Peroxisome Proliferator-activated Receptor-γ Activation Enhances Insulin-stimulated Glucose Disposal by Reducing ped/pea-15 Gene Expression in Skeletal Muscle Cells

EVIDENCE FOR INVOLVEMENT OF ACTIVATOR PROTEIN-1*

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Background: PPARγ modulation of glucoregulatory response in skeletal muscle has been only partially elucidated.

Results: PPARγ inhibits the transcription of the diabetes-associated gene ped/pea-15 via AP-1.

Conclusion: ped/pea-15 is downstream of a PPARγ-regulated inflammatory network.

Significance: These studies further elucidate the gene network responsible for inflammation-induced insulin resistance.

The gene network responsible for inflammation-induced insulin resistance remains enigmatic. In this study, we show that, in L6 cells, rosiglitazone— as well as pioglitazone-dependent activation of peroxisome proliferator-activated receptor-γ (PPARγ) represses transcription of the ped/pea-15 gene, whose increased activity impairs glucose tolerance in mice and humans. Rosiglitazone enhanced insulin-induced glucose uptake in L6 cells expressing the endogenous ped/pea-15 gene but not in cells expressing ped/pea-15 under the control of an exogenous promoter. The ability of PPARγ to affect ped/pea-15 expression was also lost in cells and in C57BL/6J transgenic mice expressing ped/pea-15 under the control of an exogenous promoter, suggesting that ped/pea-15 repression may contribute to rosiglitazone action on glucose disposal. Indeed, high fat diet mice showed insulin resistance and increased ped/pea-15 levels, although these effects were reduced by rosiglitazone treatment. Both supershift and ChIP assays revealed the presence of the AP-1 component c-Jun at the PED/PEA-15 promoter upon 12-O-tetradecanoylphorbol-13-acetate stimulation of the cells. In these experiments, rosiglitazone treatment reduced c-Jun presence at the PED/PEA-15 promoter. This effect was not associated with a decrease in c-Jun expression. In addition, c-Jun silencing in L6 cells lowered ped/pea-15 expression and caused nonresponsiveness to rosiglitazone, although c-jun overexpression enhanced the binding to the ped/pea-15 promoter and blocked the rosiglitazone effect. These results indicate that PPARγ regulates ped/pea-15 transcription by inhibiting c-Jun binding at the ped/pea-15 promoter. Thus, ped/pea-15 is downstream of a major PPARγ-regulated inflammatory network. Repression of ped/pea-15 transcription might contribute to the PPARγ regulation of muscle sensitivity to insulin.

Peroxisome proliferator-activated receptor-γ (PPARγ) is a member of the nuclear hormone receptor superfamily. In addition to other PPAR isoforms, this superfamily also includes the receptors for thyroid hormones, retinoids, steroid hormones, and vitamin D (1, 2). PPARγ regulates gene transcription by binding with the retinoid X receptors to specific DNA sequences termed peroxisome proliferator response elements (3). After ligand activation, PPARγ modifies its conformation, which facilitates the release of corepressors and subsequent binding of a distinct set of nuclear coactivators, thereby fostering PPARγ action (3). Ultimately, formation of these transcriptional complexes enables PPARγ transcriptional control of a variety of biological processes, including insulin sensitivity (4). Indeed, PPARγ exerts important modulatory actions upstream of the major inflammatory networks involving AP-1 and NF-κB transcriptional regulation (5).

PPARγ activation by thiazolidinediones (TZDs) markedly improves insulin sensitivity in type 2 diabetic patients (6–9). However, the molecular mechanisms responsible for PPARγ-mediated insulin sensitization have been only partially defined. Studies in tissue-specific PPARγ knock-out mice have generated insights into the role of different tissues in PPARγ-mediated regulation of systemic insulin-stimulated glucose metabolism (10) (2, 11). To date, the relevance of PPARγ in maintaining systemic insulin sensitivity in adipose tissues has been convincingly demonstrated. In fact, the PPARγ-mediated adipogenesis associated with the capability for fatty acid trapping has emerged as a major factor in protecting against non-adipose tissue insulin resistance (12). Skeletal muscle expression of PPARγ has further been shown to contribute to systemic insulin sensitivity by maintaining intact insulin-mediated glucose utilization in muscle (10). Indeed, PPARγ was reported to directly coordinate glucoregulatory responses in this tissue (11). However, the genetic network responsible for

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§ The abbreviations used are: PPARγ, peroxisome proliferator-activated receptor-γ; TZD, thiazolidinedione; Ptz, pioglitazone; TPA, 12-O-tetradecanoylphorbol-13-acetate; qRT-PCR, quantitative RT-PCR; Rtz, rosiglitazone; CRE, cAMP-response element.

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PPARγ and ped/pea-15 Transcription

these functions in skeletal muscle has not been completely elucidated yet.

Phosphoprotein Enriched in Diabetes/Phosphoprotein Enriched in Astrocytes (PED/PEA-15) is a scaffold cytosolic protein widely expressed in most human tissues (13, 14). Early studies indicated that PED/PEA-15 has an important role in controlling glucose disposal in the skeletal muscle by impacting on the phospholipase D/protein kinase C signaling network (15, 16). Further investigations demonstrated that PED/PEA-15 is commonly overproduced in individuals with type 2 diabetes as well as in their euglycemic offspring, causing skeletal muscle insulin resistance in these individuals (17). PED/PEA-15 cellular levels are regulated by ubiquitinylation and proteosomal degradation (18). In addition, run-on experiments in cultured cells from type 2 diabetic patients have demonstrated that PED/PEA-15 overproduction is caused, at least in part, by transcriptional abnormalities (13). More recent studies evidenced that epigenetic changes at the PED/PEA-15 gene have a major role in controlling its transcription (19). However, the molecular details responsible for regulation of PED/PEA-15 transcription as well as the abnormalities in these mechanisms occurring in type 2 diabetes remain unclear.

In this report, we demonstrate that PPARγ represses the transcription of the diabetes-associated gene ped/pea-15. We found that PPARγ repression of this gene requires displacement of the c-JUN component of the AP-1 transcriptional complex from the ped/pea-15 promoter. These findings identify ped/pea-15 as a gene downstream of major PPARγ-regulated inflammatory networks, whose control may be instrumental to PPARγ action on glucose disposal by the skeletal muscle.

EXPERIMENTAL PROCEDURES

Materials—Rosiglitazone and pioglitazone (Pzt) were purchased from Cayman Chemical (Ann Arbor, MI). 12-O-Tetradecanoylphorbol-13-acetate (TPA) and GW9662 were purchased from Sigma. Solutions were prepared in dimethyl sulfoxide (DMSO) and diluted 1:1000 into serum-free DMEM chased from Sigma. Solutions were prepared in dimethyl sulfoxide (DMSO) and diluted 1:1000 into serum-free DMEM chased from Sigma. The following oligonucleotides, including the scrambled phosphorothioate oligodeoxynucleotide and the rat c-JUN antisense (20), were synthesized by Sigma. [γ-32P]dATP and 2-[1-14C]deoxy-d-glucose, were purchased from PerkinElmer Life Sciences. The pCEFL for c-Jun-HA was provided by Dr. Musti (University of Cambridge, United Kingdom). The expression vector (pcEF) for c-Jun-HA was provided by Dr. Musti (University of Cosenza, Italy).

Cell Culture Studies—L6 skeletal muscle cells and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin solution at 37 °C in a humidified 95% air and 5% CO2 atmosphere. L6 myoblasts were plated at a density of 3 × 104 cells/ml, and differentiation of confluent cells was initiated by reducing the FBS content of the media to 2% with a medium change every 48 h. Differentiation of myoblasts into multinucleated myotubes was completed by 7 days after medium change. Fused multinucleated myotubes were incubated with the serum-depleted (0.5%) medium for 16 h and treated with TZDs at the indicated concentrations for a further 24 h, although control cells received the vehicle alone in the serum-depleted (0.5%) medium. L6 myoblasts were electroporated with the Neon Transfection System (Invitrogen), and L6 myotubes and HeLa cells were transfected using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions (15). All cell culture media and reagents were from Lonza (Basel, Switzerland).

Glucose uptake in L6 cells was assayed as reported previously (25). Briefly, the cells were incubated for 24 h in serum-free medium supplemented with 0.25% (w/v) bovine serum albumin (BSA) in the absence or presence of 1 μM Rttz. The cells were rinsed in glucose-free HEPES buffer (5 mM KCl, 120 mM NaCl, 1.2 mM MgSO4, 10 mM NaHCO3, 1.2 mM KHPO4, and 20 mM HEPES, pH 7.8, 2% albumin) and further exposed to 100 nM insulin for 30 min. 2-Deoxy-d-[14C]glucose uptake was measured over a 10-min period, with nonspecific uptake determined in the presence of cytochalasin-B.

Animal Studies—5-Week-old male C57BL/6J mice were hosted at the common facility of the University of Naples Medical School and had free access to water and food. For high fat diet treatment, mice were fed a high fat diet with ~60 cal% fat or a standard diet with ~10 cal% fat (Research Diets, New Brunswick, NJ) (26) for 12 weeks. During the last 10 days, the animals received either 10 mg/kg/die Rttz or vehicle alone (0.5% carboxymethylcellulose) by oral gavage. For insulin tolerance testing, mice were fasted for 4 h and then subjected to intraperitoneal injection with insulin (0.75 milliunits g−1 of body weight). Venous blood was subsequently drawn by tail clipping at 0, 15, 30, 45, 60, 90, and 120 min as in Ref. 27. For intraperitoneal glucose tolerance testing, mice were fasted overnight and then subjected to intraperitoneal injection with glucose (2.0 g kg−1 of body weight). Venous blood was subsequently drawn by tail clipping at 0, 15, 30, 45, 60, 90, and 120 min as in Ref. 27. In overnight fasted mice, serum insulin concentrations were measured by rat insulin RIA kit (Millipore, Billerica, MA) (27). Blood glucose levels were measured with Accu-Chek® glucometers (Roche Applied Science). In overnight fasted mice, analyses of serum triglycerides, total cholesterol, HDL cholesterol, and LDL cholesterol were done on Horiba AXB Pentra 400 Chemistry Analyzer (HORIBA ABX, Montpellier, France). The animals were sacrificed, and skeletal muscle tissues (gas-
trocnemius) were removed, rinsed with 0.9% NaCl, frozen in liquid nitrogen, and kept in −80 °C before harvesting.

Western Blot Analysis—Cells were solubilized by scraping and passed 10 times through a 25-gauge needle in ice-cold lysis buffer, supplemented with the Complete Protease Inhibitor Mixture Tablets (Roche Applied Science). Lysates were clarified by centrifugation at 16,000 × g for 10 min at 4 °C, and protein concentration was determined using the protein assay based on Bradford’s method (Bio-Rad) (28). Total cell extracts in equal amounts were separated by SDS-PAGE and blotted on nitrocellulose membranes (Millipore, Billerica, MA). Membranes were blocked in 5% BSA, and specific proteins were detected by incubation with appropriate primary and secondary antibodies (horseradish peroxidase-conjugated) in 150 mM NaCl, 50 mM Tris, 0.5% Tween 20 (TBST). Protein bands were visualized using an enhanced chemiluminescence (ECL) kit (Thermo Scientific Pierce Protein Biology, Waltham, MA) and quantified by the ImageJ software (a public domain and Java-based image processing program developed at the National Institutes of Health). Relative protein abundance was calculated after 14-3-3e normalization.

Real Time PCR Analysis—Total RNA was extracted with the TRIzol reagent (Invitrogen), according to the manufacturer’s protocol. Reverse transcription of 1 μg of total RNA was performed using SuperScript III (Invitrogen), following the manufacturer’s instructions. Quantitative real time PCR was performed in triplicate by using iQ SYBR Green Supermix on iCycler real time detection system (Bio-Rad). Relative quantification of gene expression was calculated by the ∆∆Ct method (29). Each Ct value was first normalized to the respective glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Ct value of a sample to account for variability in the concentration of RNA and in the conversion efficiency of the RT reaction. For copy number analysis, the real time PCR amplification products for ped/pea-15 and gapdh were cloned into the pGEM®-T easy vector (Promega, Madison, WI), and calibration curves were made from serial 10-fold dilutions of plasmid DNAs as described previously (30). The mean slopes of the calibration curves for the two genes were similar, −3.3 for ped/pea-15 and −3.1 for gapdh, where a slope of −3.3 ± 10% reflects an efficiency of 100 ± 10% of the PCR r, and the mean correlation coefficient (R) for both of the curves was 0.99. The equations drawn from the graph of the standard curves were used to calculate the precise number of specific cDNA molecules present in the samples (copy number variation).

Luciferase Assays—Cells were cotransfected with 2 μg of the Firefly luciferase vector (pGL3 plasmids, Promega, Madison, WI) and 1 μg of the pRSV-β-galactosidase (Promega, Madison, WI), as internal control to normalize for transfection efficiency. In each reaction, the total amount of transfected DNA was kept constant at 5 μg by using the empty expression vector pcdNA3 or PPARγL468A/E471A mutant. 24 h after transfection, cells were incubated with the serum-depleted (0.5%) medium for 16 h and then exposed to serum-depleted (0.5%) medium supplemented with vehicle (0.1% DMSO) or TPA (0.1 μM) in the absence or presence of increasing concentrations of Rtz for 4 h. Luciferase and β-galactosidase activities were measured with a luminometer (Berthold Technologies, Bad Wildbad, Germany). Luciferase activities were divided by the respective β-galactosidase activity and expressed as relative luciferase units.

Electrophoretic Mobility Gel Shift (EMSA) and Supershift Assays—Nuclear extracts from HeLa cells were prepared as described previously with minor modifications (31). Double-stranded oligonucleotides were end-labeled with [γ-32P]ATP by T4 polynucleotide kinase (Promega, Madison, WI) according to the manufacturer’s instructions. The radiolabeled probes were purified by spin columns (Roche Applied Science). 5 μg of nuclear protein extracts from control and treated cells were incubated with 100,000 cpm 32P-labeled oligonucleotide probe in 25 mM HEPES, pH 7.4, 50 mM KCl, 10% glycerol (v/v), 5 mM dithiothreitol (DTT), and 1 μg of poly(dI-dC) (GE Healthcare) for 30 min at room temperature in a final volume of 20 μl. Upon binding, protein-DNA complexes were separated on 6% nondenaturing polyacrylamide gels at 120 V in 0.5× TBE buffer. Gels were dried and further subjected to PhosphoImager analysis (Bio-Rad). In competition analysis, 25-, 50-, and 100-fold molar excess of the unlabeled double strand oligonucleotide was added to the reaction mixture prior to the addition of the labeled probe. For the antibody supershift analysis, 1 μg of antibody was added to the nuclear extracts prior to the radiolabeled oligonucleotide.

FIGURE 1. PPARγ expression and function in L6 skeletal muscle cells. A, 3T3-L1 fibroblasts, 3T3-L1 adipocytes, L6 myotubes, and HeLa cells were lysed, and total protein extracts were separated by SDS-PAGE. The antibodies used were specific for PPARγ and 14-3-3e as loading control. The autoradiograph shown is representative of four additional experiments. B, L6 myotubes were cotransfected with PPRE3-tk-luc and pRSV-β-gal constructs as described under “Experimental Procedures.” Upon transfection, the cells were incubated in the absence or presence of the indicated concentrations of Rtz. Luciferase and β-galactosidase activities were measured in cellular extracts 24 h later. β-Galactosidase activity enabled the estimation of transfection efficiency and assessment of relative luciferase values (RLU). Bars represent the means ± S.D. of three independent experiments. Asterisks indicate statistically significant differences at a p < 0.001 level.
Chromatin Immunoprecipitation Assay (ChIP)—ChIP assays were performed as reported previously (21). Briefly, upon protein-DNA cross-linking, cells were lysed and sonicated to achieve chromatin fragments ranging between 500 and 1000 bp in size. The lysates were incubated with either c-JUN antibody or a rabbit control IgG, and then complexes were isolated using protein A-agarose/salmon sperm DNA (Millipore, Billerica, MA). Immunoprecipitates were extensively washed and then eluted by freshly prepared 1% SDS, 0.1 M NaHCO₃ buffer. After reversal of cross-linking, DNA was purified by the QIAquick PCR purification kit (Qiagen, Hilden, Germany) followed by PCR amplification. PCR products were resolved by 2% agarose gel electrophoresis, revealed by ethidium bromide staining, and analyzed by densitometry using the ImageJ software (National Institutes of Health).

Statistical Analysis—All data are presented as means ± S.E. Statistical differences were determined by one- or two-way analysis of variance as appropriate, and Bonferroni post hoc testing was performed when applicable. A p value < 0.05 was considered significant.

RESULTS

PPARγ Regulates ped/pea-15 Function in Muscle Cells—We assessed PPARγ expression in the L6 skeletal muscle cell line by Western blot analysis. The PPAR-specific antibody detected both the isoforms 1 and 2, which have a predicted molecular mass of ~53 and 57 kDa, respectively. In L6 myotubes as well as in HeLa cells, the γ1 isofrom was more abundant than the γ2 isofrom, although PPARγ2 was the most highly expressed isoform in 3T3-L1 adipocytes. At variance, 3T3-L1 fibroblasts showed very low PPARγ levels (Fig. 1A). L6 cells were also transfected with a construct featuring the PPAR response element upstream from the luciferase gene (PPRE3-tk-luc). As shown in Fig. 1B, treatment of these cells with increasing amounts of the PPARγ agonist Rtz determined a significant

FIGURE 2. PPARγ repression of ped/pea-15 transcription. A, L6 myotubes were incubated in the presence of the indicated concentrations of Rtz (black bars) or Ptz (gray bars) for 24 h. Total RNA was obtained, and ped/pea-15 and gapdh mRNA levels were measured by qRT-PCR. Values were normalized for gapdh and presented as fold decrease relative to the control (untransfected cells). B, L6 myotubes were incubated in the absence or presence of Rtz for 24 h. Total protein extracts were separated by SDS-PAGE followed by immunoblotting with PED/PEA-15 or 14-3-3 antibody, as indicated. The one presented is representative of two additional experiments with very similar results. C, total RNA was obtained from gastrocnemius of C57BL/6J mice treated or not with 10 mg/kg/die Rtz for 10 days. The mRNAs were assessed by qRT-PCR. ped/pea-15 values were normalized for gapdh and expressed as fold decrease versus control. D, L6 myotubes were preincubated with 10 μM GW9662 for 1 h or transfected with the PPARγL468A/E471A mutant as described under “Experimental Procedures” and then treated with 1 μM Rtz for 24 h. Total RNA was obtained, and ped/pea-15 and gapdh mRNA levels were measured by qRT-PCR. Values were normalized for gapdh and presented as fold decrease relative to the control. Bars represent the means ± S.D. of three (A and D) and four independent experiments (B). Five mice/group were used in C. Asterisks denote statistically significant differences (*, p < 0.05; **, p < 0.01, and ***, p < 0.001).
increase in the promoter activity, indicating that PPARγ is transcriptionally active in L6 cells.

PPARγ has been reported to regulate a number of genes controlling metabolic functions (32). Interestingly, in L6 myotubes, Rtz and Ptz repressed ped/pea-15 expression in a dose-dependent manner, and this effect occurred both at mRNA (Fig. 2A) and at protein levels (Fig. 2B). A comparable decrease in ped/pea-15 mRNA levels was also observed in the skeletal muscle of Rtz-treated C57BL/6J mice (Fig. 2C). In addition, both pretreatment of L6 cells with the PPARγ antagonist GW9662 and transfection with the dominant negative PPARγ mutant, PPARγL468A/E471A, completely abolished the Rtz effect (Fig. 2D), indicating that TZDs inhibit ped/pea-15 expression via PPARγ.

The known physiological importance of PED/PEA-15 in regulating glucose tolerance prompted us to further investigate the molecular details of the TZDs action on ped/pea-15 function. To this end, we transfected L6 cells with a myc-tagged PED/PEA-15 cDNA driving the expression of the gene under the control of the cytomegalovirus (CMV) promoter, and then we exposed the cells to Rtz. As shown in Fig. 3A, Rtz did not affect the levels of the exogenous PED/PEA-15 protein (21 kDa), although it reduced the levels of the endogenous one (15 kDa). Consistent with these findings, treatment with Rtz did not significantly affect PED/PEA-15 protein levels in the skeletal muscle of transgenic mice overexpressing this gene under the control of the exogenous β-actin promoter (Tgped), although it reduced endogenous PED/PEA-15 protein levels up to 60% in their wild-type littermates (Fig. 3B). Furthermore, as shown in Fig. 3C, Rtz enhanced glucose uptake in wild-type L6 cells (L6WT) but not in those cells expressing the exogenous PED/PEA-15 (L6ped). Thus, it appeared that the endogenous ped/pea-15 promoter is necessary for PPARγ regulation of the gene.

PPARγ Silences ped/pea-15 by Interfering with AP-1 Signaling—These findings led us to explore the molecular mechanisms involved in PPARγ regulation of ped/pea-15 in greater detail and to identify the PPARγ responding region at the ped/pea-15 promoter. Previous studies in cells treated with TPA, a known activator of both AP-1 and NF-κB, revealed increased PED/PEA-15 expression (18). We have therefore addressed the hypothesis that PPARγ reduces PED/PEA-15 transcription by transrepressing one or both of these transcription factors. In silico analysis of the human PED/PEA-15 promoter revealed the presence of a TGACATCA CRE-like site between the positions −106 and −86 bp from the transcription start site (+1). This sequence is known to bind the AP-1 transcriptional complex (33). A cAGGActtt NF-κB-binding site between positions −798 and −786 bp was also identified. Furthermore, sequence alignment of the human and rat 5′-flanking regions revealed that these two sequences are highly conserved, suggesting they may perform a major role in regulating PED/PEA-15 expression and prompted us to test the significance of AP-1 and NF-κB in the PPARγ-dependent regulation of the PED/PEA-15 promoter.

To this goal, we transfected HeLa cells with a reporter vector featuring the luciferase gene downstream from the −1942 to +58 bp of the proximal 5′-flanking region of the human PED/PEA-15 gene (pPED2000). In these transfected cells, TPA up-regulated the PED/PEA-15 promoter activity, achieving its maximum effect within 4 h of exposure (Fig. 4A). Consistently, a >2-fold increase in PED/PEA-15 mRNA levels was also demonstrated by quantitative RT-PCR (qRT-PCR) analysis (Fig. 4B). Furthermore, TPA effect on both PED/PEA-15 mRNA levels (Fig. 4C) and promoter activity (Fig. 4D) was significantly inhibited by treating the cells with Rtz. In addition, the presence
of the PPARγ antagonist GW9662 as well as that of the dominant negative PPARγ mutant, PPARγ<sub>L468A/E471A</sub>, enabled full TPA effect on promoter activity during simultaneous treatment with Rtz (Fig. 4D). These findings initially supported the possibility that PPARγ regulation of PED/PEA-15 function might involve AP-1 and/or NF-κB transcriptional control.

To further explore this hypothesis, we transfected HeLa cells with either a PED/PEA-15 deletion construct lacking the NF-κB-binding site but featuring an intact CRE-like site (pPED210) or the same construct where the CRE-like site was mutagenized (pPED210<sub>MUT</sub>). Interestingly, in the former case, exposure to TPA induced a 2.5-fold enhanced transcriptional activity, which was almost completely suppressed by Rtz treatment (Fig. 5A). At variance, cells transfected with the CRE-like mutagenized construct exhibited depressed basal promoter activity with complete loss of TPA responsiveness, whether in the absence or presence of Rtz. These observations led us to hypothesize that AP-1 plays a major role in basal and PPARγ-dependent regulation of PED/PEA-15 expression. Indeed, in cells transfected with the pPED2000<sub>MUT</sub> construct, which features the intact NF-κB site but the mutated CRE-like site, Rtz treatment failed to repress TPA-induced PED/PEA-15 promoter activity (Fig. 5B), suggesting that NF-κB is not involved in the regulation of PED/PEA-15 expression by PPARγ.

EMSA also revealed increased occupancy of the CRE-like site at PED/PEA-15 promoter in lysates from TPA-treated as compared with untreated HeLa cells (Fig. 6A, left). This CRE-like site occupancy was dose-dependently inhibited by Rtz. Importantly, supershift assays with a specific c-JUN antibody revealed the presence of the AP-1 component c-JUN in the binding complex at the CRE-like site (Fig. 6A, right). c-JUN occupancy at the PED/PEA-15 promoter was also investigated in living cells by ChIP assays. In these experiments, the cross-linked chromatin was precipitated using the c-JUN antibody followed by amplification of the CRE-like site at the human PED/PEA-15 promoter. As shown in Fig. 6B, TPA exposure determined the c-JUN association to the CRE-like site, which was reduced by Rtz treatment in a dose-dependent manner.

**FIGURE 4.** Mechanisms of TPA regulation of ped/pea-15 promoter activity. A and D, HeLa cells, cotransfected with the pPED2000 and pRSV-β-gal constructs, were further transfected with the PPARγ<sub>L468A/E471A</sub> mutant or pretreated with 10 μM GW9662 for 1 h and subsequently incubated in the absence or presence of 0.1 μM TPA and 1 μM Rtz for 4 h, as indicated. Luciferase activities were measured and normalized for β-galactosidase activities. Bars represent the means ± S.D. of four independent experiments each performed in duplicate. B and C, HeLa cells were incubated in the absence or presence of 0.1 μM TPA and 1 μM Rtz for 4 h, as indicated. PED/PEA-15 mRNA levels were subsequently quantitated by qRT-PCR, and values were normalized for GAPDH of the same samples and shown as fold increase relative to untreated cells. Bars represent the means ± S.D. of three independent experiments. Asterisks denote statistically significant differences (***, p < 0.001).
This effect was not due to a decrease in TPA-induced expression of c-jun in HeLa cells (Fig. 6C).

**PPARγ Represses ped/pea-15 Expression via AP-1 in L6 Cells**—Based on ChIP assays, Rtz as well as Ptz treatment decreased c-jun binding to the CRE-like site also in L6 myotubes (Fig. 7A), confirming the major role of AP-1 in the ped/pea-15 down-regulation by PPARγ also in skeletal muscle cells. Importantly, in these experiments, the two TZDs did not affect the binding of the NF-κB subunit p65 to its binding site on the ped/pea-15 promoter (Fig. 7B).

In L6 cells, the overexpression of c-jun (Fig. 8A) slightly increased ped/pea-15 expression in untreated cells (Fig. 8B). However, higher levels of ectopic c-jun bypassed the down-regulation of ped/pea-15 due to PPARγ (Fig. 8B). In parallel, activated PPARγ was not able to displace c-jun from the CRE-like site at ped/pea-15 promoter when it was overexpressed in L6 cells (Fig. 8C). After c-jun silencing (Fig. 8A), L6 cells showed a significantly lower expression of ped/pea-15 compared with wild-type cells no longer reduced by both Rtz and Ptz (Fig. 8B), supporting the major role of AP-1 in ped/pea-15 down-regulation by PPARγ also in skeletal muscle cells.

**PPARγ Decreases ped/pea-15 Expression in High Fat Diet-fed Mice**—The in vivo significance of ped/pea-15 regulation by PPARγ agonists was further explored in mice subjected to a high fat diet regimen for 12 weeks. As shown in Table 1, these mice became dyslipidemic and developed significant hyperinsulinemia and insulin resistance. Interestingly, these changes were accompanied by a 2.5-fold increase in ped/pea-15 expression in their skeletal muscle. Further treatment of these mice...
with Rtz for 10 days improved dyslipidemia and plasma insulin levels. Based on the determination of glucose areas under the curves during the accomplishment of insulin tolerance tests, insulin sensitivity was also rescued by Rtz. Simultaneously ped/pea-15 mRNA levels were significantly reduced, further supporting the important role of ped/pea-15 in Rtz action in vivo.

**DISCUSSION**

The relevance of adipose tissue versus skeletal muscle in mediating PPARγ function in glucose tolerance has been debated (10, 11, 34). PPARγ is a master regulator of adipogenesis (7, 35) whose induction, associated with the capability for fatty acid trapping, has been shown to represent an important contributor to the maintenance of systemic insulin sensitivity (12, 36). However, studies in mice with targeted loss of PPARγ in the skeletal muscle revealed development of severe insulin resistance in these animals (2). Despite development of fat and liver insulin resistance (2, 11), evidence was also obtained that, in these mice, PPARγ directly coordinates glucoregulatory responses in the skeletal muscle and this action is needed for the beneficial effects of PPARγ agonists in ameliorating insulin resistance conditions (10). Comprehensive and unbiased mRNA profiling studies in rats revealed that PPARγ activation has coordinate effects on gene expression in multiple insulin-sensitive tissues, with regulation of specific gene panels in each of these tissues (37).

However, in the skeletal muscle, the panel of the genes regulated by PPARγ is still incomplete, making the molecular details of PPARγ action on muscle glucoregulatory function unclear. In this study, we report that in mouse skeletal muscle tissue and...
in L6 skeletal muscle cell line the PPARγ agonists Rtz and Ptz repress the expression of ped/pea-15, identifying this gene as a novel downstream target of PPARγ. Indeed, either preincubation with the PPARγ antagonist GW9662 or transfection with the dominant negative PPARγ mutant, PPARγY1468A/E1471A (24), completely blocked the Rtz effect.

Previous studies in mice and in humans demonstrated that PED/PEA-15 serves as a physiological regulator of glucose tolerance (13, 18). PED/PEA-15 is transcriptionally up-regulated in type 2 diabetics as well as in their euglycemic offspring (17), determining insulin resistance in glucose disposal in the skeletal muscle mass (15). The mechanisms of PED/PEA-15 transcriptional control have been only partially elucidated, but its repression by PPARγ agonists in skeletal muscle may contribute to the beneficial effects of these agents when administered to individuals with abnormalities in glucose tolerance. In support of these conclusions, we observed that, together with its action on ped/pea-15 transcription, Rtz improved insulin-stimulated glucose uptake in the L6 cells, although both these effects were simultaneously impaired in cells expressing ped/pea-15 under the control of an exogenous promoter. In addition, the in vivo studies reported in this work now show that high fat diet raised the expression of ped/pea-15 in parallel with the development of insulin resistance, whereas Rtz simultaneously reversed both of these effects.

The finding that ped/pea-15 is a target of PPARγ prompted us to further explore in vitro the details of PPARγ regulation of this gene. Previous studies in cells treated with TPA, a known activator of both AP-1 and NF-κB, revealed increased ped/pea-15 expression (18), supporting the hypothesis that PPARγ brakes ped/pea-15 transcription by transrepressing one or both of these transcription factors. Indeed, in the course of this study, we have functionally validated the presence of binding sequences for AP-1 and NF-κB at the promoter of PED/PEA-15, and the anti-inflammatory action of PPARγ is known to be mediated to a major extent via transrepression of these factors (39). Interestingly, supershift and ChIP experiments revealed that Rtz reduced ped/pea-15 promoter occupancy by the c-jun moiety of the AP-1 transcriptional complex. In contrast, NF-κB presence at ped/pea-15 promoter was unaffected in TZD-treated cells, indicating high specificity in the PPARγ-dependent transcriptional control of ped/pea-15. The observation that PPARγ inhibits Jun/AP-1 binding to DNA suggests a potential direct protein-protein interaction between PPARγ and c-Jun. A similar negative interference with AP-1 activity has been described for other nuclear receptors, such as the retinoic acid receptor (40) and the glucocorticoid receptor (41). In these reports, both the glucocorticoid receptor and retinoic acid receptor were shown to form a nonproductive complex with c-jun, leading to a decrease of AP-1 binding activity. Alternatively, PPARγ could block or destabilize an interaction between c-jun and a cellular factor that facilitates DNA binding. Constructs bearing mutations at the CRE-like site of PED/PEA-15 promoter, but not within the NF-κB-binding site, failed in affecting promoter activity when AP-1 and NF-κB were activated by TPA. Thus, AP-1 but not NF-κB is necessary for PPARγ control of the PED/PEA-15 transcription. Furthermore, Rtz treatment in cells upon silencing of c-jun showed no effect on ped/pea-15 expression, indicating that c-jun transrepression is sufficient for the PPARγ action. The mechanism responsible for the Rtz effect on ped/pea-15 promoter occupancy by c-jun was not based on changes in the levels of c-jun itself. Alternatively, Rtz might impair ability of c-jun to bind to the ped/pea-15 promoter, as forcing its occupancy by c-jun overexpression blocked the Rtz repression of ped/pea-15.

The chronic low grade inflammation induced by obesity is well recognized as a key trait of type 2 diabetes (42), as a number of inflammatory pathways are activated in classical insulin target tissues of these individuals (43, 44). These signaling cascades include the JNK/AP-1 network that is known to be downregulated by PPARγ (39). However, genes that are ultimately involved in the impairment of glucose tolerance induced by inflammatory stimuli are still largely unknown. We now report that ped/pea-15 expression is controlled by physiological levels of c-jun in the cells. Indeed, c-jun silencing with specific antisense impairs basal ped/pea-15 expression. In addition, PPARγ activating anti-inflammatory agents regulate ped/pea-15 by reducing c-jun occupancy at its promoter. These findings identify ped/pea-15 as a gene whose transcription may change in response to common abnormalities typical of the low grade

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**Table 1: Rosiglitazone effects on biochemical parameters of mice subjected to different dietary regimens**

| Parameter          | STD (n = 10) | HFD (n = 8) | HFD+R (n = 6) |
|--------------------|--------------|-------------|--------------|
| Body weight        | 28.51 ± 0.99 g | 39.11 ± 1.30 g<sup>a</sup> | 39.84 ± 2.27 g |
| Food intake        | 3.16 ± 0.09 g/mouse/day | 2.87 ± 0.2 g/mouse/day | 2.40 ± 0.2 g/mouse/day |
| AUC ITT            | 9934.5 ± 424.5 mg/dl120 min | 21517.5 ± 1812.7 mg/dl120 min<sup>b</sup> | 14817 ± 1038.5 mg/dl120 min<sup>b</sup> |
| PED/PEA-15         | 2.81 ± 2.1E-05 CNV | 6.77 ± 1.2E3-04 CNV<sup>b</sup> | 5.25 ± 7.7E5-05 CNV<sup>b</sup> |
| CHOL               | 102.5 ± 6.71 mg/dl | 207.33 ± 17.48 mg/dl<sup>b</sup> | 130.8 ± 15.73 mg/dl<sup>b</sup> |
| TG                 | 95.4 ± 6.64 mg/dl | 204.23 ± 26.74 mg/dl<sup>b</sup> | 83.86 ± 9.97 mg/dl<sup>b</sup> |
| HDL                | 47.16 ± 6.70 mg/dl | 68.87 ± 11.10 mg/dl | 57.74 ± 5.33 mg/dl |
| LDL                | 6.25 ± 1.10 mg/dl | 15.40 ± 2.30 mg/dl<sup>d</sup> | 6.83 ± 0.68 mg/dl |
| Glucose            | 107 ± 6.0 mg/dl | 180 ± 11 mg/dl<sup>d</sup> | 163 ± 6.0 mg/dl<sup>d</sup> |
| Insulin            | 0.36 ± 0.13 ng/ml | 1.09 ± 0.2 ng/ml<sup>d</sup> | 0.68 ± 0.32 ng/ml<sup>d</sup> |

<sup>a</sup> Significant differences were between high fat diet mice versus standard diet mice and between high fat diet mice after treatment with rosiglitazone versus high fat diet mice.

<sup>p</sup> < 0.001.

<sup>c</sup> Data are indicated by t test.

<sup>d</sup> p < 0.05.
unresolved inflammation associated with type 2 diabetes. Further studies assessing ped/pea-15 function may offer previously unrecognized opportunities to identify these abnormalities.

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