0022-3565/03/3062-763–771$7.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
JPET 306:763–771, 2003

Vol. 306, No. 2
49791/1080762
Printed in U.S.A.

PAT5A: A Partial Agonist of Peroxisome Proliferator-Activated Receptor γ Is a Potent Antidiabetic Thiazolidinedione Yet Weakly Adipogenic

PARIMAL MISRA, RANJAN CHAKRABARTI, REEBA K. VIKRAMADITHYAN, GOPALAKRISHNAN BOLUSU, SURESH JULURI, JAGADHESHAN HIRIYAN, CYNTHIA GERSHOME, ABDUL RAJJAK, PAPREDDY KASHIREDDY, SONGTAO YU, SAILESH SURAPUREDDI, CHAO QI, YI-JUN ZHU, M. SAMBASIVA RAO, JANARDAN K. REDDY, and RAJAGOPALAN RAMANUJAM

Discovery Research, Dr. Reddy's Laboratories Ltd., Miyapur, Hyderabad, India (P.M., R.C., R.K.V., B.G., J.S., H.J., G.C., A.R., R.R.); and Department of Pathology Northwestern University, The Feinberg School of Medicine, Chicago, Illinois (P.M., P.K., S.Y., S.S., C.Q., Y.J.Z., M.S.R., J.K.R.).

Received January 31, 2003; accepted April 30, 2003

ABSTRACT

PAT5A [5-[4-[*N*-(2-pyridyl)-(2*S*)-pyrrolidine-2-methoxyl]phenylmethylene[thiazolidine-2,4-dione, malic acid salt]], a chemically distinct unsaturated thiazolidinedione, activates peroxisome proliferator-activated receptor γ (PPARγ) submaximally in vitro with the binding affinity ~10 times less than that of rosiglitazone, a highly potent thiazolidinedione. PAT5A reduces plasma glucose level and improves insulin sensitivity in insulin resistant db/db mice, similar to that of rosiglitazone, while exerting a relatively weak adipogenic effect. In contrast to rosiglitazone, PAT5A inhibits cholesterol and fatty acid biosynthesis suggesting that PAT5A possesses a unique receptor-independent non-PPAR related property. PAT5A induces qualitatively similar but quantitatively different protease digestion patterns and interacts with PPARγ differently than rosiglitazone. PAT5A shows differential cofactor recruitment and gene activation than that of rosiglitazone. Thus, the partial agonism of PAT5A to PPARγ together with its receptor independent effects may contribute to its antidiabetic potency similar to rosiglitazone in vivo despite reduced affinity for PPARγ. These biological effects suggest that PAT5A is a PPARγ modulator that activates some (insulin sensitization), but not all (adipogenesis), PPARγ-signaling pathways.

The nuclear receptor superfamily consists of several ligand-regulated transcription factors that include the steroid and thyroid hormone receptors, vitamin D_3 receptor, retinoic acid receptors, and the peroxisome proliferator-activated receptors (PPARs), among others (Mangelsdorf et al., 1995; McKenna et al., 1999; Brivanlou and Darnell, 2002). The PPAR subfamily consists of three isoforms, PPARα, PPARγ, and PPARβ/δ. After ligand binding, these isoforms heterodimerize with retinoid X receptor (RXR) and bind to the peroxisome proliferator response elements (PPREs) in the promoter regions of target genes (Mangelsdorf et al., 1995; Schoonjans et al., 1997; Desvergne and Wahli, 1999; McKenna et al., 1999; Brivanlou and Darnell, 2002). PPARs play important roles in lipid metabolism, inflammation, and cancer. Of the three PPAR isoforms, PPARγ has received a lot of attention. PPARγ-activating drugs represent a novel opportunity to treat insulin resistance, a significant pathogenic factor in type-2 diabetes. The antidiabetic thiazolidinediones (TZDs) such as troglitazone, rosiglitazone, and pioglitazone bind and activate PPARγ and in the process improve insulin sensitivity and exert antidiabetic effects (Berger et al., 1996; Willson et al., 1996). The therapeutic efficacy of the current thiazolidinediones in type 2 diabetes warrants improvement because of several side effects, including liver toxicity, edema, and increase in body weight (Schoonjans and Auwerx, 2000). Thus, there is a definite need for a safe and efficacious euglycemic drug for the treatment of type-2 diabetic patients.

Recently, we have reported impressive euglycemic and hypolipidemic activities of PAT5A, a novel thiazolidinedione analog containing pyridine [5-[4-[*N*-(2-pyridyl)-(2*S*)-pyrrolidine-2-methoxyl]phenylmethylene[thiazolidine-2,4-

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
DOI: 10.1124/jpet.103.049791.

ABBREVIATIONS: PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; PPRE, peroxisome proliferator-activated response element; TZD, thiazolidinedione; PAT5A, 5-[4-[*N*-(2-pyridyl)-(2*S*)-pyrrolidine-2-methoxyl]phenylmethylene[thiazolidine-2,4-dione, malic acid salt; PCR, polymerase chain reaction; bp, base pair; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; LBD, ligand binding domain; PBP, PPAR binding protein; CBP, cAMP response element-binding protein; PRIP, PPAR-interacting protein; PGC-1, PPARγ coactivator; PEPCK, phosphoenol pyruvate kinase; SRC-1, steroid receptor coactivator-1.

dione, malic acid salt] (Vikramadithyan et al., 2000). The overall antidiabetic potency of PAT5A is much better than that of troglitazone and is comparable to that of rosiglitazone, and yet weakly adipogenic. Preliminary toxicity studies did not show any adverse effects. Although the compound exerted dose-dependent decrease in plasma glucose, triglyceride, insulin levels, and an improved glucose tolerance in vivo, it exhibited only a weak PPARγ transactivation potential (Vikramadithyan et al., 2000). Reports indicate that the in vitro activation by TZDs correlates well with their in vivo hypoglycemic activity, with the exceptions of MCC 555, troglitazone, and nonthiazolidinedione FMOC-leucine (F-L-Leu) (Reginato et al., 1998; Camp et al., 2000; Rocchi et al., 2001). In this study, we examined the mechanism of action of PAT5A and report that this compound is a modulator of PPARγ activity in that it functions as a partial (or weak) PPARγ agonist. We show that PAT5A binds to PPARγ quantitatively less compared with rosiglitazone and interacts with PPARγ in a manner different from rosiglitazone. PAT5A promotes differential recruitment of coactivators compared with that of rosiglitazone. These effects are responsible for eliciting downstream biological effects. Our observations suggest that PAT5A exhibits insulin sensitizing property through a partial agonism of PPARγ and thus explaining its antidiabetic potency similar to rosiglitazone in vivo despite reduced affinity for PPARγ.

Materials and Methods

Materials and Animals. PAT5A, rosiglitazone, and Wy-14,643 were synthesized by Discovery Chemistry (Discovery Research, Dr. Reddy's Laboratories Ltd., Miyapur, Hyderabad, India). 9-*cis*-Retinoic acid and carbacyclin were purchased from Sigma-Aldrich (St. Louis, MO). db/db mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). All experiments were approved by the DRL Institutional Animal Ethics Committee and were in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

Plasmid Constructs. pCDNA3.1~SRC1, pCMX~PBP, pCDNA3.1~PRIP, pCDNA3.1~CBP, pCMV~RXRα, 3xPPRE~Luc, and 5xUAS~Luc have been described earlier (Zhu et al., 1996; Zhu et al., 1997; Zhu et al., 2000; Misra et al., 2002). GAL4~hPPARα LBD, hPPARβLBD, hPPARγLBD, hRXRα LBD, pCDNA3.1~hPPARγ, and pCDNA3.1~hRXRα were generous gifts from Novo Nordisk (Bagsværd, Denmark). pCDNA3.1~PGC-1α-FLAG and aP2 were generous gifts from Dr. B. M. Spiegelman (Dana Farber Cancer Institute, Department of Cell Biology, Harvard Medical School, Boston, MA). LPL cDNA was generous gift from Dr. J. Auwrex (Institut de Genetique et de Biologie, Moleculaire et Cellulaire, Illkivch, France). Probes for northern blot of adipsin, resistin, and glucose-6-phosphatase were cloned by reverse transcription-PCR. Total RNA was isolated from mouse liver using TRIzol reagent (Invitrogen, Carlsbad, CA), and 5 μg of total RNA was utilized for reverse transcription using oligo (dT) primers (200 ng each) with Moloney murine leukemia virus reverse transcriptase (Invitrogen). The following PCR primers were used: 1) mouse adipsin—sense: 5′-*ctccgtgtacttcgtggctctg*-3′, antisense: 5′-*agccacgtgtctctggtgtcc*-3′ (GenBank sequence NM_013459); 2) mouse resistin—sense: 5′-*aagtcctctgccacgtacccac*-3′, antisense: 5′-*tgtgtgcttgtgtgtggattcg*-3′ (GenBank sequence NM_022984); and 3) mouse glucose-6-phosphatase—sense: 5′-*gggctgtgcagctgaacgtctg*-3′, antisense: 5′-*cttcctggcaaatgcatggtgc*-3′ (GenBank sequence BC013448). PCR was performed using denaturing at 94°C for 2 min, followed by 30 cycles of denaturing at 94°C for 10 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. PCR-amplified cDNA fragments were cloned into pGEMT-easy vector (Promega, Charbonnières, France) and sequenced to confirm their authenticity. cDNA fragments for adipsin (795 bp), resistin (494 bp), and glucose-6-phosphatase (950 bp) were digested with *EcoR*I, extracted, and used as probes.

Transient Transfection Assay. 293T cells/CV1 cells, maintained in high glucose Dulbecco's modified Eagle's medium containing 10% charcoal dextran-treated fetal bovine serum (Hyclone Laboratories, Logan, UT), were transiently transfected with expression vectors for the respective receptor chimeras, coactivators, reporter constructs, and Renilla luciferase as internal control as indicated by Superfect/Polyfect (QIAGEN, Valencia, Germany). Approximately 42 h after transfection, cells were placed in phenol red-free Dulbecco's modified Eagle's medium containing 10% delipidated serum and treated for 18 h with a test compound or vehicle. For antagonism assay, different concentrations of PAT5A were added to the culture 30 min before the addition of rosiglitazone. Cells were lysed and luciferase activity determined (Packard/Promega) as the -fold activation relative to untreated cells after normalization with Renilla luciferase values.

Binding Assay. Ligand binding assay was performed essentially has been described (Reginato et al., 1998). Briefly, GST-PPARγ fusion protein isolated from *Escherichia coli* DH5α cells were pelleted and lysed by sonication in Tris-EDTA-NaCl buffer (10 mM Tris-HCl, pH8.0, 1 mM EDTA, and 100 mM NaCl). For competition binding assays, bacterial extracts (~200 μg of protein) were incubated at 4°C for 2 h with 200 nM [^{3}H] rosiglitazone (specific activity, 23 Ci/mmol) in the absence or presence of unlabeled TZD in buffer containing 10 mM Tris, pH 8.0, 50 mM KCl, and 10 mM dithiothreitol. Bound radioactivity was separated from free radioactivity by elution through a 1-ml Sephadex G-25 desalting column (Amersham Biosciences, Inc., Piscataway, NJ) and quantitated using a liquid scintillation counter.

Limited Protease Sensitivity Assay. Two microliters of in vitro synthesized [^{35}S]methionine-labeled, full-length human PPARγ was preincubated with 50 μM rosiglitazone or PAT5A in 25 mM KCl, 10 mM Tris-HCl, pH 8.0, and 10 mM dithiothreitol for 20 min. Trypsin was added to a final concentration of 50, 100, or 200 μg/ml and incubated for 30 min. The reaction was terminated by adding 2× SDS sample loading buffer and resolved in 12% SDS-PAGE. Labeled PPARγ was visualized by autoradiography.

Docking Study of PAT5A. Rosiglitazone and PAT5A were sketched in SYBYL 6.8 (molecular modeling software; Tripos Associates, Inc., St. Louis, MO) and geometry optimized using an MMFF94 force field. The structures were aligned using rosiglitazone as template by FlexS (Lemmen and Lengauer, 1997) method. The aligned molecules were then docked using FlexX (Rarey et al., 1996, 1997) method in the active site of PPARγ LBD crystal structure (PDB code: 2PRG) (Nolte et al., 1998). The active site was defined as a sphere of radius 6.5 Å surrounding the ligand (rosiglitazone). After docking, hydrogen atoms were added to the protein followed by minimization. The minimized protein-ligand complex was solvated with water molecules and subjected to 1-ps molecular dynamics simulation with step size of 1 fs at 300°K using distance-dependent dielectric with a constant of 1. The structure obtained after dynamics was further minimized for 1000 cycles using an MMFF94 force field and charges. This final structure was then analyzed for various parameters: interaction energy, strain energy, H-bonding distance between Tyr-473, and TZD ring and root mean square deviation with the starting structure.

GST Pull-Down Assay. Full-length PPARγ expressed in *E. coli* as GST tagged fusion protein was isolated and partially purified using glutathione agarose (Amersham Biosciences). Full-length SRC1, PBP, CBP, PRIP, PGC-1α were labeled with [^{35}S]methionine in a coupled in vitro transcription/translation system (Promega). The coactivators binding assay were carried out as follows: 5 to 10 μl of [^{35}S]methionine-labeled coactivators were incubated with the immobilized GST or GST-PPARγ fusion proteins in GST-binding buffer

(100 mM KCl, 20 mM Tris-HCl, pH 7.9, 1 mM EDTA, 0.05% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) in presence of indicated ligands. The mixtures were incubated for 2 h at 4°C with gentle rocking. The beads were washed 4 times with 1 ml of GST binding buffer containing 0.1% Nonidet P-40. Bound proteins were eluted in 20 μl of 2× SDS loading buffer, run on a 10% SDS-PAGE and analyzed by autoradiography.

Triglyceride Measurement in 3T3L1 cells. The 3T3L1 cells were plated at 20,000 cells/well in 24-well plate, and 24 h after confluence, 3T3L1 cells were treated with PAT5A (0.01 to 0 30 μM) and rosiglitazone (0.01 to 0.10 μM), 1 μM dexamethasone, and 10 μg/ml insulin in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum daily. After 7 days of treatment, the cells were rinsed with PBS and lysed with 0.1% NP-40 in 0.1 N NaOH. To 100 μl of lysate was added the same volume of triglyceride assay reagent (Pointe Scientific) and plates were incubated for 10 min at 37°C. The enzymatic color reaction was measured at 540 nm.

Northern Analysis. Male db/db mice (8 weeks old) (n = 2) were administered PAT5A and rosiglitazone (10 mg/kg and 30 mg/kg) by gavage daily for 8 consecutive days. On day 9, mice were sacrificed and total RNA was isolated from epididymal fat pads and livers using a QIAGEN Midi RNA isolation system (QIAGEN). Northern blot hybridization was done at 42°C overnight in the presence of cDNA probe labeled using random primers and autoradiographed at −80°C for 24.

The 3T3L1 cells were plated at 20,000 cells/well in 24-well plates, and 24 h after confluence, they were treated with 1.0 μM PAT5A or rosiglitazone, 1.0 μM dexamethasone, and 10 μg/ml insulin in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum daily. After 7 days of treatment, cells were rinsed with PBS and RNA extracted for Northern blotting.

Measurements of Liver PEPCK activity. db/db mice (n = 5) were treated with rosiglitazone (10 mg/kg) or PAT5A (10 mg/kg) or vehicle (0.25% carboxymethyl cellulose) for 10 days by gavage. The animals were sacrificed. Livers were removed and placed immediately in ice-cold homogenization buffer (10 mM Tris-HCl, pH 7.5 with 0.35 M sucrose). The tissues were homogenized and then centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was centrifuged at 25,000 rpm for 1 h at 4°C. The supernatant obtained was used as the enzyme source. PEPCK activity was measured by monitoring NADH oxidation at 340 nm. The assays were conducted at 30°C in a 1.0-ml reaction mixture containing 56 mM Hepes buffer (pH 7.5), 47 mM $NaHCO_3$, 2 mM phosphoenolpyruvate, 2 mM $MnCl_2$, 1 mM IDP, 0.25 mM NADH, and 20 units of malate dehydrogenase. Reactions were initiated by addition of enzyme. The change in absorbance was measured for 5 min. Activity units are expressed as micromoles of NADH oxidized per milligram of protein per minute (Gallwitz et al., 1988).

Results

PAT5A Is a Potent Antidiabetic Thiazolidinedione. PAT5A is a novel unsaturated thiazolidinedione analog containing pyridine [5-[4-[*N*-(2-pyridyl)-(2*S*)-pyrrolidine-2 methoxy]phenyl-methylene]thiazolidine-2,4-dione, malic acid salt] as a key heteroatomic moiety. The structure of PAT5A is shown and compared with that of rosiglitazone and other TZDs in Fig. 1. As mentioned earlier (Vikramadithyan et al., 2000), PAT5A and rosiglitazone showed similar plasma glucose lowering effect (ED_{50} = ~10 and 8.8 mg/kg) in db/db mice. Both compounds did not show any effect on food consumption, and PAT5A showed significantly less body weight increase compared with rosiglitazone (Vikramadithyan et al., 2000).

PAT5A Is a Dual Activator of PPARα and PPARγ. To determine whether PAT5A has transactivation potential of nuclear receptors like PPARα, PPARγ, PPARδ, and RXR, transactivation assays were done using Gal4 constructs of these receptors in HEK-293 cells. The results show that PAT5A has less transactivation potential of PPARγ compared with rosiglitazone (~5.0- versus ~14.0-fold) but has slightly better PPARα transactivation potential (~3.0-fold) compared with rosiglitazone (~2.0-fold) at 50 μM concentration (Fig. 2). At this concentration, the PPARα agonist Wy-14,643 showed an ~8.0-fold activation, indicating that both

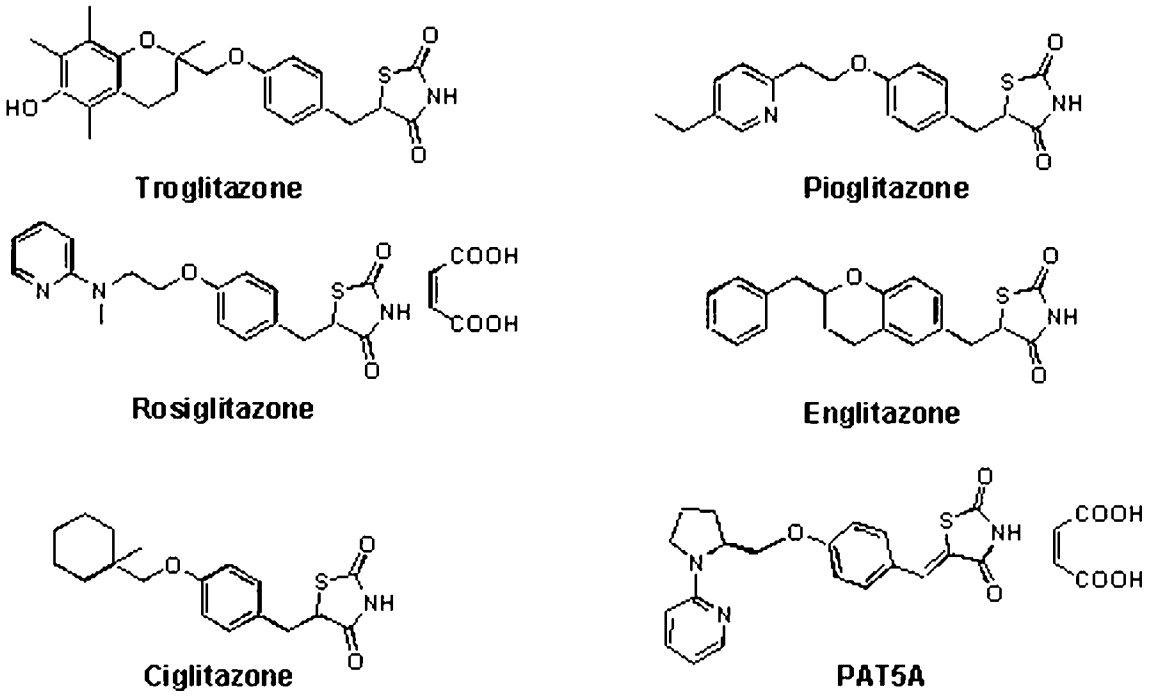

Fig. 1. Chemical structure of PAT5A compared with other TZDs.

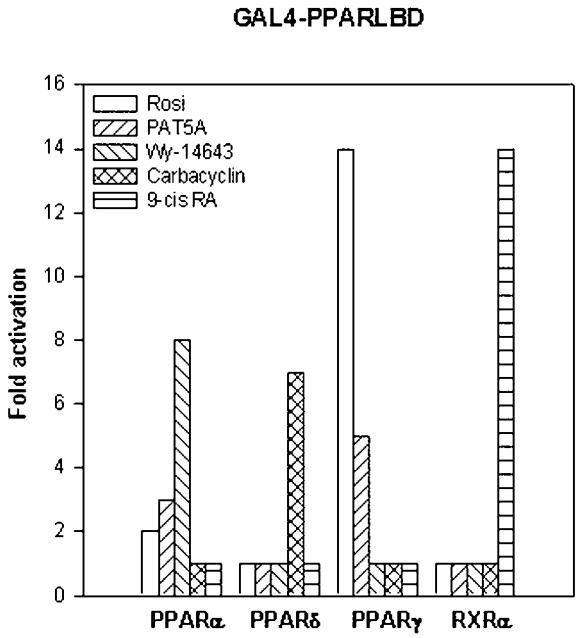

Fig. 2. PAT5A is a dual activator of PPARα and PPARγ. HEK-293T cells were transfected with 2 μg of reporter plasmid (Gal4 binding sites x5)-SV40-luciferase; 0.25 μg of Gal 4-hPPARα, γ, δ, or RXRα LBD, respectively, and treated with 50 μM of indicated ligands.

PAT5A and rosiglitazone are likely to have minimal hypolipidemic activity compared with Wy-14,643. Neither of these compounds showed any transactivation potential of PPARδ or RXRα (Fig. 2).

PAT5A Is a Partial Agonist of PPARγ. Antidiabetic TZDs have been shown to function as activating ligands for PPARγ. To determine whether the unsaturated TZD PAT5A can activate full-length PPARγ, transactivation assays were performed in HEK-293 cells using the well characterized PPAR binding sites from the acyl-coenzyme-A oxidase gene, upstream of a luciferase reporter driven by thymidine kinase promoter, in the presence of PPARγ heterodimer partner RXR. Even at saturating concentration, PAT5A activates PPARγ submaximally (data not shown). The effects of rosiglitazone and PAT5A were specific to PPARγ because both compounds did not exert any effect on RXR by themselves, whereas a dramatic effect of 9-*cis*-retinoic acid on RXR was noted (Fig. 2). Despite its almost equal hypoglycemic activity, however, PAT5A was less potent than rosiglitazone in this activity. It showed an EC_{50} of ~3-fold, indicating poor correlation of in vitro transactivation potential and in vivo hypoglycemic potency of this TZD (data not shown).

We have compared earlier the properties of PAT5A and rosiglitazone in the context of Gal4-PPARγ LBD, a chimeric fusion protein used to drive the expression of a reporter under the control of Gal4 binding sites in HEK-293 cells. The maximal efficacy achieved by PAT5A (5×10^{-5} M) was ~15% of that observed with rosiglitazone (10^{-5} M), indicating PAT5A is less potent than rosiglitazone (Vikramadithyan et al., 2000). Comparison of in vitro transactivation potential of PAT5A with rosiglitazone in the context of an another cell line, CV1, using Gal4-PPARγ LBD construct revealed that at two different doses, 1 and 10 μM, PAT5A showed lower activation potential compared with rosiglitazone (data not shown). Thus, in all three transactivation assays employed, namely, 1) full-length PPARγ, 2) Gal4-PPARγ LBD in HEK-293 cell, and 3) Gal4-PPARγ LBD in CV-1 cell, PAT5A functioned as a partial agonist of PPARγ transactivation. These results predicted that at a high concentration, PAT5A would displace rosiglitazone and functions as an inhibitor of rosiglitazone-stimulated activation of Gal4-PPARγ. Indeed, rightward shift of the rosiglitazone dose-response curve of PPARγ-dependent activation in the presence of a high relative concentration of PAT5A (10, 25 and 50 μM) indicated antagonistic property of PAT5A on rosiglitazone-dependent activation (Fig. 3).

PAT5A Binds to PPARγ Weakly and Its Interaction with the PPARγ Binding Pocket Is Different Compared with Rosiglitazone. To confirm the transactivation results, we sought to obtain direct proof of PAT5A binding to PPARγ by testing PAT5A and rosiglitazone as inhibitors of radiolabeled rosiglitazone bound to GST-PPARγ. It appears that PAT5A is a low-affinity PPARγ ligand, with a K_i value in the micromolar range (Fig. 4A) and a binding affinity of the PAT5A ~10-fold less than that observed for rosiglitazone (K_i ~200 nM).

The binding pattern of PAT5A and rosiglitazone to PPARγ was also assessed by the generation of protease resistant bands following limited digestion of recombinant receptor. [^{35}S]Methionine-labeled full-length PPARγ was preincubated with dimethyl sulfoxide (vehicle), 10 μM of rosiglitazone, or PAT5A and digested with increasing concentration of trypsin (0, 50, 100, 200 μg/ml) for 30 min. We found that PAT5A produced a protease protection patterns qualitatively similar to that of control and rosiglitazone, but quantitatively different to that of rosiglitazone, indicating higher affinity of rosiglitazone for the receptor (Fig. 4B).

This result prompted us to determine the interaction of

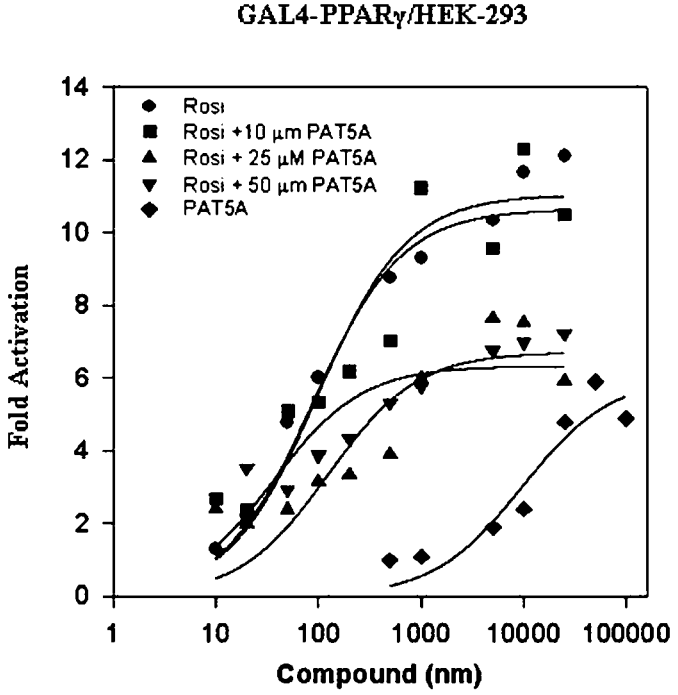

Fig. 3. PAT5A is a partial agonist of PPARγ. Effects of increasing doses of PAT5A on Gal4~PPARγ activation by rosiglitazone. 293T cells were transfected with 2 μg of reporter plasmid (Gal 4 $\times$ 5); SV40-luciferase, 0.25 μg of Gal4~hPPARγ LBD, and a different concentration of PAT5A were added 30 min before the addition of different doses of rosiglitazone.

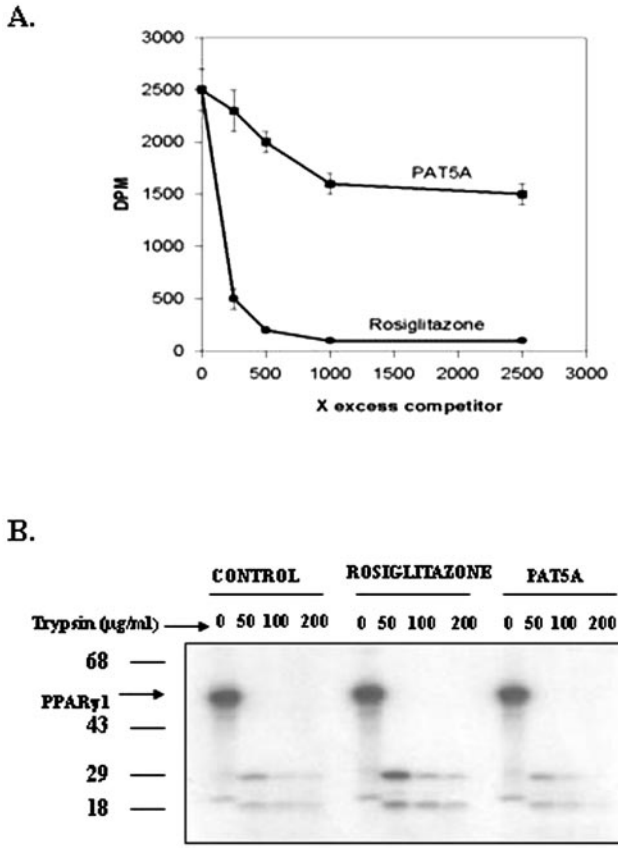

Fig. 4. PAT5A binds to PPARγ. A, PAT5A binds to PPARγ with a severalfold lower affinity than rosiglitazone. Competition binding assays were performed using bacterial extracts containing GST-mPPARγ LBD and 200 nM [^{3}H]rosiglitazone in the presence of cold rosiglitazone or PAT5A. Data are presented as mean of triplicate points and were normalized to reactions performed with vehicle alone (0.5% dimethyl sulfoxide). B, PAT5A induces qualitatively similar but quantitatively different protease digestion patterns. Autoradiogram of a SDS-PAGE showing [^{35}S]methionine-labeled full-length PPARγ digested with an increasing concentration of trypsin (0, 50, 100, and 200 μg/ml) for a period of 30 min. The receptor was preincubated with dimethyl sulfoxide (vehicle), 10 μM rosiglitazone or PAT5A each. ⋆, trypsin-resistant protein fragments.

PAT5A with the binding pocket of PPARγ. We docked PAT5A and rosiglitazone into the crystal structure of PPARγ LBD (2PRG) and evaluated by molecular dynamics. The root mean square deviations of the final structures compared with the crystal structure (all atoms) are 1.74 and 1.71 Å for rosiglitazone and PAT5A, respectively. Analysis showed that rosiglitazone interacts with Tyr-473, Ser-289, and His-323, while PAT5A interacts with His-449 only (Fig. 5). The TZD ring of PAT5A is slightly away from Tyr-473 of AF2 helix. The distance between Tyr-473 and thiazolidinedione ring N in PAT5A is 5.6 Å, while in rosiglitazone, it is 3.2 Å indicating that there is no H-bond between PAT5A and Tyr-473. Interaction energy for rosiglitazone is −28.13 kcal/mol, while it is −25.14 kcal/mol for PAT5A. Strain energy for rosiglitazone and PAT5A is 0.693 and 2.041 kcal/mol, respectively. These observations suggest that PAT5A interacts with the binding pocket of PPARγ in a manner different from that of rosiglitazone.

PAT5A Functions as a Full Agonist in 3T3L1 Cells But Has Weaker Adipogenic Activity Compared with Rosiglitazone. It is possible that reporter assays might not reflect the true potency of PAT5A since the in vitro transactivation assays did not give clear picture of the promoter specific modulation of PPARγ target genes following treatment with different TZDs. Therefore, we assessed the ability of PAT5A to induce adipocyte differentiation of 3T3-L1 cells, a well established property of TZDs that is mediated by PPARγ.

Both PAT5A and rosiglitazone induced adipocyte differentiation in a dose-dependent manner and reaches a plateau (Fig. 6A). PAT5A at 300 nM and rosiglitazone at 100 nM doses exhibited equal and maximum adipogenesis as measured by triglyceride levels (Fig. 6A). Northern analysis revealed that 3T3L1 cells had high levels of lipoprotein lipase mRNA, a PPARγ-responsive gene, after treatment with PAT5A and rosiglitazone (Fig. 6B). These observations suggest that in this cell context PAT5A behaves as a full agonist like rosiglitazone, although the EC_{50} of PAT5A is ~3 times less than that of rosiglitazone, implying weaker adipogenic

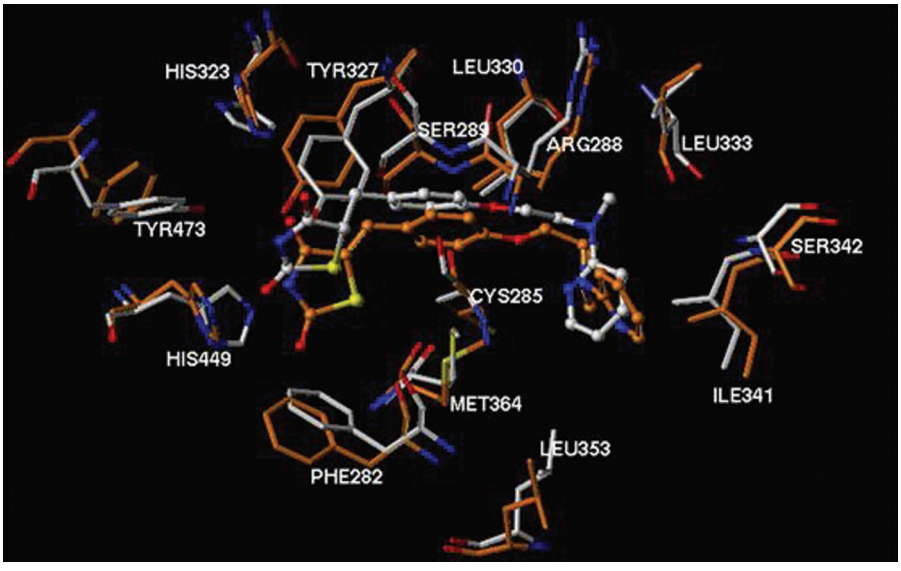

Fig. 5. PAT5A interacts with the binding pocket of PPARγ in slightly different way compared with rosiglitazone. Superimposition of rosiglitazone (gray) and PAT5A (golden-orange) within their binding pockets after dynamics.

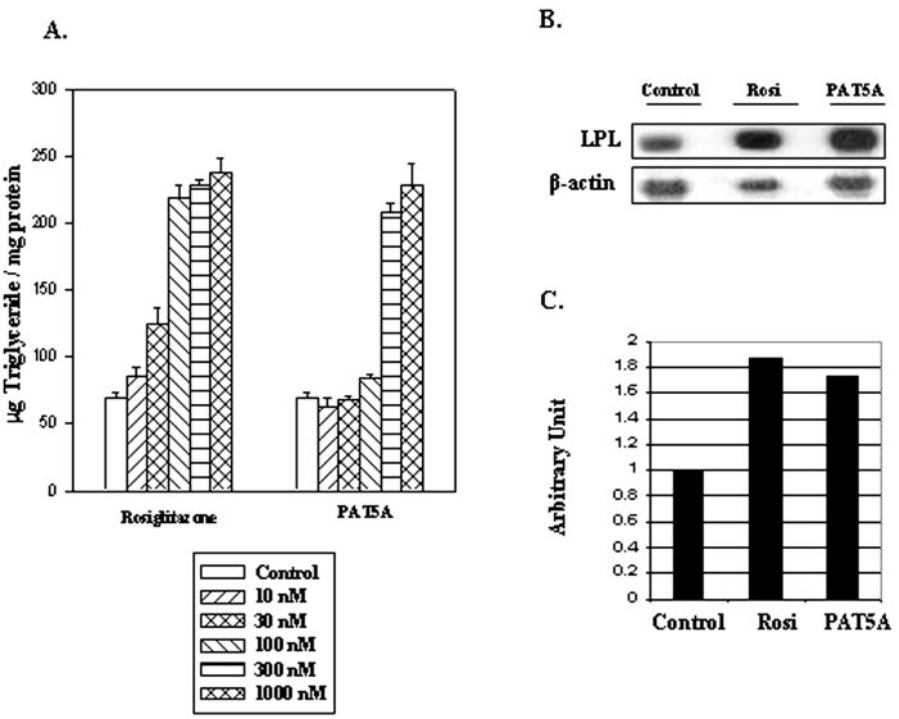

Fig. 6. PAT5A functions as a full agonist in 3T3-L1 cells but has weaker adipogenic potential compared with rosiglitazone. A and B, PAT5A enhances adipocyte differentiation as revealed by measurement of triglyceride (A) or expression of adipocyte marker LPL (B). 3T3L1 cells were exposed to different concentration of TZDs, as indicated in presence of 1 μM dexamethasone and 10 μg/ml insulin. After 7 days of treatment, the cells were lysed, and TG was measured by a TG assay kit (Pointe Scientific), or Northern analysis was done using 15 μg of total RNA isolated from the above-mentioned differentiating 3T3L1 cells treated with 1 μM of indicated TZDs and probed with cDNAs for LPL and β-actin (internal control). C, -fold activation has been shown after normalization of the values with internal control.

potential of PAT5A. This in vitro data correlates well with previously reported in vivo adipogenic data obtained in db/db mice as they gained less body weight after treatment with PAT5A when compared with rosiglitazone (Vikramadithyan et al., 2000).

PAT5A Functions as a Partial Agonist in Enhancing the Interaction of PPARγ with Coactivators SRC-1, CBP, PBP, and PRIP but as a Full-Agonist with PGC-1α. Ligand binding to PPARγ causes conformational changes within the receptor, which promotes the recruitment of coactivators such as SRC1, CBP, PBP, PRIP, and PGC-1α. It has been postulated that these coactivators act as bridges to transmit the regulatory signal from nuclear receptor to the basal transcription machinery of relevant genes (Rosenfeld and Glass, 2001). Figure 7A shows the recruitment of different coactivators to PPARγ by PAT5A and rosiglitazone. As predicted from the reduced affinity of PAT5A for the PPARγ, even at saturating concentration, the amount of SRC-1, CBP, PBP, and PRIP recruited by GST-PPARγ by PAT5A is significantly less than that recruited by saturating concentration of rosiglitazone. On the other hand, we found that PGC-1α recruitment was similar with PAT5A and rosiglitazone. In transactivation assays, using full-length PPARγ and its heterodimeric partner RXR, PAT5A showed less transactivation potential compared with that of rosiglitazone in the presence of equal amount of SRC-1, CBP, PBP, and PRIP, but at the same concentration both compounds exhibited equal and robust transactivation potential in the presence of PGC-1α (Fig. 7B). Furthermore, transactivation assays performed with different amounts of PGC-1α and saturating concentration of PAT5A or rosiglitazone showed equal or better potency with PAT5A compared with rosiglitazone. Likewise, dose response study with both compounds in the presence of equal amount of PGC-1α also revealed that PAT5A functions as a full agonist in enhancing the interaction of PPARγ. These observations show a good correlation between the in vitro coactivator recruitment data with functional data. These findings indicate PAT5A functions as a partial agonist in enhancing the interaction of PPARγ with coactivators SRC-1, CBP, PBP, and PRIP but as a full-agonist with PGC-1α (Fig. 7, C and D). It is possible that PAT5A probably induces PPARγ-responsive gene(s) in vivo to the same level compared with rosiglitazone by the recruitment of PGC-1α. This explains partly the in vivo hypoglycemic potency of PAT5A, which is similar to that of rosiglitazone despite its reduced affinity for PPARγ.

Differential Regulation of PPARγ-Responsive Genes from Adipose Tissue of PAT5A-Treated db/db Mice. We examined epididymal adipose tissue of db/db mice for the expression of PPARγ genes. We chose aP2, LPL, and adipsin, which are up-regulated by PPARγ ligands, and resistin as genes that are down-regulated by PPARγ ligands. Figure 8 shows that aP2 and LPL were induced from their already high levels by treatment with PAT5A and rosiglitazone. As expected, resistin expression level was decreased by rosiglitazone but not altered by PAT5A. In contrast, the level of adipsin was increased by PAT5A ~2.0-fold more compared with that of rosiglitazone demonstrating differential gene regulations by PAT5A and rosiglitazone.

Discussion

We have shown earlier that the overall antidiabetic potency of PAT5A is much better than that of troglitazone and comparable to that of rosiglitazone, although PAT5A is a weak PPARγ ligand (Vikramadithyan et al., 2000). Reports indicate that the in vitro activation of TZDs correlates well with their in vivo hypoglycemic activity, with the exceptions of MCC 555 and troglitazone (Reginato et al., 1998; Camp et al., 2000). In this study, we have carried out detailed experiments to elucidate the molecular mechanism(s) of action of this novel thiazolidinedione and provide evidence supporting

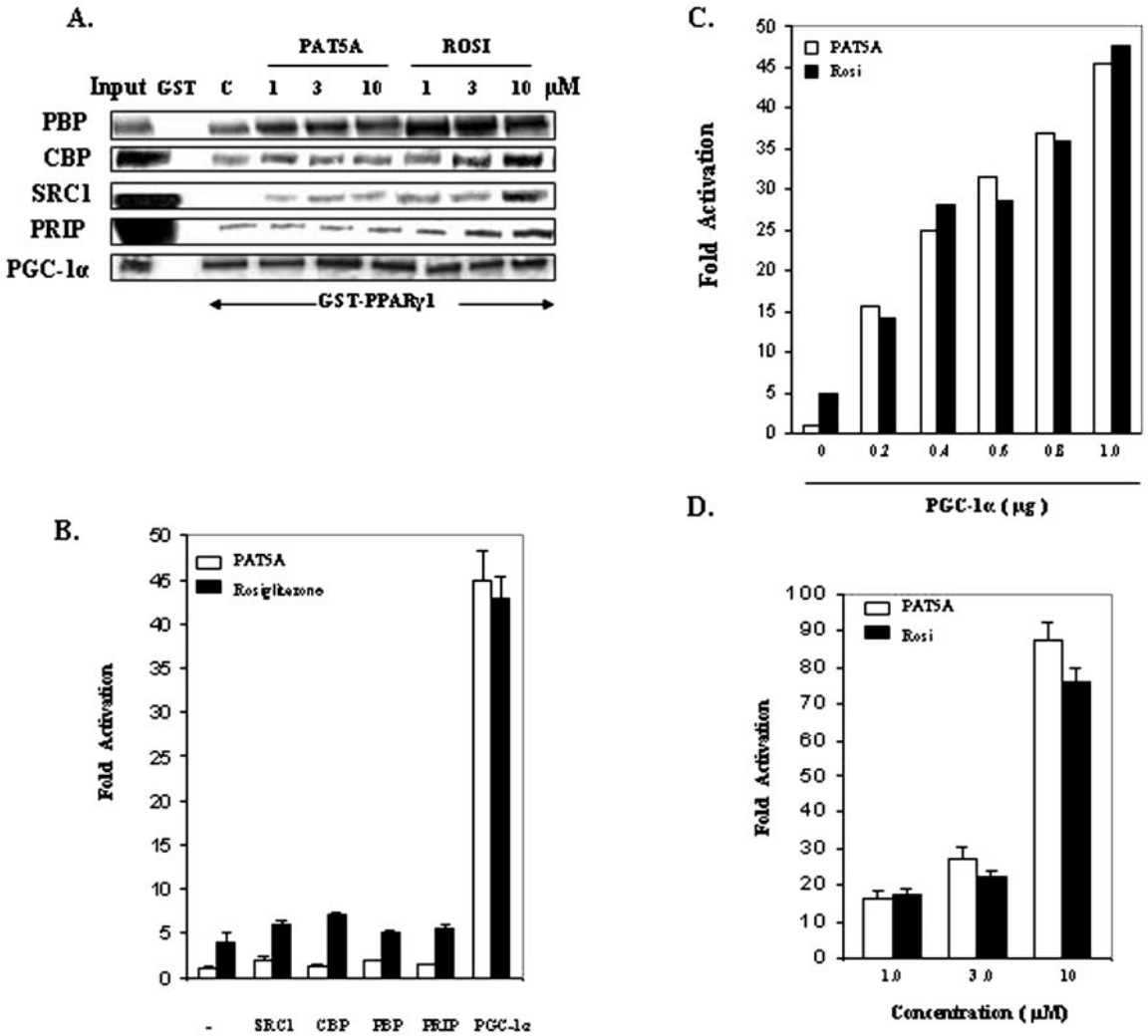

Fig. 7. PAT5A functions as a partial agonist in enhancing the interaction of PPARγ with the coactivators SRC-1, CBP, PBP, and PRIP but as a full-agonist with PGC-1α. A, In coactivator recruitment assays, coactivator-dependent receptor ligand interaction was determined using GST~PPARγ incubated with 5 μl of radiolabeled in vitro translated different coactivators in the presence of the indicated ligands at 4°C for 4 h. After vigorous washing, bound proteins were eluted and analyzed by SDS-PAGE. B–D, transactivation assay. 293T cells were transfected with 2 μg of reporter plasmid (acyl-coenzyme-A PPREx3) ~tk~luciferase, 0.25 μg of pCDNA3.1~ PPARγ, and pCDNA3.1~RXRα with or without 0.5 μg of different coactivators and treated with indicated 10 μM TZDs (B), with different amount of pCDNA3.1~PGC-1α and treated with 10 μM of indicated TZDs (C), or with 0.5 μg of pCDNA3.1~PGC-1α and indicated concentration of TZDs (D). Normalized luciferase activity was plotted as the -fold activation relative to untreated cells.

that PAT5A is a partial (or weak) PPARγ agonist. In two different cell systems transfected either with Gal4-PPARγ LBD or with full-length PPARγ, PAT5A induced a submaximal PPARγ transcriptional response compared with the more potent ligand rosiglitazone. Cotreatment of transfected cells with these ligands demonstrated that PAT5A antagonized rosiglitazone induced PPARγ transcriptional activity (Fig. 3). Competition binding assays using bacterial extracts containing GST-PPARγ LBD showed that PAT5A does bind to PPARγ although binding affinity of the PAT5A is ~10-fold less than that observed with rosiglitazone (Fig. 4A).

In contrast to these in vitro results, PAT5A appeared to behave as a full agonist in the context of 3T3-L1 adipocyte differentiation function, although it has weaker adipogenic potential compared with rosiglitazone. Furthermore, PAT5A and rosiglitazone exerted similar inductive effects on PPARγ-responsive genes, aP2, and LPL in the adipose tissue of db/db mice, but differential regulation of adipsin and resistin genes. In the db/db mouse liver, both PAT5A and rosiglitazone are equally potent in inhibiting the expression of glucose-6-phosphatase or PEPCK activity. In HepG2 cells, PAT5A inhibits cholesterol and fatty acid biosynthesis, but rosiglitazone showed no effects (data not shown). We believe that this effect of PAT5A on lipid biosynthesis may be a direct one and not mediated through PPARγ. Other investigators have also reported such activity of another TZD troglitazone (Wang et al., 1999). Further studies are required to evaluate the mechanism of action of PAT5A.

In an effort to understand the molecular basis for the observed differences in the transcriptional activities of PAT5A and rosiglitazone, we entertained the possibility that the two ligands cause differential cofactor recruitment. It is now well established that the immediate consequence of ligand binding is a conformation change in the receptor that increases its affinity for the transcriptional coactivator complexes, which act as a bridge to transmit the regulatory signal from nuclear receptor to the basal transcription machinery of relevant genes. In this study, we have shown that

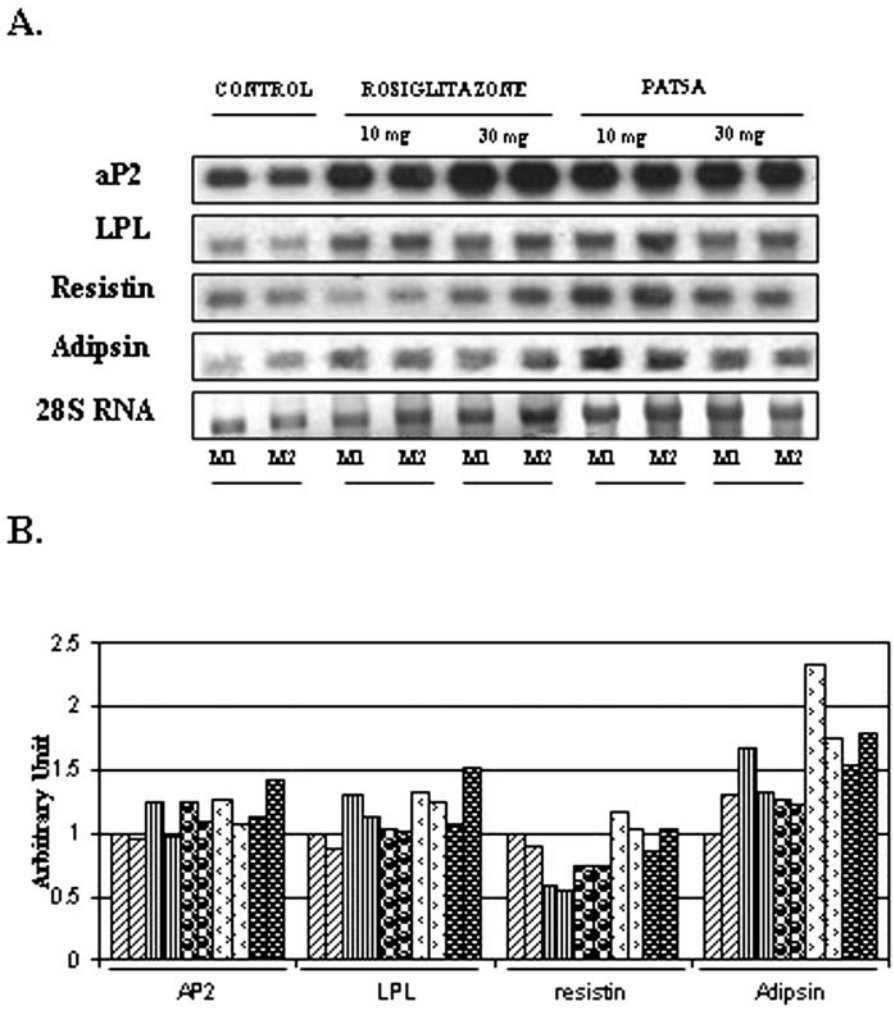

Fig. 8. Regulation of PPARγ-responsive genes from adipose tissue of PAT5A-treated db/db mice. A, Northern analysis of epididymal white adipose tissue of db/db mice that were treated with rosiglitazone or PAT5A (10 and 30 mg/kg/day) for 9 days. Total RNA (20 μg) was probed with cDNA for aP2, LPL, adipsin, and resistin. 28S rRNA (ethidium bromide staining) is also as evidence of equal loading. B, -fold activation has been shown after normalization of the values with the internal control.

four coactivators, CBP, SRC-1 (both with histone acetyl transferase activity) PBP, and PRIP (having no HAT activity) appear to have a lower affinity for PPARγ-bound to PAT5A compared with rosiglitazone (Fig. 7A), which correlates well with the transactivation and binding data. The observation that PAT5A behaves as full agonist in 3T3L1 cells suggests that there may be additional coactivator(s) in this cell type that interact with PAT5A-PPARγ complex. We have indeed shown that PPARγ after occupancy either with PAT5A or with rosiglitazone interacts equally with PGC1. Many of the coactivators studied here are expressed ubiquitously, although a cell type specific (adipocyte) coactivator, PGC1, has been described (Puigserver et al., 1998). Thus, a model can be constructed in which PAT5A acts as a partial agonist in cell lines containing primarily coactivators such as CBP, SRC1, PBP, or PRIP and behaves as a full agonist in cell lines containing PGC1.

Several investigators have shown that binding of ligand to PPARγ induce the interaction of coactivators like CBP, SRC-1, PBP, and PRIP with the ligand binding domain of PPARγ, whereas interaction of PGC-1 occurs with the DNA binding domain, and this interaction did not require L*XX*LL signature motif to bind to PPARγ (Zhu et al., 1996, 1997, 2000; Puigserver et al., 1998). It is hypothesized that upon ligand binding, PPARγ undergoes a conformation change, which induces the interaction of coactivators with PPARγ (Berger et al., 1996). Our findings suggest that conformation of the ligand binding domain may be somewhat different in PAT5A-bound PPARγ compared with the rosiglitazone-bound receptor, but the conformation of DNA binding domain remains same. Limited protease protection assay has been used as a tool to study the change in conformation of PPARγ LBD upon ligand binding by several investigators (Berger et al., 1996; Camp et al., 2000; Rocchi et al., 2001). Our data with PAT5A and rosiglitazone indicate that the two ligands induce protease digestion patterns qualitatively similar but quantitatively different upon receptor occupation, indicating higher affinity of rosiglitazone for receptor (Fig. 4B). Molecular docking data suggests that PAT5A binds to the PPARγ in the same pocket but the binding orientation and interaction energy is slightly different. The crucial H-bond between Tyr-473 O in the AF2 helix of PPARγ LBD and thiazolidinedione ring nitrogen of PAT5A is absent, supporting our interpretation of difference in conformation of PPARγ LBD after occupancy of PAT5A and rosiglitazone (Fig. 5). Further studies with the use of NMR spectroscopy techniques and/or X-ray crystallography to identify altered receptor conformations with PAT5A versus rosiglitazone and PAT5A-PPARγ cocrystal structure are needed to confirm our interpretation.

Finally, the molecular basis for the differential regulation of adipsin and resistin genes by PAT5A and rosiglitazone in the adipose tissue may be in the assembly of the transcrip-

tional complex specific to the context of a given gene and its promoter. As per the SPPARM model hypothesized on the basis of SERM concept (Brozowski et al., 1997), it is possible that PPARγ complexes may be different with different ligands leading to variations in the covalent modification of transcriptional machinery resulting in the alteration of transcriptional regulation (Olefsky, 2000). The differential gene regulation of adipocyte-specific target genes also explains the mechanism of the weak adipogenic potential of PAT5A in animals, demonstrating the beneficial role of this novel thiazolidinedione in the treatment of type-2 diabetes, a disorder aggravated by obesity. These results indicate that PAT5A selectively modulates PPARγ activity, triggering only certain PPARγ-controlled pathways, such as insulin sensitization, yet being much less efficient in activating other pathways, such as the stimulation of adipocyte differentiation and fat deposition.

References

Berger J, Bailey P, Biswas C, Cullinan CA, Doebber TW, Hayes NS, Saperstein R, Smith RG, and Leibowitz MD (1996) Thiazolidinediones produce a conformational change in peroxisomal proliferator-activated receptor-gamma: binding and activation correlate with antidiabetic actions in db/db mice. *Endocrinology* **137:**4189–4195.

Brivanlou AH and Darnell JE (2002) Signal transduction and the control of gene expression. *Science (Wash DC)* **295:**813–818.

Brzozowski AM, Pike ACW, Dauter Z, Hubbard RE, Bonn T, Engstrom O, Ohman L, Greene GL, Gustafsson J-A, and Carlquist M (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature (Lond)* **389:**753–758.

Camp HS, Li O, Wise SC, Hong YH, Frankowski CL, Shen X, Vanbogelen R, and Leff T (2000) Differential activation of peroxisome proliferator-activated receptor-gamma by troglitazone and rosiglitazone. *Diabetes* **49:**539–547.

Desvergne B and Wahli W (1999) Peroxisome proliferator-activated receptors: nuclear control of metabolism. *Endocr Rev* **20:**649–688.

Gallwitz WE, Jacoby GH, Ray PD, and Lambeth DO (1988) Purification and characterization of the isozymes of phosphoenolpyruvate carboxykinase from rabbit liver. *Biochimi Biophys Acta* **964:**36–45.

Lemmen C and Lengauer T (1997) Time-efficient flexible superposition of medium-sized molecules. *J Comput-Aided Mol Des* **11:**357–368.

Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schultz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, and Evans RM (1995) The nuclear receptor superfamily: the second decade. *Cell* **83:**835–839.

McKenna NJ, Lanz RB, and O'Malley BW (1999) Nuclear receptor coregulators: cellular and molecular biology. *Endocr Rev* **20:**321–344.

Misra P, Qi C, Yu S, Shah SH, Cao WQ, Rao MS, Thimmapaya B, Zhu Y, and Reddy JK (2002) Interaction of PIMT with transcriptional coactivators CBP, p300 and PBP differential role in transcriptional regulation. *J Biol Chem* **277:**20011–20019.

Nolte RT, Wisely GB, Westin S, Cobb JE, Lambert MH, Kurokawa R, Rosenfeld MG, Willson TM, Glass CK, and Milburn MV (1998) Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor-γ. *Nature (Lond)* **395:**137–143.

Olefsky JM (2000) Treatment of insulin resistance with peroxisome proliferator-activated receptor gamma agonists. *J Clin Investig* **106:**467–472.

Puigserver P, Wu Z, Park CW, Graves R, Wright R, Wright M, and Spiegelman BM (1998) A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell* **92:**829–839.

Rarey M, Kramer B, and Lengauer T (1997) Multiple automatic base selection: protein-ligand docking based on incremental construction without manual intervention. *J Comput-Aided Mol Des* **11:**369–384.

Rarey M, Kramer B, Lengauer T, and Klebe G (1996) A fast flexible docking method using an incremental construction algorithm. *J Mol Biol* **261:**470–489.

Reginato MJ, Bailey ST, Krakow SL, Minami C, Ishii S, Tanaka H, and Lazar MA (1998) A potent antidiabetic thiazolidinedione with unique peroxisome proliferator-activated receptor gamma-activating properties. *J Biol Chem* **273:**32679–32684.

Rosenfeld MG and Glass CK (2001) Coregulator codes of transcriptional regulation by nuclear receptors. *J Biol Chem* **276:**36865–36868.

Schoonjans K and Auwerx J (2000) Thiazolidinediones: an update. *Lancet* **355:**1008–1010.

Schoonjans K, Martin G, Staels B, and Auwerx J (1997) Peroxisome proliferator-activated receptors, orphans with ligands and functions. *Curr Opin Lipidol* **8:**159–166.

Rocchi S, Picard F, Vamecq J, Gelman L, Potier N, Zeyer D, Dubuquoy L, Bac P, Champy M-F, Plunket KD, Leesnitzer LM, et al. (2001) A unique PPARγ ligand with potent insulin-sensitizing yet weak adipogenic activity. *Molecular Cell* **8:**737–747.

Vikramadithyan RK, Chakrabarti R, Misra P, Premkumar M, Kumar SK, Rao CS, Ghosh A, Reddy KN, Uma C, and Rajagopalan R (2000) Euglycemic and hypolipidemic activity of PAT5A: a unique thiazolidinedione with weak peroxisome proliferator activated receptor gamma activity. *Metabolism* **49:**1417–1423.

Wang M, Wise SC, Leff T, and Su TZ (1999) Troglitazone, an antidiabetic agent, inhibits cholesterol biosynthesis through a mechanism independent of peroxisome proliferator-activated receptor-γ. *Diabetes* **48:**254–260.

Willson TM, Cobb JE, Cowan DJ, Wiethe RW, Correa ID, Prakash SR, Beck KD, Moore LB, Kliewer SA, and Lehmann JM (1996) The structure-activity relationship between peroxisome proliferator-activated receptor γ agonism and the antihyperglycemic activity of thiazolidinediones. *J Med Chem* **39:**665–668.

Zhu Y, Kan L, Qi C, Kanwar YS, Yeldandi AV, Rao MS, and Reddy JK (2000) Isolation and characterization of peroxisome proliferator-activated receptor (PPAR) interacting protein (PRIP) as a coactivator for PPAR. *J Biol Chem* **275:**13510–13516.

Zhu Y, Qi C, Calandra C, Rao MS, Reddy JK (1996) Cloning and identification of mouse steroid receptor coactivator-1 (mSRC-1), as a coactivator of peroxisome proliferator-activated receptor gamma. *Gene Expr* **6:**185–195.

Zhu Y, Qi C, Jain S, Rao MS, and Reddy JK (1997) Isolation and characterization of PBP, a protein that Interacts with peroxisome proliferator-activated receptor. *J Biol Chem* **272:**25500–25506.

Address correspondence to: Dr. R. Rajagopalan, Discovery Research, Dr. Reddy's Laboratories Ltd, Bollaram Road, Miyapur, Hyderabad: 500050, India. E-mail: rajagopalanr@drreddys.com