Cruz, CA). As a positive control, protein extracts of HEK-293 cells infected with the adenovirus driving murine PGC-1α (40 μg/lane) were run in parallel (see below). Incubation with an anti-β-actin antibody (Sigma, A5441) was performed to establish equal loading of protein samples.

Plasmids and transfection assays

The plasmid −2553-PGC-1α-Luc, in which the region −2553 to +78 of the mouse PGC-1α gene drives the promoterless luciferase gene from firefly, was a gift from Dr. B. Spiegelman (Dana-Farber Cancer Institute and Department of Cell Biology, Harvard Medical School, Boston, MA). The plasmid −2553-PPREmut-PGC-1α-Luc, a version containing point mutations at the putative PPAR-responsive element (PPRE) (GT instead of AG at sites −2043 and −2044, and GCT instead of AGG at sites −2050 to −2052) was generated using the Quikchange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and checked by direct DNA sequencing. The plasmid in which the proximal region containing the cAMP-responsive element at −146/−129 (19) had been removed (−2553-CREmut-PGC-1α-Luc) was obtained by digestion of the parental plasmid −2553-PGC-1α-Luc with *Pvu*II and *Zra*I and further ligation. Plasmid expression vectors pSG5-PPARγ (29), pRSV-RXRα (30), pSV-PGC-1α (7), and SRα-PKA (31) are described elsewhere. HIB-1B cells were transiently transfected using FuGENE-6 (Roche Molecular Bio-

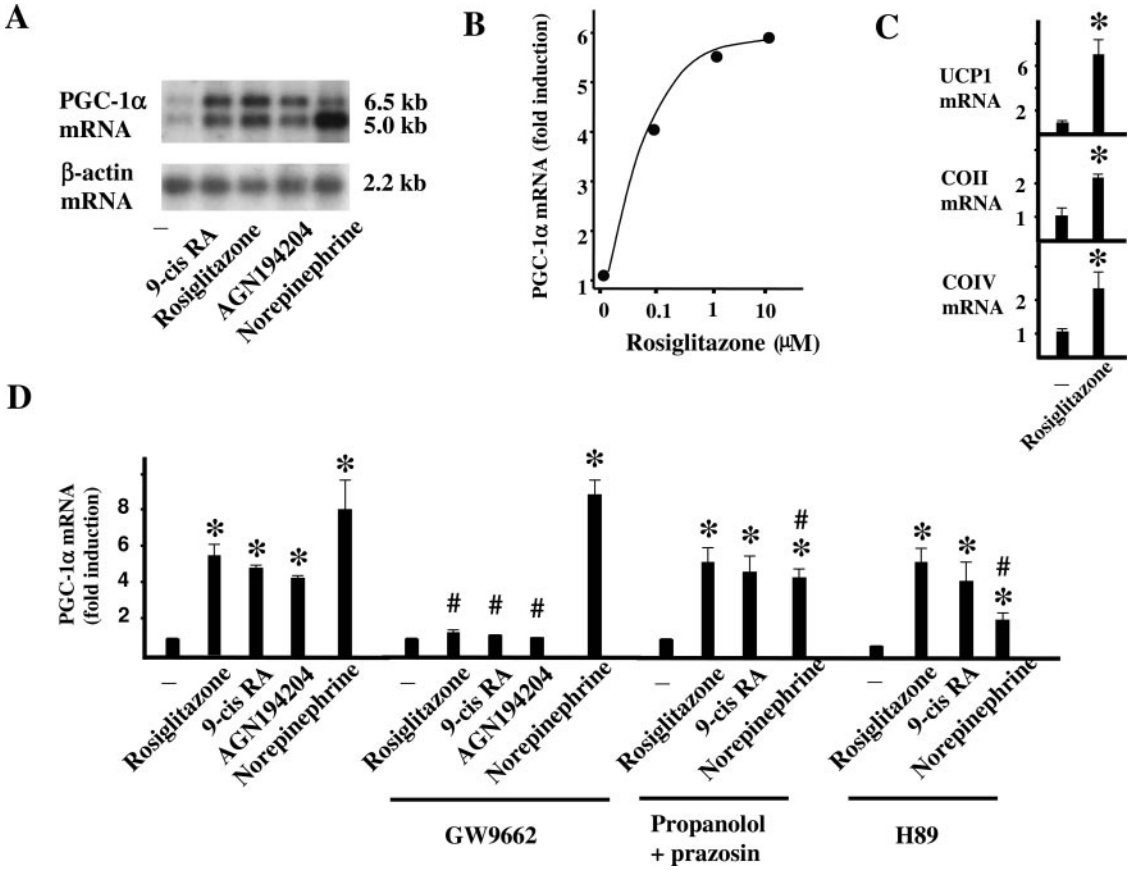

FIG. 1. Effects of rosiglitazone, 9-cis-RA, and norepinephrine on PGC-1α mRNA in brown adipocytes in primary culture. Murine brown adipocytes differentiated in primary culture were used for all figure panels. Cells were treated 24 h with 10 μM or the other indicated concentrations of rosiglitazone, 1 μM *9*-cis RA, 1 μM AGN 194204, 30 μM GW9662, 10 μM propanolol plus prazosin or for 6 h with 0.5 μM norepinephrine. A, Example of Northern blot analysis PGC-1α mRNA expression. B, Dose response of the effects of rosiglitazone on PGC-1α mRNA levels. C, Effects of 10 μM rosiglitazone on UCP1 mRNA, COII mRNA, and COIV mRNA expression. D, Effects of the PPARγ inhibitor GW 9662, the adrenergic inhibitors propanolol plus prazosin, and the PKA inhibitor H89 on the induction of PGC-1α mRNA by rosiglitazone, 9-cis RA, or norepinephrine. Results are the mean ± SEM of four to five independent experiments. *, Significant differences ($P < 0.05$) with respect to controls; #, Significant differences due to GW9662, propanolol plus prazosin, or H89 for each experimental situation.

chemicals, Indianapolis, IN) for 16 h. Unless otherwise indicated, each transfection contained 1.5 μg of −2553-PGC-1α-Luc, 0.3 μg of plasmid expression vectors, and 3 ng of pRL-CMV (Promega Corp., Madison, WI), an expression vector of Renilla reniformis luciferase. Cells were incubated for 48 h after transfection and, when indicated, were treated for 24 h before harvest with or without 10 μM rosiglitazone or 1 μM 9-cis RA. Luciferase activities were measured in a Turner Designs luminometer TD20/20 using the dual luciferase reporter assay system (Promega). Luciferase activity elicited by wild-type and mutant −2553-PGC-1α-Luc constructs was normalized for variation in transfection efficiency using Renilla luciferase as internal standard.

EMSA experiments

Nuclear proteins were isolated from rat brown adipose tissue or differentiated primary brown adipocytes as described elsewhere (32). cDNAs for mouse PPARγ and human RXRα were transcribed and translated *in vitro* using the TNT quick-coupled transcription/translation systems (Promega). The PGC-1α-PPRE double-stranded oligonucleotide used in EMSAs corresponds to positions −2036 to −2062 of the mouse PGC-1α gene, and its sequence is 5′-GTAAAATTCAGGACAAAGGTCATGGGCTC-3′ (wild type). The mutated version of PGC-1α-PPRE designed to have impaired PPARγ/RXR binding had GT instead of AG at sites −2043 and −2044 and GCT instead of AGG at sites −2050 to −2052. The ^{32}P-labeled PGC-1α-PPRE wild-type or mutated oligonucleotides (10,000–20,000 cpm) were incubated for 30 min at 25 C with 5 μg of nuclear protein extract or 5 μl of *in vitro*-transcribed/translated proteins. Reactions were carried out in a volume of 20 μl containing 10 mM Tris-HCl (pH 8.0), 0.05% Nonidet P-40, 1 mM dithiothreitol, 40 mM KCl, 6% glycerol, and 2 μg of poly(dI)/(dC). Samples were analyzed by electrophoresis at 4 C in nondenaturing 5% polyacrylamide gels in 0.5 × 44.5 mM Tris, 44.5 mM borate, and 1 mM EDTA. In the competition experiments, 100-fold molar excess of unlabeled oligonucleotide was included. When indicated, 1 μl of antiserum against PPARγ (sc-7196; Santa Cruz Biotechnology) or CCAAT/enhancer-binding protein (C/EBP)δ (sc-151; Santa Cruz Biotechnology) was used.

Chromatin immunoprecipitation (ChIP) assay

HIB-1B cells were transfected with the PPARγ expression vector and exposed to 10 μM rosiglitazone as described above. When indicated, cells were transfected with −2553-PGC-1α-Luc or −2553-PPREmut-PGC-1α-Luc. Cells were chilled at 4 C for 10 min and treated with 5 mM HEPES-KOH (pH 8.0), 0.1 mM EDTA, 0.05 mM EGTA, 10 mM NaCl, and 1.1% formaldehyde. After centrifugation (600 g, 10 min at 4 C), cell pellets were lysed in ice-cold lysis buffer [50 mM HEPES-KOH (pH 7.4), 1 mM EDTA, 0.5 mM EGTA, 140 mM NaCl, 10% glycerol, 0.5% Nonidet P-40, 0.25% Triton X-100] supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 1 μg/ml each of aprotinin, leupeptin, and pepstatin A). After incubation at 4 C for 10 min, crude nuclei were collected by centrifugation (600 × *g*, for 5 min at 4 C). Nuclei were resuspended in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5 mM EGTA, and 200 mM NaCl plus protease inhibitors, collected as above, and resuspended in radioimmunoprecipitation assay buffer [10 mM Tris-

HCl (pH 8.0), 1 mM EDTA, 0.5 mM EGTA, 140 mM NaCl, 5% glycerol, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% Triton X-100, supplemented with protease inhibitors]. Samples were sonicated with Branson Sonifier 250 and lysates were cleared by centrifugation (16,000 × *g*, 15 min at 4 C) and used for immunoprecipitation with 2 μg of anti-PPARγ antibody (sc-7196, Santa Cruz Biotechnology) or an equal amount of an unrelated immunoglobulin (sc-9314, Santa Cruz Biotechnology).

Immunoprecipitations were performed for 16 h at 4 C, and immune complexes were collected by nutating the lysates for 1 h at 4 C with 30 μl/sample of 50% slurry protein G agarose beads (Santa Cruz Biotechnology) preincubated with 100 μg/ml salmon sperm DNA (Life Technologies). The beads were washed with radioimmunoprecipitation assay buffer supplemented with 1 mM phenylmethylsulfonyl fluoride and 100 μg/ml yeast tRNA (Life Technologies). The beads were then incubated in 100 μl of Tris/EDTA buffer, 0.5% sodium dodecyl sulfate, and 200 μg/ml proteinase K (Sigma) for 3 h at 55 C, and cross-links were reversed for 6 h at 65 C. After phenol-chloroform extraction, DNA was used for PCR analysis. Primers for amplifying a 378-bp fragment encompassing the putative PPRE in the PGC-1α gene were: forward, 5′GTATCAGTTACCATCAGG 3′ and reverse, 5′AACAAGATGGCCAACAGC3′; those amplifying a 237-bp fragment of the murine cyclophilin A gene, used as control, were: forward, 5′ AAGACTGAATGGCTGGATGG 3′ and reverse, 5′ TTACAGGACATTGCGAGCAG 3′. After 35 cycles of amplification, PCR products were electrophoresed in a 1.5% agarose gel and visualized by ethidium bromide staining.

Transduction of brown adipocytes with an adenoviral vector driving PGC-1α

Differentiated brown adipocytes were infected with an adenoviral vector driving murine PGC-1α (AdCMV-PGC-1α, provided by B. Spiegelman) or AdCMV-GFP (control) at a multiplicity of infection of 400 for 4 h in DMEM/F12 medium. Further culture of cells with regular culture medium for brown adipocytes (21) was performed for 48 h. This treatment led to an efficiency of transduction of about 80%, on the basis of assessment of green fluorescent protein (GFP) fluorescence. In these conditions, transduction increased the 120-kDa immunoblot signal for PGC-1α protein at least 10-fold with respect to controls.

Statistical analysis were performed using the Student's *t* test.

Results

TZDs and rexinoids induce PGC-1α gene expression in brown adipocytes

Rosiglitazone increased PGC-1α mRNA expression in mouse primary brown adipocytes differentiated in culture (Fig. 1A). This effect was dose and time dependent (Fig. 1B). PGC-1α mRNA expression was significantly higher than the controls 4 h after exposure to 10 μM rosiglitazone (4.8 ± 0.5-fold induction), peaked 24 h after exposure (see Fig. 1B), and remained high for 48 h (not shown). Other TZDs, such as troglitazone or ciglitazone, had similar effects. Thus, 10 μM troglitazone caused a 5.3 ± 0.4-fold induction in PGC-1α mRNA levels ($P < 0.05$); and 30 μM ciglitazone caused a 3.9 ± 0.3-fold induction in PGC-1α mRNA ($P < 0.05$). Treatment of cells with 9-cis RA also induced PGC-1α mRNA expression (Fig. 1A). Similar activation by AGN194204, a specific RXR agonist (Fig. 1A), but not TTNPB, a retinoic acid receptor activator (data not shown), indicated that RXR activation was responsible for the effects of 9-cis RA. Parallel experiments in which brown adipocytes were exposed to norepinephrine indicated a robust induction, consistent with the known adrenergic, cAMP-dependent, activation of PGC-1α gene transcription (18). However, whereas the induction elicited by norepinephrine occurred mainly for the 5.0-kb PGC-1α mRNA transcript, changes elicited by TZDs and rexinoids occurred equally for the 5.0- and 6.5-kb transcripts. A concomitant increase in the mRNAs for the genes encoding the mitochondrial proteins UCP1, COII, and COIV, known targets of PGC-1α effects, was observed in response to rosiglitazone (Fig. 1C). Rosiglitazone also increased significantly PGC-1α mRNA in HIB-1B cells, a cell line representative of brown adipocytes (7.3 ± 0.9-fold induction, $P < 0.05$) as did 9-cis RA, albeit to a lesser extent (3.4 ± 0.5-fold induction, $P < 0.05$).

The induction of PGC-1α by TZDs and rexinoids is mediated by a PPARγ-dependent pathway

The effects of both rosiglitazone and 9-cis RA on PGC-1α gene expression were blunted in the presence of GW9662, a specific PPARγ antagonist, thus indicating that rosiglitazone acted through PPARγ and that 9-cis RA effects via RXR also required an active PPARγ (Fig.1D). This indicates that PPARγ/RXR heterodimers mediate TZD and 9-cis RA effects on PGC-1α gene expression. The PPARγ antagonist GW9662 did not alter the induction of PGC-1α mRNA levels by norepinephrine, thus indicating that TZDs and adrenergic, cAMP-mediated activation of PGC-1α gene expression take place through distinct mechanisms. This was further established by the use of a mixture of β- and α-adrenergic antagonists, propanolol plus prazosin, which reduced the norepinephrine effects on PGC-1α mRNA expression, whereas it did not alter the action of rosiglitazone or 9-cis RA (Fig.1D). The PKA inhibitor H89 also reduced the action of norepinephrine on PGC-1α mRNA expression, whereas it did not have any effect on rosiglitazone- and 9-cis RA-dependent induction (Fig.1D). Cycloheximide did not affect the induction of PGC-1α by rosiglitazone (5.8 ± 0.7-fold induction by rosiglitazone in control cells, 6.5 ± 0.9-fold induction by rosiglitazone in cycloheximide-treated cells), thus indicating that rosiglitazone acts on PGC-1α gene expression through protein synthesis-independent mechanisms, compatible with a ligand-dependent activation of PPARγ.

TZDs induce the expression of the PGC-1α protein in brown adipocytes

We determined whether the observed induction of PGC-1α mRNA levels by TZDs result in an increase in the PGC-1α protein in brown adipocytes. A robust time-dependent induction of PGC-1α protein abundance in brown adipocyte protein extracts was observed as a consequence of treatment with rosiglitazone (Fig. 2).

TZDs and rexinoids activate PGC-1α gene expression in white adipocytes

The effects of rosiglitazone were analyzed in differentiated 3T3-L1 white adipocytes. As expected, PGC-1α mRNA levels were much lower (~100-fold less) than in brown adipocytes and required a more sensitive method (real-time PCR) of quantification. Rosiglitazone induced PGC-1α mRNA expression as did 9-cis RA, albeit to a lesser extent (Fig. 3). The effects of rosiglitazone and 9-cis RA were also analyzed in human white adipocytes differentiated in primary culture. As for 3T3-L1 cells, PGC-1α mRNA abundance was in a

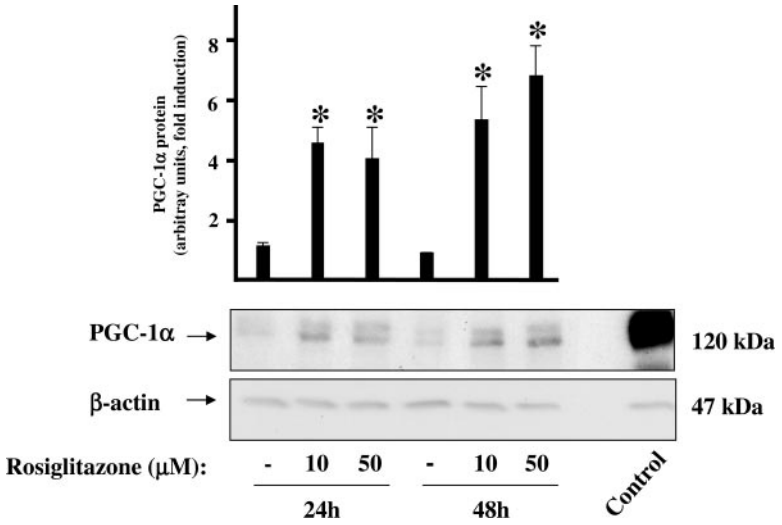

FIG. 2. Effects of rosiglitazone on PGC-1α protein levels in brown adipocytes in primary culture. Murine brown adipocytes differentiated in primary culture were treated with rosiglitazone at the indicated times and concentration. Data are means ± SEM of at least three independent experiments and are shown as the ratio of the densitometric intensity of the immunoreactive signal of PGC-1α protein normalized by the immunoreactive signal for β-actin (*top*). *, Significant differences ($P < 0.05$) with respect to controls. Representative immunoblot analysis of PGC-1α protein levels (*bottom*). Control corresponds to the immunoreactive signal using a protein extract of HEK-293 cells stably transduced with a cytomegalovirus-driven adenoviral construct for murine PGC-1α expression.

range much lower than brown adipocytes. However, rosiglitazone and 9-cis RA induced significantly PGC-1α mRNA levels (Fig. 3). Known targets of PGC-1α such as UCP1 mRNA and COII mRNA were also significantly induced ($P < 0.05$) in response to rosiglitazone (2.6 ± 0.3-fold induction and 1.5 ± 0.1-fold induction, respectively) and 9-cis RA (2.8 ± 0.2-fold induction and 1.48 ± 0.1-fold induction, respectively).

The PGC-1α gene is a target of PPARγ-mediated transcriptional activation via a PPARγ-responsive element in the promoter region

To determine whether the PGC-1α gene is a target of transcriptional regulation by TZDs, HIB-1B cells were transiently transfected using the −2553-PGC-1α-Luc plasmid construct. Rosiglitazone induced a dose-dependent increase in the PGC-1α gene promoter activity (see Fig. 4A). When cells were cotransfected with PPARγ and rosiglitazone was added, an enhancement in the dose-dependent induction was observed. Parallel experiments indicated a more than

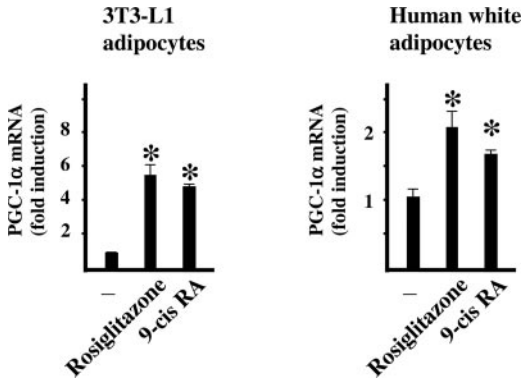

FIG. 3. Effects of rosiglitazone and 9-cis RA on PGC-1α mRNA in white adipocytes. Murine 3T3-L1 adipocytes and human white adipocytes differentiated in primary culture were treated for 24 h with 10 μM rosiglitazone or 1 μM 9-cis RA. Relative abundance of PGC-1α mRNA was determined by quantitative real-time PCR (see *Materials and Methods*). Results are the mean ± SEM of three to four independent experiments. *, Significant differences ($P < 0.05$) with respect to controls.

2-fold induction by 9-cis RA, and this was enhanced to around 6-fold induction by cotransfection with PPARγ or RXRα (Fig. 4B). Maximal induction was achieved with PPARγ plus RXRα cotransfection and addition of rosiglitazone. This result indicates that rosiglitazone acts on the PGC-1α gene via ligand-dependent activation of PPARγ/RXR heterodimers. Cotransfection with an expression vector driving the catalytic subunit of PKA led to a significant induction of the PGC-1α promoter activity, in the range of that achieved via rosiglitazone activation of PPARγ/RXRα heterodimers. This is consistent with the presence of cAMP-responsive elements in the PGC-1α promoter (18, 19). Moreover, as for endogenous PGC-1α gene expression, the activation of the PGC-1α promoter was also observed for other TZDs. Troglitazone and ciglitazone led to a 2.7 ± 0.4- and 2.3 ± 0.3-fold induction of PGC-1α promoter activity, respectively, in the absence of cotransfected PPARγ, and 3.8 ± 0.5- and 2.9 ± 0.4-fold induction when PPARγ had been cotransfected.

Computer-assisted analysis of the sequence of the mouse PGC-1α promoter region (MatInspector) revealed the presence of a direct repeat with one bp spacing, *i.e.* a potential PPARγ-responsive element, at position −2043 to −2055. The sequence of this element was practically identical with consensus PPARγ-response elements, and it was fully conserved in the promoter of human PGC-1α gene and PGC-1α genes from the other mammalian species (rat, bovine) in which the 5′ noncoding region sequence is available in databases (accession no. NT_078473 for mouse, NM_031347 for rat, NT_006316 for human, AY547550 for bovine). Position around −2 kb of the transcription initiation is also conserved among species (Fig. 5A). A point-mutation construction that disrupts PPARγ binding capacity at that site was designed, and, when transfected into HIB-1B cells, it showed a diminished capacity to be activated by rosiglitazone either alone or in the presence of cotransfected PPARγ (Fig. 5B). Both the wild-type and the PPRE mutant forms of the promoter were significantly activated by PKA, in agreement with the location of a cAMP-responsive element, CREB, and ATF-2 binding site, in the proximal region of the PGC-1α promoter (18, 19). In contrast, a deletion mutant lacking the cAMP-respon-

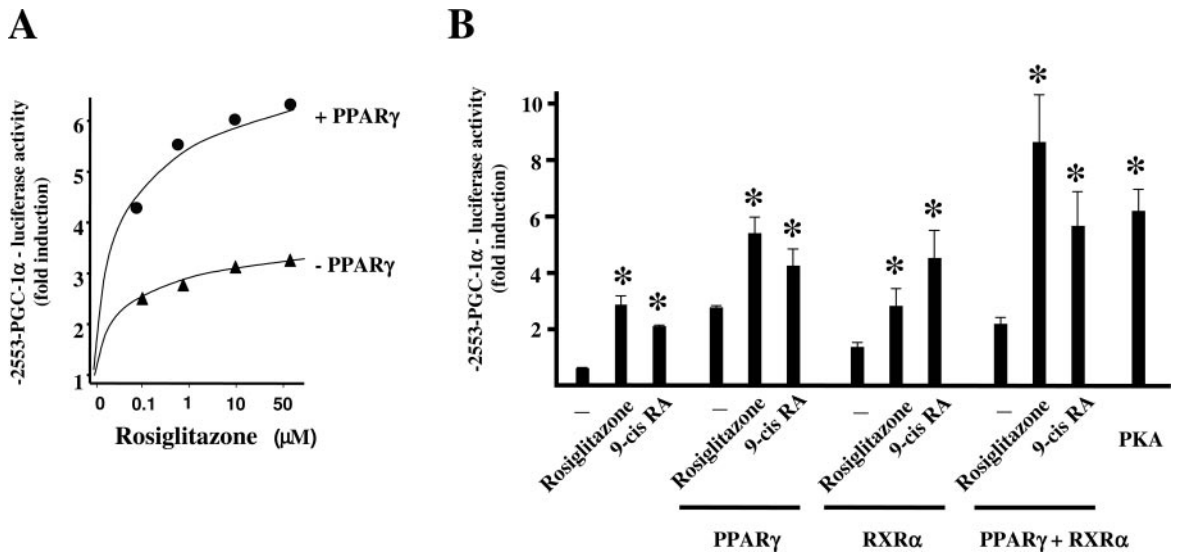

FIG. 4. Effects of rosiglitazone, 9-cis RA, and PPARγ on PGC-1α gene promoter activity. Transient transfection experiments were performed in HIB-1B cells with −2553 PGC-1α-Luc. A, Dose-response effects of rosiglitazone on −2553 PGC-1α-Luc activity in the absence or presence of cotransfected PPARγ. B, When indicated, the expression vectors for PPARγ, RXRα, or for constitutively active PKA were cotransfected and 24 h previous to harvest 10 μM rosiglitazone or 1 μM 9-cis RA were added to the cells. B, Results show the mean ± SEM of at least three independent experiments done in triplicate. *, Statistically significant differences with respect to untreated controls ($P < 0.05$).

sive element showed impaired PKA-dependent activation but remained completely sensitive to activation by rosiglitazone (Fig. 5B). The separate actions of cAMP-dependent pathways and TZD effects on PGC-1α gene transcription are consistent with the distant placement of their respective *cis*-acting elements in the PGC-1α promoter.

To determine whether PPARγ binds the PGC-1α promoter, EMSA was performed using as probe an oligonucleotide corresponding to the −2036 to −2062 region. Incubation with a mixture of PPARγ and RXRα translation products led to the appearance of a specific retarded band. (Fig. 6, *left panel*) When the labeled probe was incubated with nuclear extracts from brown adipose tissue, several specific bands appeared. Incubation with the antibody against PPARγ led to the appearance of a novel, low-mobility, band (supershift) associated with a marked reduction in the intensity of two bands of higher mobility (Fig. 6, *center panel*). Incubation with an unrelated antibody (against C/EBPδ), used as a negative control, did not show any effect on the pattern of retarded bands. When a parallel EMSA was performed using nuclear extracts from brown adipocytes differentiated in primary culture, results were identical (Fig. 6, *right panel*). These findings indicate that the PPARγ-responsive sequence in the PGC-1α promoter binds PPARγ present in brown adipocyte nuclei. EMSA of an oligonucleotide corresponding to the mutated form used to disrupt PPARγ responsiveness confirmed the loss of binding of the PPARγ-containing complexes when incubated with either PPARγ/RXRα translation products (Fig. 6, *left panel*) or differentiated brown adipocyte nuclear extracts (Fig. 6, *right panel*).

To further examine the interaction of PPARγ with the PGC-1α distal promoter region *in vivo*, ChIP experiments were performed in HIB-1B cells. Immunoprecipitation of protein-DNA complexes with the PPARγ antibody caused a dramatic enrichment in the 378-bp PCR product corresponding to the −2172/−1794 region of the endogenous PGC-1α gene containing the PPRE (Fig. 7A). To establish the involvement of the PPRE element, ChIP experiments were performed comparing HIB-1B cells transfected with the −2553-PGC-1α-Luc or the −2553-PPREmut-PGC-1α-Luc plasmids. After immunoprecipitation with the PPARγ antibody, PCR amplification detected enrichment in the 378-bp fragment of DNA region containing the PPRE in cells transfected with −2553-PGC-1α-Luc (Fig. 7B). Conversely, when the −2553-PPREmut-PGC-1α-Luc was transfected, immunoprecipitation of that DNA fragment with the PPARγ antibody was not enriched, compared with a similarly handled sample in the absence of the specific antibody. These results confirm the interaction of PPARγ with the PPRE element in the distal region of the PGC-1α gene promoter.

PGC-1α coactivate the PPARγ-dependent activation of its own gene in response to TZDs

The identification of PGC-1α as a target gene of PPARγ-mediated TZDs activation suggested the possibility that PGC-1α, as a coactivator of PPARγ, could be involved in the regulation of its own gene expression. To check this possibility, primary cultures of brown adipocytes were transduced with an adenoviral vector driving PGC-1α expression. Northern blot analyses using the PGC-1α cDNA as probe allowed discrimination between the adenoviral-driven recombinant PGC-1α transcript (3.0 kb) and the endogenous PGC-1α transcripts (6.5 and 5.0 kb) (Fig. 8A). In the absence of exogenous PGC-1α, rosiglitazone caused a 5.4 ± 0.6-fold induction of PGC-1α mRNA (Fig. 8A), and when exogenous PGC-1α was overexpressed, the induction became significantly higher (9.1 ± 1.2-fold, $P < 0.05$). PGC-1α alone did not modify endogenous PGC-1α mRNA expression in the absence of rosiglitazone. The same results were obtained in HIB-1B cells (not shown). For comparison, we analyzed the expression of UCP1 mRNA, a bona-fide target of PGC-1α

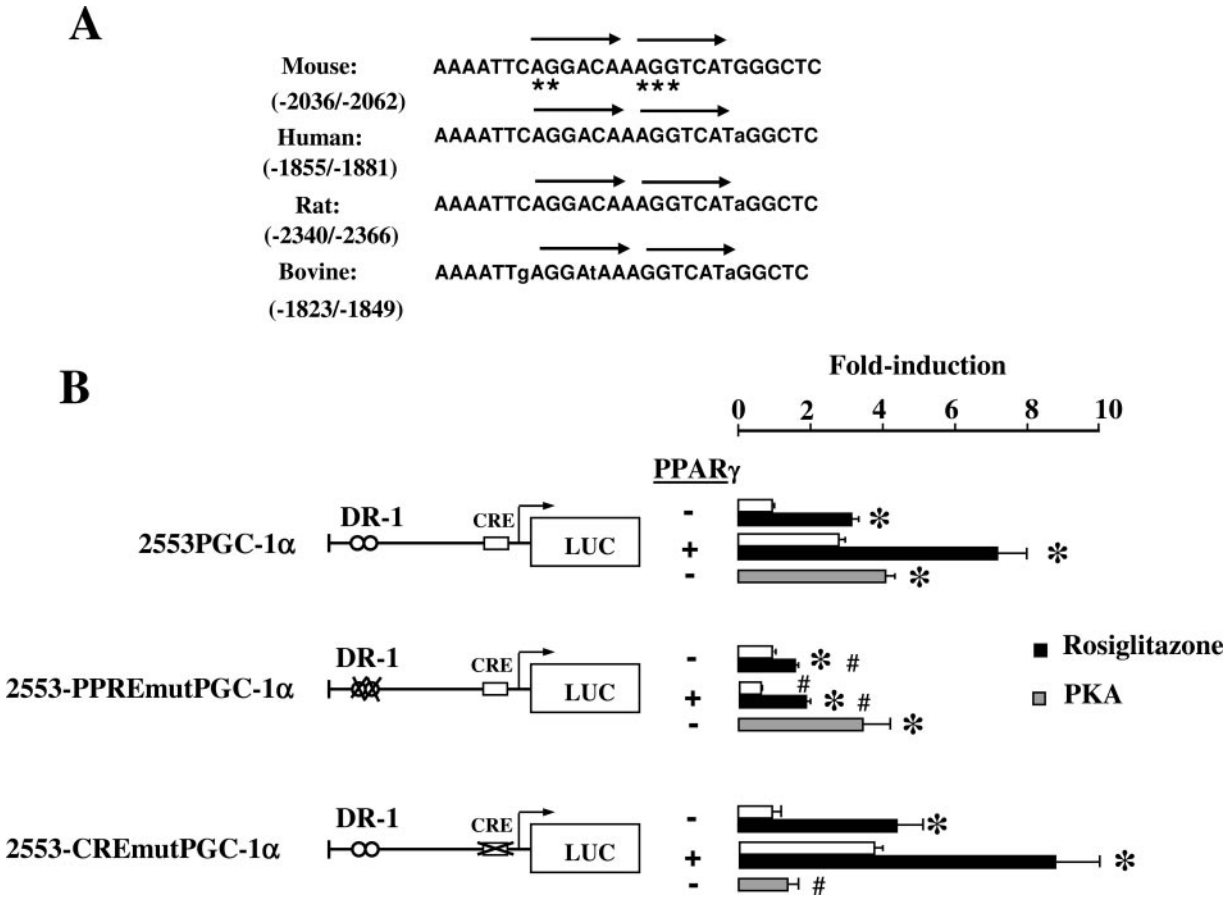

FIG. 5. Identification of a PPARγ-responsive element in the PGC-1α gene promoter. A, Sequence of the PPARγ-response element in the mouse PGC-1α gene promoter and comparison with similar sequences in PGC-1α promoters from other species. *Arrows* indicate one-base spaced direct repeat alignment. B, Effects of points mutations in the PPARγ-response element (shown as *asterisks* in A) or deletion mutation of the proximal cAMP response element (see *Materials and Methods*) on the responsiveness of the PGC-1α gene promoter activity to rosiglitazone, PPARγ, and PKA. The wild-type (2553 PGC-1α-Luc) and mutated (2553-PPREmutPGC-1α-Luc, 2553-CREmutPGC-1α-Luc) plasmid constructs were transfected and treated as in Fig. 4. Results show the mean ± SEM of three to five independent experiments done in triplicate. *, Statistically significant differences ($P < 0.05$) due to rosiglitazone treatment or PKA cotransfection; #, Statistically significant differences ($P < 0.05$) observed after comparing either constructs at equal conditions of cotransfection or treatment.

coactivation of PPARγ-dependent regulation (7). As for the endogenous PGC-1α mRNA, the stimulus of UCP1 mRNA expression by rosiglitazone was enhanced by PGC-1α overexpression (Fig. 8A). However, in this case PGC-1α alone also induced UCP1 mRNA. In contrast, aP2/FABP mRNA was induced by rosiglitazone, but this was not enhanced by PGC-1α overexpression. To establish whether the coactivation of PGC-1α gene expression by itself involves the transcriptional activation of the promoter in response to TZDs, HIB-1B cells were transfected with −2553-PGC-1α-Luc and exposed to rosiglitazone in the presence or absence of a cotransfected expression vector for PGC-1α. Cotransfection with PGC-1α increased the expression of PGC-1α promoter activity in response to rosiglitazone in the presence of PPARγ (Fig. 8B).

Discussion

Present findings indicate that the PGC-1α gene is a direct target of TZD in brown and white adipocytes, and this is due to the presence of a PPARγ-responsive site in the distal PGC-1α promoter region. This constitutes a novel regulatory pathway of the PGC-1α gene, distinct from the adrenergic, cAMP-mediated regulation. This induction of PGC-1α expression would be amplified by the occurrence of an autoregulatory loop mediated by the coactivation of the PPARγ action on PGC-1α gene transcription by PGC-1α itself, a mechanism of feed-forward regulation in response to TZDs. This capacity of PGC-1α to coactivate the action of PPARγ to the PPAR-responsive element in the PGC-1α gene in response to TZDs is similar to that of other genes, such as the brown fat UCP1 gene (present results and Refs. 7 and 33). However, it contrasts with the behavior of other genes such as aP2/FABP, which, in accordance with previous reports (7), is insensitive to PGC-1α coactivation, although it is also PPARγ responsive. Differential control of the transcriptional activity of PPARγ and TZDs on target genes expressed in adipocytes has recently been shown to involve selective dissociation of corepressors (34). During the present study, it was reported that TZDs induce PGC-1α in white adipocytes, in agreement with our present findings, and this has been proposed to contribute to the regulation by TZDs of the expression of genes such as glycerol kinase (34).

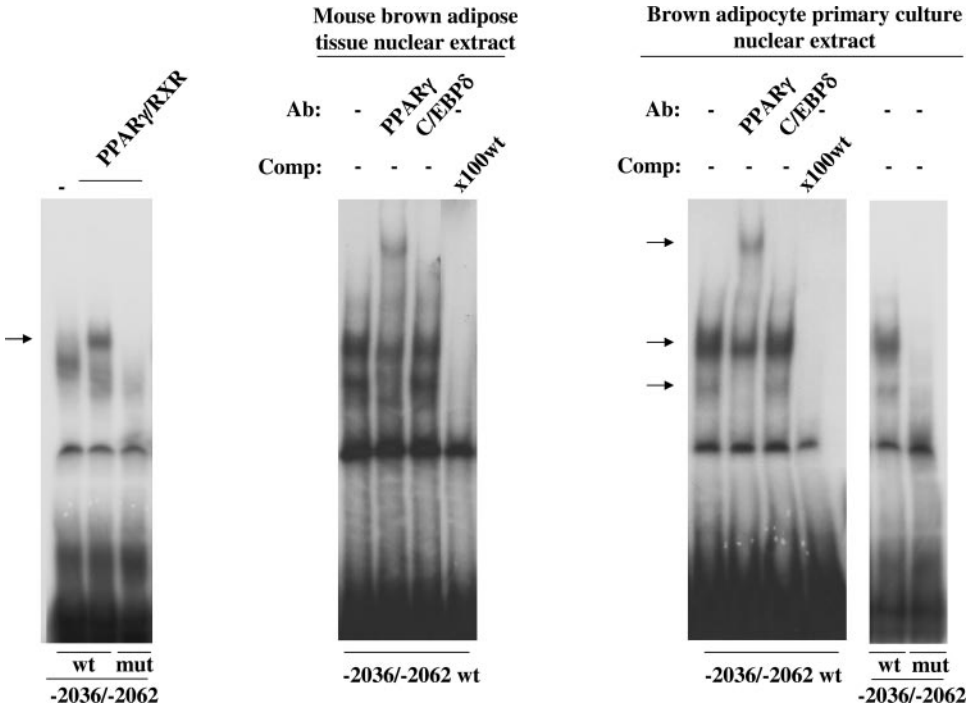

FIG. 6. EMSA of PPARγ binding to the PPARγ-response element in the PGC-1α gene promoter. Double-stranded oligonucleotides corresponding to −2036/−2062 region of the mouse PGC-1α gene promoter (−2036/−2062 wt) or a mutated version designed to impair PPARγ/RXR binding (−2036/−2062 mut) (see *Materials and Methods*) were used as a labeled probes. Five microliters of *in vitro*-transcribed/translated PPARγ plus RXRα protein extract or unprogrammed lysate (*left*), 5 μg of nuclear protein extracts from mouse brown adipose tissue (*center*), or 5 μg of nuclear protein extracts from brown adipocytes in primary culture (*right*) were incubated as described in *Materials and Methods* and when indicated, antibodies (Ab) against PPARγ and C/EBPδ (negative control) were added. × 100 wt means addition of 100-fold excess unlabeled nonmutated probe (Comp). *Arrows* indicate super-shifted (up) or weakened (down) bands.

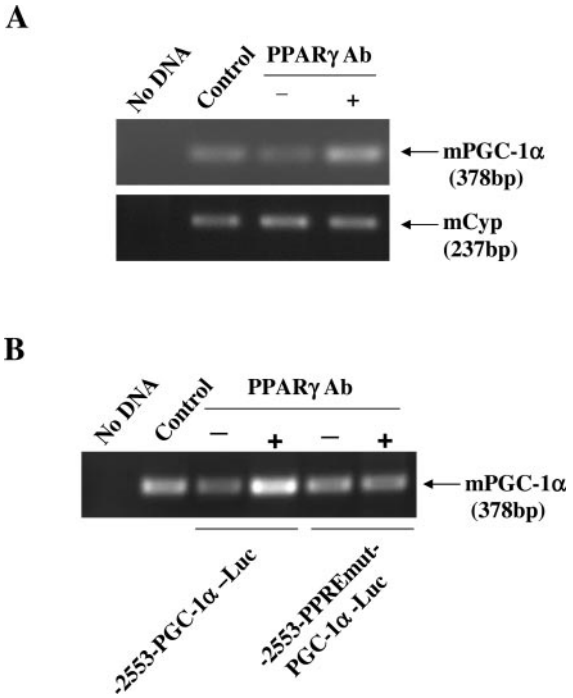

FIG. 7. ChIP analysis of PPARγ binding to the PGC-1α gene promoter. PPARγ antibody (+) or an unrelated Ig (−) was used to immunoprecipitate the protein-DNA complexes (see *Materials and Methods*). A, ChIP analysis of the PPARγ binding to the endogenous PGC-1α gene promoter in HIB-1B cells transfected with the PPARγ expression vector and treated with 10 μM rosiglitazone. *Arrows* indicate the 378-bp PGC-1α and 237-bp cyclophylin A (Cyp) PCR products. No DNA and control represent negative and positive PCR controls. B, HIB-1B cells were transfected with the plasmids −2553-PGC-1α-Luc and −2553-PPREmut-PGC-1α-Luc always in the presence of PPARγ expression vector and 10 μM rosiglitazone. Immunoprecipitation and PCR amplification were performed as in A.

The presence of a PPARγ-responsive element in the PGC-1α gene explains the induction of PGC-1α gene expression in white adipose tissue depots after chronic treatment of rodents with TZDs (14) and even with non-TZD PPARγ activators (35). Moreover, the presence of a PPARγ-responsive element in the PGC-1α gene that binds PPARγ/RXR heterodimers is consistent with the observed induction of the PGC-1α gene by rexinoids, selective RXR activators, considering the known ligand-dependent sensitivity of the RXR moiety in the PPARγ/RXR heterodimers (36). Treatment of experimental animals with rexinoids mimics some of the effects of PPARγ activation by TZDs, including insulin sensitization. However, the more pleiotropic effects of rexinoids caused by the capacity of RXR to dimerize with members of the nuclear hormone receptor superfamily other than PPARγ have precluded to date their clinical use in type 2 diabetes (37). Finally, the presence of a PPARγ-responsive element in the PGC-1α gene raises the possibility that this element could also mediate regulation by other PPAR subtypes. In some target genes of PPARγ, such as lipoprotein lipase gene, the PPARγ-responsive element mediates response to TZDs in adipose tissue but also response to fibrates in liver, via PPARα-dependent activation (38). It has been reported that PPARδ activators induce PGC-1α gene expression in differentiated muscle cells, whereas activators of PPARα do not (39). Further research will be needed to assess the action of PPAR subtypes on the PGC-1α gene and their relation to the tissue-specific effects of drugs capable of activating these receptors.

The effects of PGC-1α induction by TZDs in adipocytes may entail enhancement of oxidative capacity in adipose tissue, which could constitute a major therapeutic mechanism of TZD action. PGC-1α overexpression in rodent or human white adipose cells in culture promotes mitochondriogenesis (6, 9). Moreover, the present findings may ex-

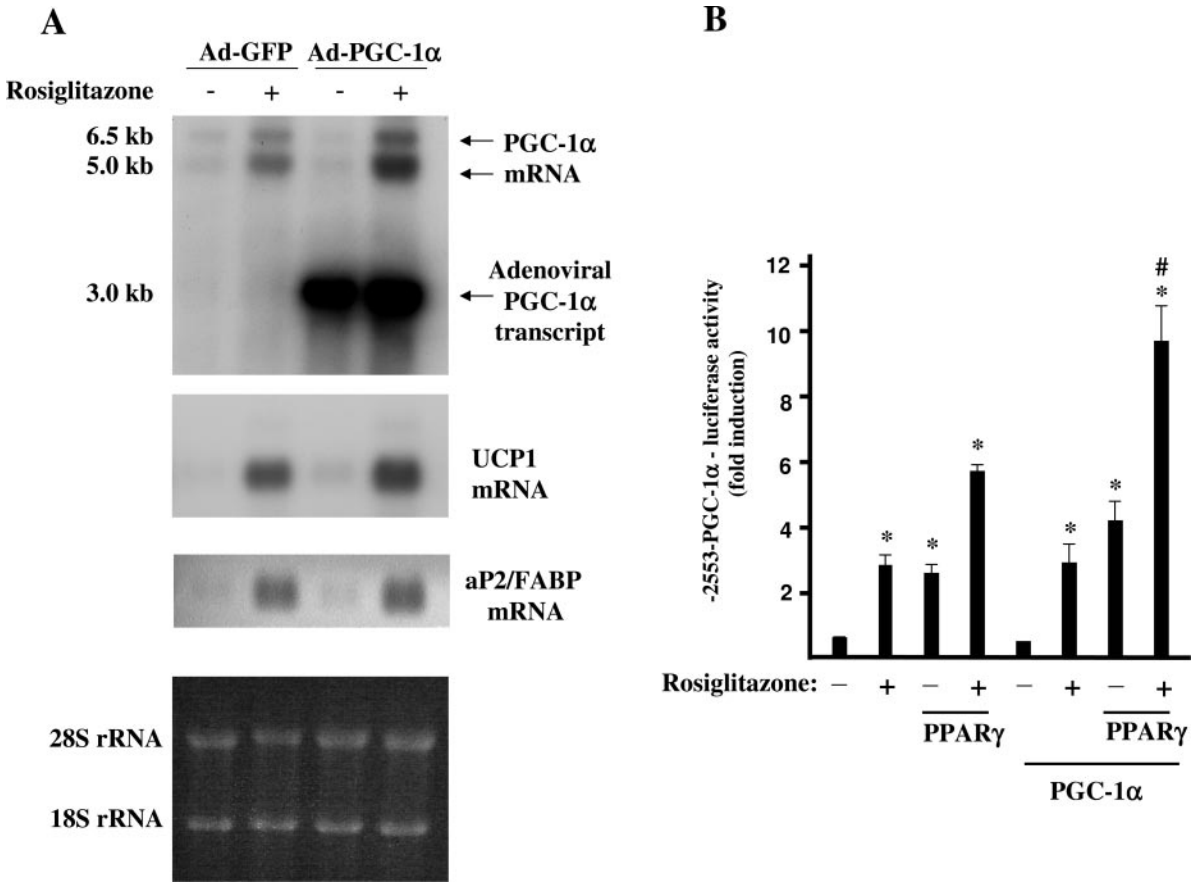

FIG. 8. PGC-1α coactivate the PPARγ-mediated effects of rosiglitazone on its own gene expression. A, Representative Northern blot analysis of RNA from murine brown adipocytes in primary culture transduced with an adenoviral vector driving recombinant PGC-1α expression (Ad-PGC-1α) or a control vector (Ad-GFP), treated or not with 10 μM rosiglitazone. Blot was probed with PGC-1α cDNA as well as with the UCP1 cDNA and aP2/FABP cDNA probes to detect recombinant, adenoviral mediated PGC-1α transcript and the endogenous PGC-1α mRNA. B, Effects of cotransfection with a PGC-1α expression vector on PGC-1α promoter activity in response to rosiglitazone and cotransfected PPARγ. Experimental details were as in Fig. 5. Results show the mean ± SEM of at least three independent experiments done in triplicate. *, Statistically significant differences ($P < 0.05$) with respect to controls; #, Statistically significant differences ($P < 0.05$) due to PGC-1α cotransfection.

plain recent reports indicating that TZDs induce a coordinate up-regulation of genes encoding the mitochondrial oxidation machinery in human adipose tissue from diabetic patients (3, 40). Accordingly, experimental treatments in rodents had already evidenced mitochondrial remodeling and enhanced energy expenditure in white fat in response to TZDs (15), including increased PGC-1α expression (14). Impaired mitochondrial oxidative capacity in skeletal muscle, particularly of genes that are targets of PGC-1α, has been identified as an early event associated with insulin resistance and type 2 diabetes (11, 12). Although this has not been unequivocally established in adipose tissue, experimental interventions promoting white adipose tissue oxidative capacity, *i.e.* through ectopic expression of the brown fat UCP1, improve overall energy expenditure and protect against diet-induced obesity (41). In fact, some of the biological effects of TZDs acting via the induction of PGC-1α gene expression would consist in promoting a brown fat-like phenotype in adipose depots. It has recently been demonstrated that reduced expression of marker genes characteristic of the brown adipocyte phenotype is associated with insulin resistance in patients with type 2 diabetes (16). Enhancement of brown fat-like features in adipose depots, *i.e.* high PGC-1α expression and promotion of mitochondrial oxidative activity and energy expenditure in response to TZDs, could be a major element in the insulin sensitizing action of these drugs because it would enhance fatty acid oxidation inside the adipose depots, thus contributing to the lowering in free fatty acids and subsequent prevention of lipotoxicity.

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