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Distinct Properties and Advantages of a Novel Peroxisome Proliferator-Activated Protein γ Selective Modulator

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Antidiabetic thiazolidinediones (TZDs) and non-TZD compounds have been shown to serve as agonists of the peroxisome proliferator-activated receptor γ (PPARγ). Here, we report the identification and characterization of a novel non-TZD selective PPARγ modulator (nTZDpa). nTZDpa bound potently to PPARγ with high selectivity *vs.* PPARα or PPARδ. In cell-based assays for transcriptional activation, nTZDpa served as a selective, potent PPARγ partial agonist and was able to antagonize the activity of PPARγ full agonists. nTZDpa also displayed partial agonist effects when its ability to promote adipogenesis in 3T3-L1 cells was evaluated. Assessment of protein conformation using protease protection or solution nuclear magnetic resonance spectroscopy methods showed that nTZDpa produced altered PPARγ conformational stability *vs.* full agonists, thereby establishing a physical basis for its observed partial agonism. DNA microarray analysis of RNA from 3T3-L1 adipocytes treated with nTZDpa or several structurally diverse PPARγ full agonists demonstrated qualitative differences in the affected gene expression profile for nTZDpa. Chronic treatment of fat-fed, C57BL/6J mice with nTZDpa or a TZD full agonist ameliorated hyperglycemia and hyperinsulinemia. However, unlike the TZD, nTZDpa caused reductions in weight gain and adipose depot size. Feed efficiency was also substantially diminished. Unlike TZDs, nTZDpa did not cause cardiac hypertrophy in mice. When a panel of PPARγ target genes was examined in white adipose tissue, nTZDpa produced a different *in vivo* expression pattern *vs.* the full agonist. These findings establish that novel selective PPARγ modulators can produce altered receptor conformational stability leading to distinctive gene expression profiles, reduced adipogenic cellular effects, and potentially improved *in vivo* biological responses. Such compounds may lead to preferred therapies for diabetes, obesity, or metabolic syndrome. (*Molecular Endocrinology* 17: 662–676, 2003)

PEROXISOME PROLIFERATOR-activated receptors (PPARs) comprise a subclass of the nuclear hormone receptor superfamily of ligand-dependent transcription factors (1). To date, three receptor subtypes have been identified: PPARγ, PPARα, and PPARδ. The PPARγ isoform has been shown to play a critical role in adipocyte gene expression and differentiation. Thus, PPARγ is expressed at high levels in adipose cells, and forced expression of the receptor is sufficient to cause the differentiation of pluripotent cell lines into adipocytes (2). PPARγ null cells cannot differentiate into adipocytes and PPARγ (+/−) mice display reduced adiposity (3–5). The adipogenic effects of PPARγ activation appear to occur through the transactivation of adipocyte gene promoters. A number of these gene promoters have been shown to contain peroxisome proliferator response elements (6).

It has been demonstrated that insulin-sensitizing agents of several diverse structural classes serve as agonists of PPARγ (7). The thiazolidinedione (TZD) class of PPARγ agonists (*e.g.* pioglitazone and rosiglitazone) have proven to be efficacious as insulin sensitizing agents in the treatment of humans suffering from type 2 or non-insulin-dependent diabetes mellitus (1, 8). Importantly, the *in vivo* efficacy of the TZDs

Abbreviations: ACS, Acyl-CoA synthase; aP2, adipose fatty acid binding protein; d, deionized; EWAT, epididymal white adipose tissue; GST, glutathione-*S*-transferase; h, human; HF, high fat; K_i, inhibition constant; LBD, ligand-binding domain; LF, low fat; LPL, lipoprotein lipase; nTZDpa, non-TZD selective PPARγ modulator; NMR, nuclear magnetic resonance; PPAR, peroxisome proliferator-activated protein; SPA, scintillation proximity assay; TZD, thiazolidinedione; TZDfa, TZD full agonist.

(and several newer non-TZD insulin sensitizers) has been shown to correlate with their potency as PPARγ agonists *in vitro* (7, 9, 10). This fact, along with other lines of evidence, strongly indicates that the mechanism of insulin sensitization by which TZDs work is predominantly via binding to, and activation of, PPARγ (1, 11). Unfortunately, PPARγ ligands that function as receptor agonists can promote enhanced adipogenesis, both *in vitro* and *in vivo* (10, 11). As a result, it is now quite clear that TZDs cause increased adiposity in both animal models of insulin resistance and in human patients with type 2 diabetes (12, 13). This undesirable action may result in diminished efficacy of PPARγ agonists with extended use since obesity itself is known to be a major cause of insulin resistance.

In both animal models and humans, PPARγ agonists are also known to have toxic effects that include plasma volume expansion, hemodilution, and edema (1, 13, 14). In addition, it has been shown that PPARγ agonists can cause substantial cardiac hypertrophy in several preclinical animal species (14). Effects on fluid balance and the potential for drug-related cases of congestive heart failure have prompted strong warnings against indiscriminate use of these agents in patients who may have early underlying heart disease as can exist in a large percentage of the diabetic population (15). Given the importance of insulin resistance as a major aspect of diabetes pathogenesis and the shortcomings of TZD insulin sensitizers noted above, a desperate need exists for improved PPARγ ligands that could retain metabolic efficacy with some reduction in deleterious effects.

Years of work characterizing ligands of the steroid receptor subclass of nuclear receptors has resulted in the identification of selective modulators, partial agonists, or antagonists with novel tissue-, cell-, and even gene-specific effects (16). Such compounds have demonstrated variable efficacy in the treatment of distinct maladies based upon their particular activity profile. They also have shown significant decreases in receptor-mediated toxicity. Thus, in contrast to the native estrogen receptor agonist ligand, estradiol, potent and selective synthetic ligands such as tamoxifen or raloxifene have antagonist activities in breast but estradiol-like effects on bone (17). Similarly, gene-selective transcriptional effects have been reported with novel glucocorticoid receptor ligands (18). With these observations in mind, we and others (19) have hypothesized that novel PPARγ ligands, which might serve as insulin-sensitizing agents lacking the undesirable effects mediated by full agonists, could be identified and ultimately developed as new therapies for metabolic disease.

Here we describe a novel, potent PPARγ selective ligand, nTZDpa, that was a partial agonist in both transactivation and adipogenesis assays. nTZDpa was also able to antagonize the activity of a full agonist in both of these assays. Through the use of protease protection and nuclear magnetic resonance (NMR) spectroscopy approaches, we discovered that this compound had a different effect on the conformational stability of PPARγ than full agonists, thereby providing a physical basis for its partial agonist activity. DNA microarray analysis demonstrated that nTZDpa differentially affected adipocyte gene expression in comparison with several full agonists. Both nTZDpa and a PPARγ full agonist, TZDfa, proved efficacious in ameliorating diet-induced insulin resistance and hyperglycemia. Interestingly, only the partial agonist caused a decrease in the weight gain, feed efficiency, and adiposity in comparison with HF-fed control mice. In addition, the insulin-sensitizing actions of nTZDpa occurred in the absence of the induction of cardiac hypertrophy as was seen with TZDfa. While both ligands altered expression of one group of PPARγ-responsive genes, expression of another set of genes was only induced by the full agonist.

RESULTS

Identification of a Novel Synthetic Partial Agonist for PPARγ

TZD agonists of PPARγ are efficacious in the treatment of insulin resistance in animal models and humans (1, 7, 8, 11). Through the use of directed chemical synthesis and molecular modeling, we identified a novel, potent, acyl indole PPARγ ligand, nTZDpa (Fig. 1). The structure of nTZDpa differs significantly from typical TZDs, and additional PPAR ligands used in the studies described here (Table 1).

To efficiently characterize PPAR ligands, scintillation proximity PPAR binding assays were developed using [$^3H_2$]nTZD3 as a radioligand for human (h) PPARγ and PPARα and [$^3H_2$]nTZD4 as a radioligand for hPPARδ PPARδ. These compounds had previously been identified as agonists of these receptors in cell based transactivation screens (Ref. 20 and unpublished data). nTZD3 was found to be a potent ligand for

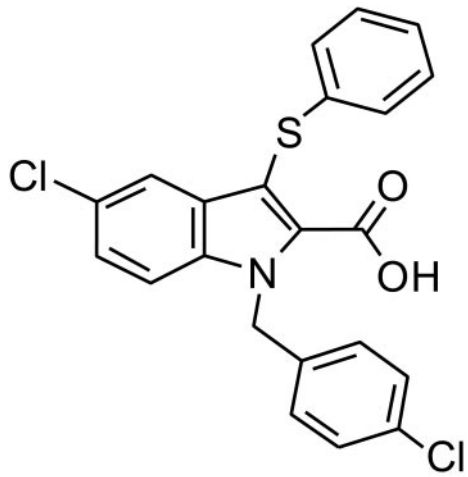
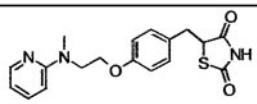
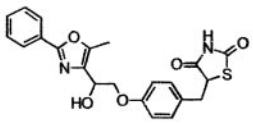
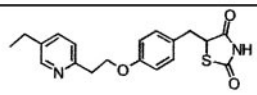
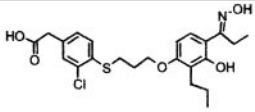
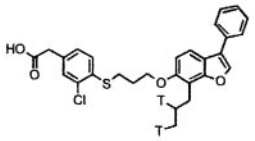
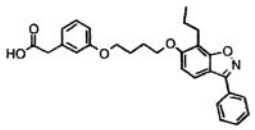
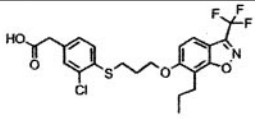
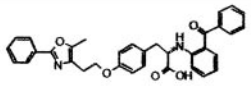


Fig. 1. Structure of Selective PPARγ Modulator nTZDpa.

Table 1. PPARγ Ligand Structures

Compound	Structure	Reference
rosiglitazone		(9)
TZDfa		(20)
pioglitazone		(9)
nTZD1		Not previously published
nTZD2		(20)
nTZD3		Not previously published
nTZD4		(20)
nTZD5		(46)

PPARγ and PPARα with inhibition constant (K_i) values of 3 nM and 4 nM, respectively. nTZD4 was a potent ligand for PPARδ with a K_i value of 2 nM.

Utilizing these scintillation proximity assay (SPA)-based receptor binding assays, nTZDpa was found to displace [$^3H_2$]nTZD3 from full-length PPARγ with a K_i = 9.7 nM (Fig. 2). In comparison, the TZD agonist, rosiglitazone, binds PPARγ with a K_i = 131 under the same assay conditions (data not shown). In contrast to its potent PPARγ binding activity, nTZDpa was unable to displace [$^3H_2$] radioligand binding to either PPARδ or PPARα when titrated up to concentrations of 1.5 μM (Fig. 2). Rosiglitazone was also unable to displace radioligand from PPARδ or PPARα (data not shown).

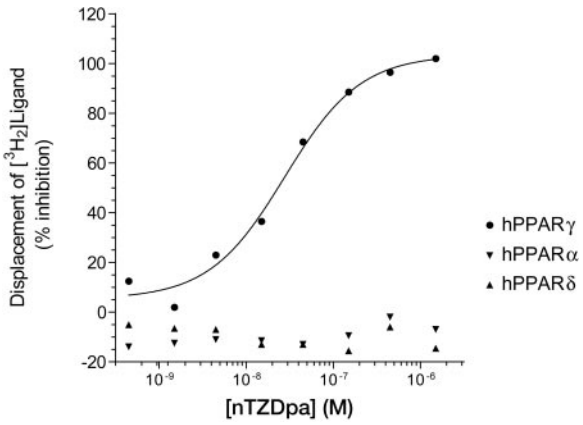


Fig. 2. nTZDpa Specifically Binds PPARγ

Competition curves generated by incubation of 5 nM [$^3H_2$]nTZD3 with GST-hPPARγ or GST-hPPARα or 2.5 nM [$^3H_2$]nTZD4 with GST-hPPARδ. The displacement of radioligand after incubation in the presence of the indicated concentration of nTZDpa for approximately 16 h is plotted. Similar results were obtained in at least two independent experiments performed in duplicate.

To examine its activity in a cell-based assay, nTZDpa was incubated with COS-1 cells that had been cotransfected with mammalian expression vectors containing chimeric receptors composed of the GAL4 DNA-binding domain and an hPPAR ligand-binding domain (LBD) along with a GAL4-responsive reporter gene plasmid. nTZDpa served as a potent (EC_{50} = 57 nM), selective PPARγ partial agonist, activating the receptor to approximately 25% of the maximum efficacy attained with the full agonists rosiglitazone and TZDfa (Fig. 3A) as well as pioglitazone and nTZD5 (data not shown). As predicted from binding assays, nTZDpa showed no agonist activity on PPARδ and PPARα (Fig. 3A). nTZDpa was also able to antagonize the activity of the PPARγ full agonist rosiglitazone by approximately 60% with an IC_{50} of approximately 285 nM (Fig. 3B). In line with our binding results, nTZDpa did not antagonize activation of PPARδ and PPARα by nTZD1 (Table 1). This novel ligand had demonstrated potent agonist activity on both of these PPAR isoforms in preliminary transactivation studies (data not shown).

Effect of nTZDpa on 3T3-L1 Cells

It has been demonstrated that PPARγ is necessary and sufficient for adipocyte differentiation (21) and that PPARγ agonists induce adipogenesis (1, 11). To examine the effect of nTZDpa on adipocyte differentiation, we determined the levels of mouse adipose fatty acid binding protein (aP2) mRNA expression in differentiating murine 3T3-L1 preadipocytes. The level of aP2 mRNA has been shown to correlate well with the development of adipocyte morphology and induction of other adipocyte

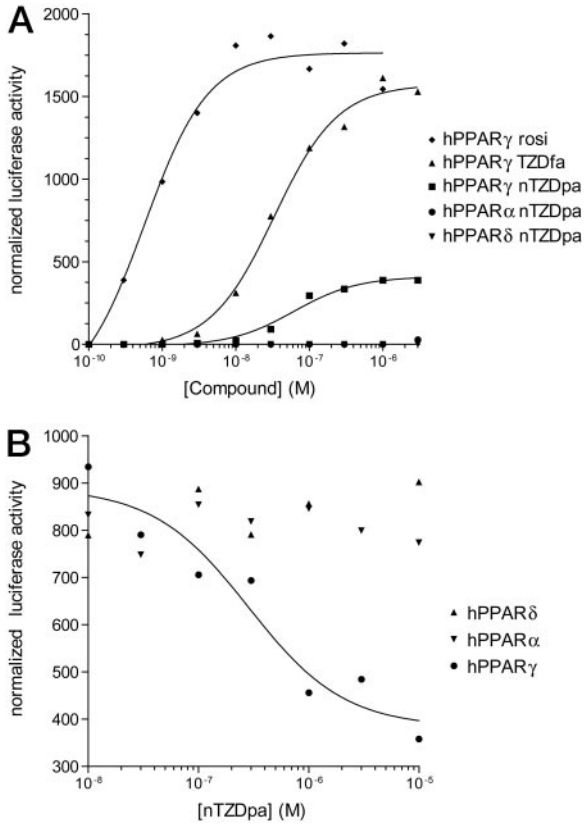


Fig. 3. nTZDpa Is a PPARγ Partial Agonist

A, Activation of hPPARγ by nTZDpa, rosiglitazone, and TZDfa. COS-1 cells were transiently cotransfected with pSG5-hPPARγ/GAL4, pSG5-hPPARδ/GAL4, or pSG5-hPPARα/GAL4 and both pUAS(5X)-tk-luciferase and pCMV-*lac*z then incubated with the indicated concentrations of rosiglitazone, TZDfa, or nTZDpa for 48 h. Cell lysates were produced, and luciferase and β-galactosidase activity in cell extracts was determined as described in *Materials and Methods*. B, Competitive antagonism of rosiglitazone activity on hPPARγ by nTZDpa. COS-1 cells were transiently cotransfected with pSG5-hPPARγ/GAL4, pSG5-hPPARδ/GAL4, or pSG5-hPPARα/GAL4 and both pUAS(5X)-tk-luciferase and pCMV-*lac*z, then incubated with 100 nM rosiglitazone (hPPARγ), 30 nM nTZD1 (hPPARδ), or 100 nM nTZD1 (hPPARα) and the indicated concentrations of nTZDpa for 48 h. Cell lysates were produced, and luciferase and β-galactosidase activity in cell extracts was determined as described in *Materials and Methods*. The concentrations of rosiglitazone or nTZD1 used in these competition assays fell within the linear dose range of their agonist activities (~EC_{50}). Basal activity of each of the three PPAR isoforms is approximately 10 U of normalized luciferase activity. Therefore, nTZDpa inhibited rosiglitazone activation of hPPARγ by approximately 60%.

genes and therefore serves as a sensitive assay of adipogenesis (22). We found that nTZDpa served as a potent (EC_{50} = 250 nM) partial agonist in augmenting the differentiation of 3T3-L1 preadipocytes. However, the acyl indole induced expression of aP2 mRNA to only approximately 40% of the maximum level observed with the PPARγ full agonist rosiglitazone (Fig. 4). In addition,

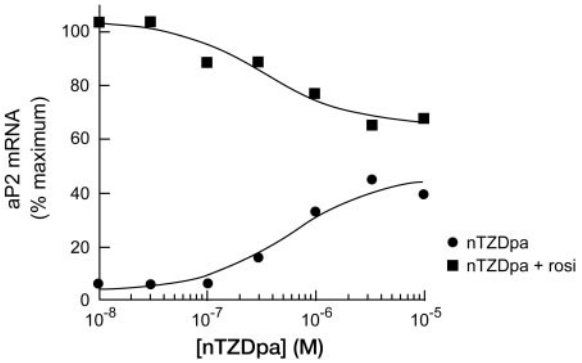


Fig. 4. nTZDpa Has Differential Effects on Adipogenesis

Confluent, differentiating 3T3-L1 preadipocytes were incubated with the indicated concentrations nTZDpa in the absence or presence of 100 nM rosiglitazone for 4 d. Total RNA was isolated and 20 μg of denatured RNA was analyzed by slot blot using a radiolabeled mouse aP2 cDNA probe. The means of normalized aP2 mRNA levels from triplicate samples of a representative experiment are plotted.

nTZDpa partially antagonized agonist-induced 3T3-L1 cell adipogenesis, blocking rosiglitazone stimulated aP2 expression with an IC_{50} = 550 nM (Fig. 4). In additional experiments (data not shown), we examined lipid content of 3T3-L1 cells using a nonquantitative method: Oil red O staining. During the course of normal adipocyte differentiation using an 8-d protocol with the complete methylisobutylxanthine, dexamethasone, and insulin differentiation medium (see *Materials and Methods*), nTZDpa (1 μM) did not appear to inhibit differentiation. However, lipid accumulation was only partially augmented *vs.* a more pronounced effect seen with rosiglitazone (1 μM). We have also observed that 48 h incubation with higher concentrations (10 μM) of nTZDpa could decrease net lipid content of fully differentiated 3T3-L1 adipocytes.

Distinct Effects of nTZDpa on PPARγ Conformation

We have previously used protease protection assays to demonstrate that PPARγ agonists alter the conformation of the receptor so that it becomes more resistant to proteolytic degradation (10, 20, 23–25). Such alterations in conformation appear to be critical to the induction of nuclear receptor activity by their agonists. Here, radiolabeled hPPARγ was synthesized in an *in vitro* transcription/translation system. The receptor was preincubated alone or with saturating concentrations of rosiglitazone or nTZDpa and then incubated with increasing concentrations of trypsin. As demonstrated in Fig. 5, in the absence of ligand, PPARγ was largely degraded by the increasing concentrations of trypsin. In contrast, both rosiglitazone and nTZDpa rendered the receptor LBD less sensitive to proteolysis. Interestingly, the trypsin resistant receptor fragments differed considerably between ligands. The full agonist rosiglitazone protected major bands at 22 and

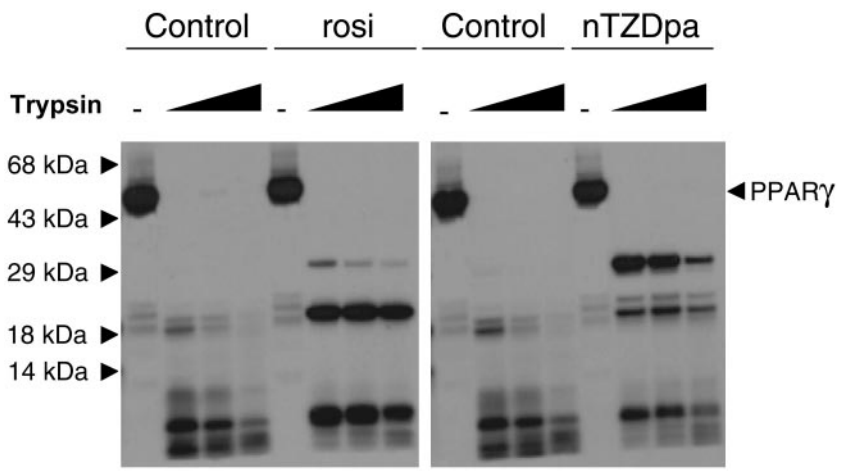


Fig. 5. nTZDpa and Rosiglitazone Produce Different PPARγ LBD Conformations

[^{35}S]hPPARγ was synthesized *in vitro* in a coupled transcription/translation system. Labeled receptor was subsequently incubated with 0.1% dimethylsulfoxide (Control) or 10 μM rosiglitazone or nTZDpa followed by incubation with dH_2O or increasing concentrations (50, 100, or 250 μg/ml) of trypsin. Digestion products were analyzed by SDS-PAGE followed by autoradiography.

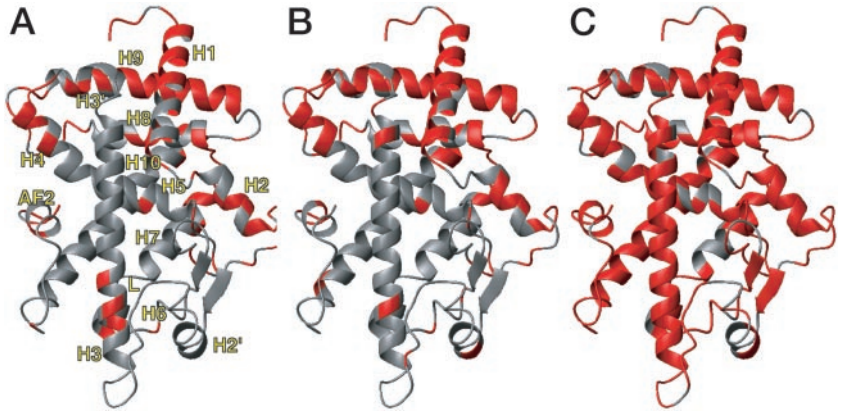


Fig. 6. NMR Spectroscopy Demonstrates that nTZDpa and Rosiglitazone Differentially Affect the Stability of the PPARγ LBD

The three-dimensional x-ray crystallographic structure of the hPPARγ LBD colored by the presence of cross peaks in the three-dimensional HNCO spectra. Stabilized, assigned amino acid residues are colored in *red*. Helices (H1-H10, AF2) and the approximate center of the ligand binding site (L) are labeled in *yellow*. A, Apo-hPPARγ LBD. B, hPPARγ LBD-nTZDpa complex. C, hPPARγ LBD-rosiglitazone complex. The figure was made with MolMol analysis software.

8 kDa as well as a minor band at 29 kDa. In contrast, nTZDpa protected a major band at 29 kDa and minor bands at 8, 22, and 25 kDa. In addition, the major PPARγ LBD fragments remained more resistant to degradation by increasing concentrations of trypsin in the presence of rosiglitazone than with nTZDpa.

To examine the effects of these ligands on the conformation of the PPARγ LBD at higher resolution, triple labeled (^{15}N, ^{13}C, and D_2O) glutathione-*S*-transferase (GST)-hPPARγ LBD was generated in cultured BL21 cells. After LBD purification, solution NMR protein spectroscopy was performed on the receptor LBD alone or when complexed with saturating concentrations of rosiglitazone or nTZDpa. We have previously shown, via NMR spectroscopy, that the binding of rosiglitazone to PPARγ greatly stabilizes the conformation of the receptor LBD in comparison with the apo receptor LBD as defined by an increase in the number of amino acid residues producing observable cross-peaks in the HNCO three-dimensional spectra of the agonist-bound receptor LBD (26). As demonstrated in Fig. 6, we were able to reconfirm that observation here. In addition, we found that nTZDpa only afforded a partial increase in receptor LBD stability compared with that produced by the full agonist. Taken together, our data support the conclusion that nTZDpa induces a distinct and less stable conformation (or subset of conformations) of PPARγ than the full agonist rosiglitazone.

Regulation of Adipocyte Gene Expression by nTZDpa

We have previously shown that the agonist activation of PPARγ alters the expression of numerous genes in adi-

pocytes (27). Studies of other nuclear receptors have demonstrated that quantitative and qualitative differences can exist in the regulation of genes by full agonists and selective ligands (16, 28). We therefore hypothesized that nTZDpa might have a different effect on cellular gene expression than PPARγ full agonists. To test this hypothesis, 3T3-L1 adipocytes were treated for 24 h with nTZDpa or each of 4 PPARγ agonists, TZDfa, rosiglitazone, pioglitazone, or nTZD5, at concentrations 20-fold greater than their respective EC_{50}s in the 3T3-L1 cell differentiation assay described above. Such treatment should cause maximal activation of PPARγ in the cells by the ligands. Gene expression profiles were then obtained from cell-derived RNA using high-density oligonucleotide microarrays. As shown in Fig. 7A, application of a two-dimensional clustering algorithm for the assessment of gene expression data (compounds *vs.* gene expression) demonstrated that treatment of adipocytes with the PPARγ agonists altered the expression of 132 genes, either up (33 genes; *red*) or down (99 genes; *green*), 1.5-fold or greater. The expression of the great majority of the up-regulated genes increased 3-fold or less while that of approximately 70% of the down-regulated genes decreased by 50% or more. Notably, the four full agonists clustered close together, whereas the partial agonist, nTZDpa, was found to exhibit a distinctly different gene expression profile and therefore occupied a separate node. This separation occurred, in part, because nTZDpa did not alter the expression of as many genes as the full agonists. In particular, the partial agonist did not significantly affect the expression of 41 genes that were effected by each of the full agonists (*gray* regions). Furthermore, the partial agonist did not modify the expression of numerous genes to the same degree as the full agonists. Among the 94 genes whose expression was significantly affected by nTZDpa, 58 were modulated to a lesser extent by the partial agonist than by any of the 4 full agonists. This result is demonstrated qualitatively by the overall dimmer hue of the nTZDpa row in comparison with those of the full agonists.

In separate experiments, we used an alternative approach (quantitative fluorescent real-time PCR) to assess whether nTZDpa could differentially regulate selected genes in 3T3-L1 adipocytes in comparison to two full agonists. Results shown in Fig. 7B are generally consistent with findings from the microarray study in that the expression pattern for four PPARγ target genes was similar for rosiglitazone and TZDfa but distinct from the effects of TZDpa. For CD36 the results were similar to the data from the microarray study (1.3- and 1.2-fold induction by rosiglitazone and TZDfa, respectively, *vs.* 0.9-fold change with nTZDpa). Phosphoenolypyruvate carboxykinase, c-cbl-associated protein, and muscle carnitine palmitoyl transferase-1 were not included in the genes represented in the microarray.

In Vivo Studies

PPARγ agonists have proven efficacious as insulin-sensitizing agents in insulin-resistant humans and animals (1). It has been demonstrated that their *in vivo* efficacy correlates with their potency *in vitro* as PPARγ agonists (7, 9, 10). Here, we compared the ability of a potent PPARγ full agonist, TZDfa, with that of nTZDpa to affect various aspects of diet-induced metabolic syndrome in mice. C57BL/6J mice were fed low-fat (LF; 11%), high-fat (HF; 58%), HF + 3 mg/kg·d TZDfa, or HF + 50 mg/kg·d nTZDpa diets for 74 d from the age of 18 d. At the end of that time, the animals on the HF diet suffered from hyperinsulinemia, modest hyperglycemia and hyperleptinemia (Table 2). Treatment of the mice with either of the PPARγ ligands essentially normalized their insulin levels while causing significant decreases in their elevated glucose and leptin levels (Table 2). HF mice also had elevated plasma triglyceride levels; however, neither PPARγ ligand was effective in lowering triglycerides. Free fatty acids levels were not significantly elevated in HF *vs.* LF mice and were not affected by PPARγ ligand treatment (Table 2). Mice on the LF diet gained only 8 g during the course of the experiment, whereas those on the HF and HF+ TZDfa diets gained more than 22 and 23 g, respectively (Fig. 8A). In contrast, HF + nTZDpa mice gained only 14 g. Therefore, these data describe a greater than 50% decrease in excess HF-induced weight gain in the HF+ nTZDpa group relative to the HF and HF + TZDfa groups. Increases in body weight seen in the HF and HF + TZDfa animals can be explained, in large part, by the greater than 2-fold increases in feed efficiency (defined as grams of weight gained per grams of food ingested) seen in these groups (Fig. 8B). In contrast, HF mice administered nTZDpa displayed nearly a 50% decrease in feed efficiency relative to these two groups of mice. It is also noteworthy that the HF, HF + TZDfa, and HF + nTZDpa mice consumed greater levels of calories compared with the LF controls. However, this effect was modestly attenuated in response to nTZDpa treatment in comparison with the HF + TZDfa or HF control groups (Table 2). The increases in body weight seen in the HF and HF + TZDfa mice appear to result from increased adiposity. Animals in both groups demonstrated increases in whole body lipid content and decreases in lean mass in comparison with the LF mice (Fig. 8, C and D). In contrast, mice treated with nTZDpa showed no change in body protein compared with the LF mice and exhibited significantly lower body fat levels than the HF and HF + TZDfa mice. With respect to the location of fat deposition, both HF and HF + TZDfa mice were found to have increases in the net weight of multiple adipose depots relative to the LF mice (Table 2). Interestingly, TZDfa treatment further increased the weight of intrascapular brown adipose tissue and the inguinal white adipose depot while decreasing that of the epididymal and mesenteric white adipose depots relative to the HF control group. In contrast, all of the adipose depots examined in the HF + nTZDpa mice were greatly diminished in comparison with the HF group. In fact, brown fat and mesenteric depots were not significantly greater than corresponding depots in the LF cohort. One undesirable effect of PPARγ full agonists that has been noted in preclinical species is cardiac

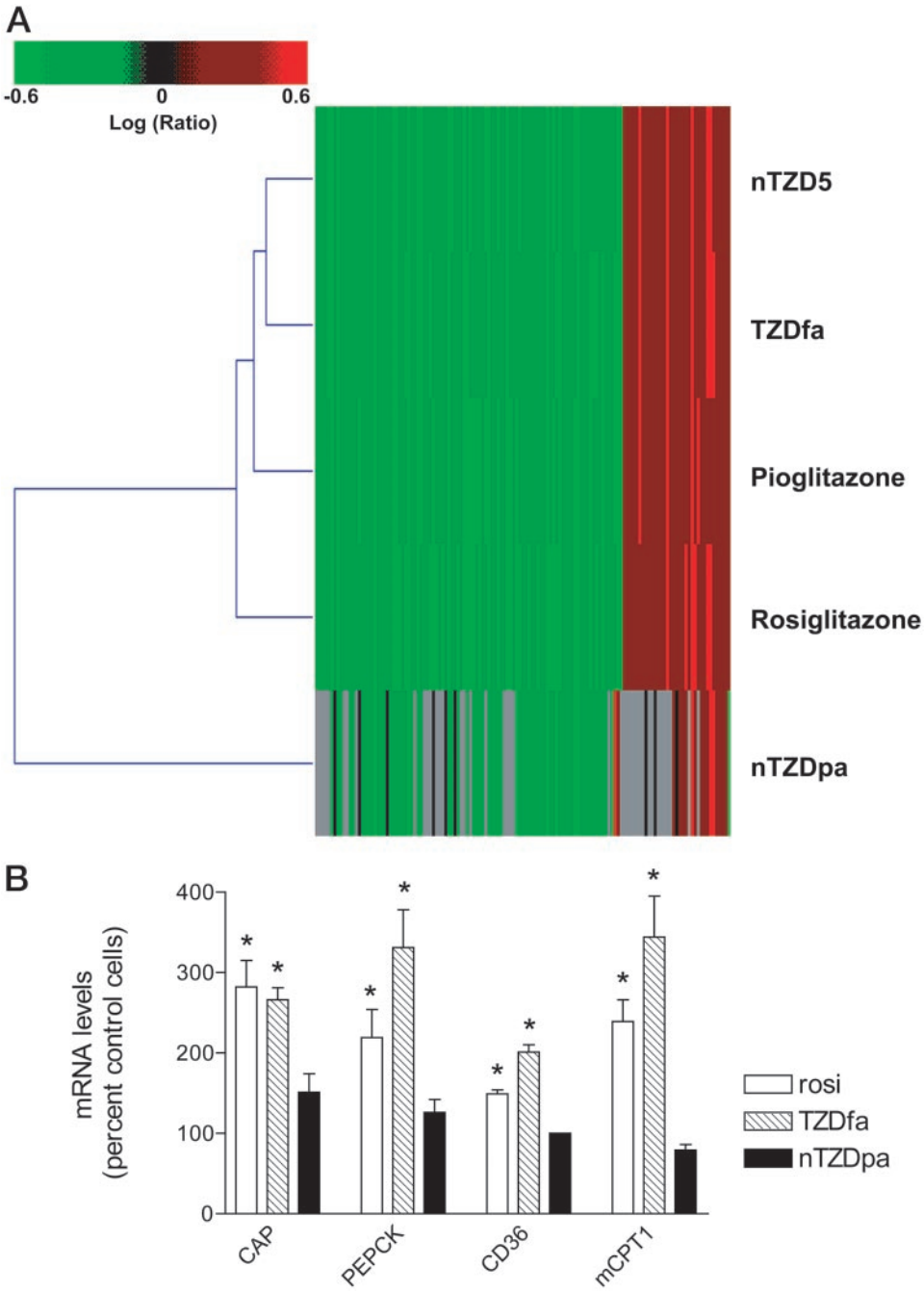


Fig. 7. Differential Regulation of Adipocyte Gene Expression by nTZDpa and PPARγ Full Agonists

3T3-L1 cells were grown to confluence then differentiated into adipocytes. The cells were then treated for 24 h ± TZDfa (20 nM), rosiglitazone (360 nM), pioglitazone (5000 nM), nTZD5 (6 nM), or nTZDpa (5000 nM). At the end of the treatment period, total RNA was isolated as described in *Materials and Methods*. A, Microarray analysis of adipocyte gene expression. Adipocyte RNA was processed and analyzed on high-density Affymetrix oligonucleotide microarrays as described in *Materials and Methods*. Gene expression data from the chips was clustered in two dimensions (compounds *vs.* gene expression) using the Agglomerative clustering program (threshold 1.5-fold, $P < 0.1$) within Resolver software (Rosetta, Kirkland, WA). B, Quantitative fluorescent real-time PCR analysis of adipocyte gene expression. Adipocyte RNA was reverse transcribed and quantitative fluorescent real-time PCR was performed on the newly synthesized cDNA using primer/probe sets for c-cbl-associated protein (CAP), phosphoenolypyruvate carboxykinase (PEPCK), CD36, and muscle carnitine palmitoyl transferase-1 (mCPT1) as described in *Materials and Methods*. The figure plots the mean ± SEM of triplicate samples. *, $P < 0.05$ *vs.* control adipocytes.

Table 2. Selected Parameters Measured in C57BL/6 Mice

Parameter	LF	HF	HF + TZDfa	HF + nTZDpa
Cumulative food intake (kcal)	820 ± 23	1145 ± 16[a]	1159 ± 27[a]	924 ± 22[a,b]
Plasma measurements				
Insulin (μU/ml)	35 ± 5	155 ± 32[a]	40 ± 7[b]	64 ± 7[b]
Glucose (mg/dl)	119 ± 3	193 ± 4[a]	167 ± 4[a,b]	166 ± 4[a,b]
Leptin (ng/ml)	10 ± 2	37 ± 2[a]	30 ± 2[a,b]	22 ± 1[a,b]
Fatty acids (mEq/liter)	0.72 ± 0.06	0.88 ± 0.06	0.86 ± 0.07	0.80 ± 0.06
Triglycerides (mg/dl)	98 ± 6	175 ± 7[a]	163 ± 9[a]	173 ± 9[a]
Adipose depot weights (% of LF control)				
IBAT	100 ± 8	208 ± 17[a]	475 ± 75[a,b]	158 ± 17[b]
EWAT	100 ± 9	456 ± 28[a]	303 ± 22[a,b]	248 ± 19[a,b]
ING	100 ± 13	503 ± 36[a]	695 ± 69[a,b]	210 ± 18[a,b]
MES	100 ± 8	385 ± 46[a]	181 ± 31[a,b]	115 ± 15[b]
Heart weight (g)	0.14 ± 0.01	0.17 ± 0.01[a]	0.23 ± 0.02[a,b]	0.15 ± 0.01[b]

Groups of C57BL/6J mice (n = 12, each) were exposed to LF diet with control vehicle treatment (LF), HF diet with control vehicle treatment (HF), HF diet with TZD full agonist treatment (HF + TZD), or HF diet with nTZDpa treatment (HF + nTZDpa). Plasma and tissue samples were obtained under *ad libitum*-fed conditions at the end of the 91-d study. Selected adipose depots included: IBAT, EWAT, ING, and MES. [a] $P < 0.05$ *vs.* LF; [b] $P < 0.05$ *vs.* HF.

hypertrophy (29). The mechanistic basis of this action is as yet unknown. As shown in Table 2, HF mice showed a small but significant increase in heart weight in comparison with the LF animals. Treatment of HF mice with TZDfa led to a robust further induction in heart weight. In contrast, HF + nTZDpa mice did not display significant cardiac hypertrophy relative to the LF group.

On the basis of the above *in vivo* data and our previous results demonstrating the differing effects of nTZDpa and PPARγ full agonists, including TZDfa, on adipocyte gene expression *in vitro*, it seemed likely that the two PPARγ effectors might also differentially regulate gene expression *in vivo*. We therefore examined the expression of several known PPARγ-responsive genes in epididymal white adipose tissue (EWAT) from mice in each of the four treatment groups. All of these genes were dysregulated in the HF mice (either up- or down-regulated in comparison with LF mice). We found that both ligands affected expression of a subset of these genes in a similar manner. Acrp30 was up-regulated by TZDfa and nTZDpa, whereas TNFα and leptin were down-regulated by both ligands (Fig. 9A). As a result, the expression of these three genes was normalized (or nearly normalized) to the levels seen in LF mice. It should be noted that circulating hyperleptinemia was only partially lowered toward normal *vs.* the apparent normalization of leptin mRNA expression in EWAT. This may suggest lessor degrees of reduced leptin gene expression in other adipose depots. In contrast to the similar effects of both ligands on Acrp30, TNFα, and leptin mRNAs, the full agonist was able to induce expression of several genes involved in lipid uptake, or metabolism, including aP2, fatty acid transport protein (FATP), CD36, lipoprotein lipase (LPL) and acyl-CoA synthase (ACS), whereas nTZDpa had little or no effect upon their expression (Fig. 9B). These results clearly demonstrated that nTZDpa differentially affected *in vivo* expression of adipose genes in comparison with a PPARγ full agonist.

DISCUSSION

Treatment of obese and insulin-resistant type 2 diabetes patients using PPARγ agonists such as rosiglitazone or pioglitazone is akin to the use of a double-edged sword. Although insulin sensitization and improved glycemic control can be achieved via this mechanism, exacerbation of weight gain and a number of worrisome adverse effects are frequently encountered (30, 31). Therefore, the identification and development of improved PPARγ ligands that retain metabolic efficacy remains a pressing need. Recent results obtained with hemizygous PPARγ null mice in comparison with PPARγ agonist (rosiglitazone)-treated wild-type mice suggest that either a net decrease or a net increase in PPARγ tone can have favorable effects that augment *in vivo* insulin sensitivity (12). On the other hand, stronger degrees of PPARγ loss-of-function, as are likely to exist in patients with dominant-negative PPARγ mutations, appears to result in insulin resistance and the development of overt diabetes (32). Given these considerations, neither full agonism nor complete antagonism of PPARγ may be viewed as optimal approaches for therapy of metabolic disorders.

Several unique and potentially interesting PPARγ ligands have recently been described in the literature. Oberfield *et al.* (33) described a compound (GW0072) that partially activated PPARγ but served to inhibit PPARγ agonist driven adipocyte differentiation *in vitro*. A second PPARγ ligand (LG100641) was shown to antagonize the activation of the receptor by the PPARγ agonist rosiglitazone (34). This compound was

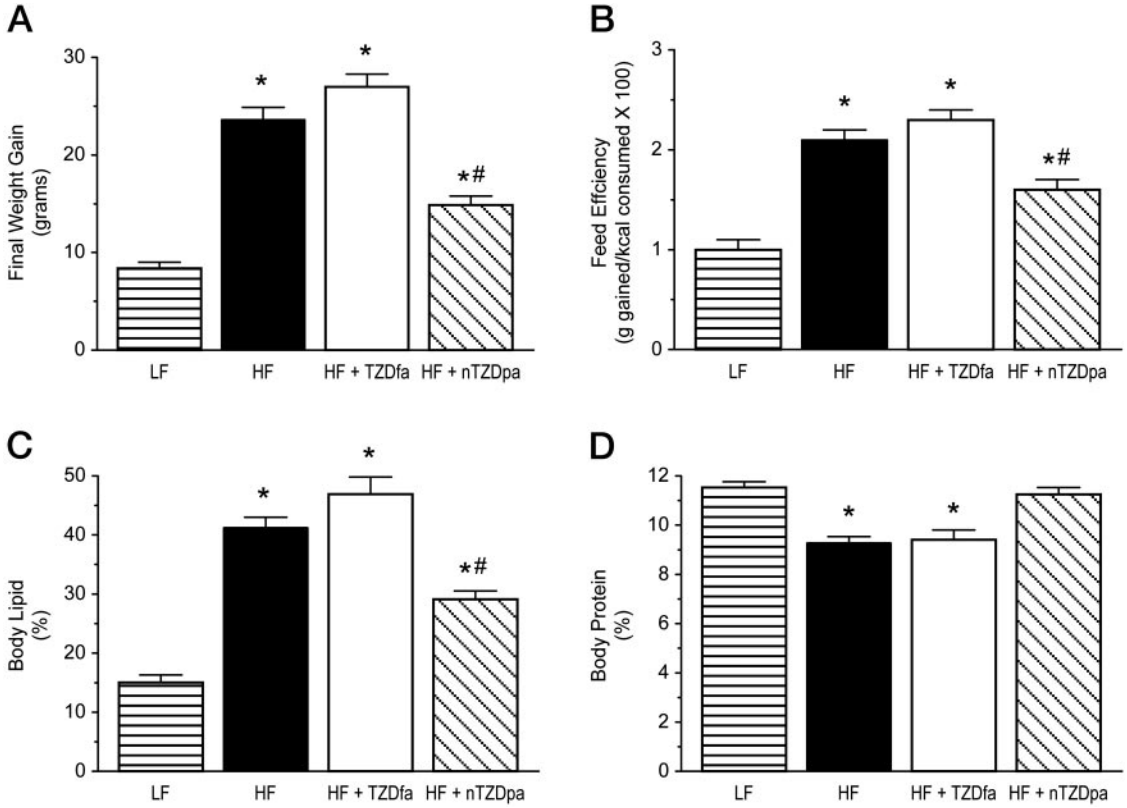


Fig. 8. nTZDpa and TZD Differentially Affect Weight Gain, Feed Efficiency, and Body Composition in HF-Fed C57BL/6J Mice

C57BL/6J mice (n = 12, in each group) were exposed to LF diet with control vehicle treatment (LF), HF diet with control vehicle treatment (HF), HF diet with TZDfa treatment (HF + TZDfa), or HF diet with nTZDpa treatment (HF + nTZDpa). Body weights and food intake were determined throughout the experiment until termination at 91 d. Subsequently, animal carcasses were digested in acidic ethanol. Lipid and protein levels in the digests were determined as described in *Materials and Methods*. A, Weight gain. B, Feed efficiency. C, Percent body lipid. D, Percent body protein. The figure plots the mean ± SEM of 12 replicate samples from each treatment group. *, $P < 0.05$ *vs.* LF; #, $P < 0.05$ *vs.* HF.

also shown to antagonize adipocyte differentiation *in vitro*. An additional and structurally unique compound, BADGE, was shown to have weak PPARγ antagonist activity and to also inhibit adipogenesis when studied under several *in vitro* conditions (35). Unfortunately, no *in vivo* data was ever provided for any of these three novel PPARγ ligands. During the preparation of this manuscript, Rocchi *et al.* (36) described a newly characterized and relatively weak PPARγ agonist of some interest, FMOC-L-Leu. In comparison with classical PPARγ agonists, FMOC-L-Leu displayed more modest adipogenic activity. Surprisingly, FMOC-L-Leu did not antagonize, but actually augmented, the activity of PPARγ agonists in transactivation and gene expression studies thereby making its interaction with the receptor somewhat obscure. *In vivo*, this ligand was found to improve insulin sensitivity in rodent models of diabetes; however, no significant difference in weight was seen between animals treated with FMOC-L-Leu or rosiglitazone.

In the studies presented here, we describe the identification and extensive characterization of a novel selective PPARγ modulator, nTZDpa. nTZDpa was a potent and selective PPARγ ligand; in this manner, it did not differ from previously described TZD and non-TZD PPARγ full agonists. However, in cell-based assays, nTZDpa differed markedly from the aforementioned ligands in that it was only able to partially activate PPARγ and could also antagonize transcriptional effects of full agonists. nTZDpa also differed with respect to its effects in 3T3-L1 cells. Relative to rosiglitazone, nTZDpa only partially augmented adipogenesis.

The physical basis for the activation of a nuclear receptor by its ligands appears to be the stabilization of the ligand receptor complex into a conformation (or limited number of conformations) that can bind nuclear receptor coactivator proteins with increased affinity (28, 37). These multimers then interact with the cellular transcriptional machinery in a manner that increases the rate of transcription initiation. Here we found by both protease protection and NMR spectroscopy techniques that the binding of our selective PPARγ ligand produced receptor LBD conformations of greater stability relative to the apo receptor LBD, but that LBD protein stability was diminished *vs.* LBD bound to a classical full agonist, rosiglitazone. The hypothesis that PPARγ partial agonists may produce

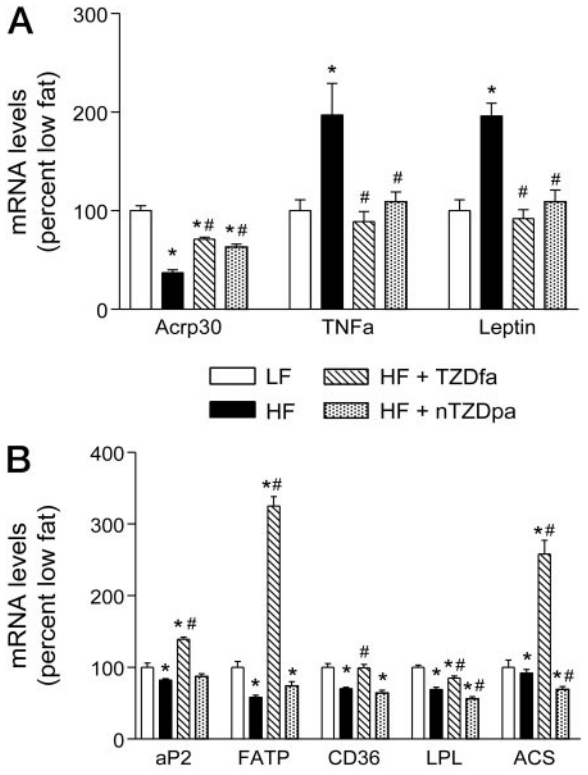


Fig. 9. Differential Regulation of Adipose Gene Expression by nTZDpa and TZDfa in HF-Fed C57BL/6J Mice

C57BL/6J mice were treated as described in the legend for Fig. 8. At the termination of the treatment period, EWAT was removed and total RNA was isolated from the tissue. Subsequently, the RNA was reverse transcribed and quantitative fluorescent real-time PCR was performed on the newly synthesized cDNA as described in *Materials and Methods*. For each of the genes, the figure plots the mean ± SEM of 12 replicate samples from each treatment group. *, $P < 0.05$ *vs.* LF; #, $P < 0.05$ *vs.* HF.

altered receptor conformation(s) is also supported by the finding that GW0072 exhibited an altered PPARγ binding mode when characterized via x-ray crystallography (33). Recently, we have also observed that the binding of partial agonists results in an apparently reduced affinity for the interaction of PPARγ with transcriptional coactivators *vs.* full agonist effects (unpublished observations). It therefore seems reasonable to conclude that nuclear receptor ligands that differ in their ability to produce stable receptor conformations will also differ in their ability to induce receptor interactions with available coactivators leading to altered gene expression profiles in cells. A corollary of this conclusion is that ligands that do not stabilize a nuclear receptor in its maximally active conformation(s) can be detected as partial agonists in standardized cell-based functional assays. This appears to be the case with nTZDpa.

Although a DNA microarray approach was recently attempted to study the gene regulation patterns of estradiol *vs.* the estrogen receptor partial agonist, tamoxifen, no differentially expressed genes derived from this effort were reported (38); nor has this approach been used to profile the effects of full *vs.* partial PPARγ agonists. Here, we treated 3T3-L1 adipocytes with nTZDpa or each of four PPARγ full agonists and then analyzed changes in mRNA expression using high-density oligonucleotide microarrays. Analysis of gene expression patterns demonstrated that nTZDpa clustered well away from the full agonists. This separation was the result of two distinctive effects on gene expression by the partial agonist. First, whereas all five compounds did regulate the expression of numerous genes (both up and down) in a similar manner, nTZDpa did not alter the expression of as many genes as the full agonists did. Second, when nTZDpa did alter expression of the same genes as the full agonists, it often did so to a lesser degree. Prior studies showed that GW0072 failed to modulate the expression of two candidate target genes (aP2 and adipsin) in 3T3L1 adipocytes (33). Similarly, LG100641 failed to induce aP2, adipsin, and PPARγ mRNAs in cultured 10T1/2 preadipocytes (34).

As noted above, we now demonstrate that a subset of genes responsive to full agonists can also be (at least partially) modulated by a partial agonist in fully differentiated adipocytes. Therefore, these results serve as a reminder that the terms full agonist, partial agonist, and antagonist are relative ones of limited value when used to describe the ability of ligands to alter gene expression because they are dependent upon the often reductionist assay used in the characterization process. We have now entered an era in which panoramic gene profiling data will be used to more fully describe nuclear receptor modulators and delineate the molecular bases of their varying cellular and physiological effects.

HF-fed C57BL/6J mice have been shown to serve as a useful model of obesity and insulin-resistant diabetes that possess many of characteristics found in humans suffering from these maladies (39, 40). As previously described, we found that when placed on a diet in which 58% of their caloric intake is provided by fat, the mice become hyperglycemic, hyperinsulinemic, and hyperleptinemic. Treatment with either TZDfa or nTZDpa significantly improved these undesirable metabolic states, apparently also attenuating insulin resistance so as to ameliorate hyperglycemia. In contrast to these similar *in vivo* actions of the two PPARγ ligands, they also showed important differences. Although HF and HF + TZDfa mice demonstrated robust weight gain, as a result of increased caloric intake and augmented feed efficiency, the HF + nTZDpa animals gained considerably less weight in large measure due to a significant diminution in feed efficiency. The HF + nTZDpa mice also took in more calories than the LF mice but HF + nTZDpa mice showed a modest decline in caloric intake in comparison with the HF and HF + TZDfa cohorts. When four adipose depots of the mice were weighed, all of them were found to be larger in HF than LF mice. The depot-specific effects of treat-

ment with the full agonist (Table 2) were consistent with previous reports showing TZD effects to increase subcutaneous fat with concomitant reductions in visceral fat in human diabetics and rodent models (41, 42). In contrast, all four measured adipose depots were reduced in size after treatment of fat-fed mice with nTZDpa. These differences in adipose tissue weights were reflected in the changes seen in percent body lipid. The HF and HF + TZDfa mice showed sizeable increases in whole-body lipid content in comparison with the LF mice, whereas the HF + nTZDpa mice had lower body lipid levels than mice in the two other HF groups. Conversely, HF and HF + TZDfa mice displayed decreased mean percent body protein *vs.* the LF group, whereas the HF + nTZDpa mice showed protein levels that were indistinguishable from those on the LF diet.

In a first attempt to identify molecular mechanisms that might be invoked to explain the similarities and differences between the *in vivo* actions of TZDfa and nTZDpa, their effects on the regulation of a limited set of white adipose tissue genes was examined. We found that the expression of genes encoding biologically active proteins that are secreted by adipose tissue were regulated in a similar manner by both PPARγ effectors. Acrp30 (adiponectin), which was down-regulated in the HF mice, was up-regulated by both ligands. This protein was recently shown to have beneficial *in vivo* metabolic effects on glucose and lipid metabolism (43, 44). Similarly, TNFα, which was robustly up-regulated in the obese mice, was normalized by TZDfa or nTZDpa treatment. TNFα has previously been shown to contribute to peripheral insulin resistance *in vivo* (45). These data support the hypothesis that altered expression of critical endocrine or paracrine factors expressed by adipose tissue may be an important means by which the insulin sensitizing actions of PPARγ effectors is mediated. In contrast to their actions upon the aforementioned modulators of insulin action, TZDfa and nTZDpa had differential regulatory effects on a group of genes encoding for proteins involved in adipocyte lipid metabolism—FATP, CD36, LPL, aP2, and ACS—that were all down-regulated in fat-fed mice. TZDfa treatment induced expression of these genes to normal or supernormal levels, whereas the nTZDpa regimen either had no significant effect (aP2, CD36, FATP) or modestly down-regulated (LPL, ACS) their expression. It is intriguing to speculate that such gene expression differences might be pointing toward an explanation for the divergent effects of full *vs.* selective PPARγ modulators on weight gain and adiposity. Additional studies will be required to test this conjecture.

Treatment of type 2 diabetics with efficacious PPARγ agonists has been show to result in weight gain along with improved insulin sensitivity (30, 31). Because many people suffering from type 2 diabetes are already obese, treatment with agents that exacerbate obesity is clearly suboptimal. It is plausible that the beneficial effects of PPARγ full agonists may be limited by further increases in body mass as obesity is a major cause of insulin resistance. Here, we demonstrated that a selective PPARγ modulator was able to produce apparent insulin sensitization in the absence of concomitant increases in weight and adiposity, thereby demonstrating that these pharmacological actions are separable. In addition, nTZDpa was found to be unique because, unlike all the full agonist TZDs, it did not cause an increase in heart weight. Although PPARγ agonists have not been shown to induce cardiac hypertrophy in the clinic, most have been shown to increase cardiac weight in nonhuman species (29). Such observations are cause for concern, especially for a class of drugs being used to treat patients with an increased incidence of cardiac disease. Thus, apprehension regarding the long-term effects of PPARγ-targeted drugs would be assuaged if newer ligands that do not promote adiposity or cardiac hypertrophy became available as therapies for human metabolic disease.

In sum, we have described a novel and potent selective modulator of PPARγ. Relative to classical PPARγ full agonists, a differential gene expression profile was demonstrated *in vitro* and *in vivo* along with potential antiobesity effects that were evident in HF-fed mice. Importantly, this agent also retained beneficial *in vivo* metabolic effects without provoking cardiac toxicity. The observed differences in the ability of nTZDpa to confer conformational changes in the PPARγ LBD provides a potential molecular mechanism for partial agonism and unique regulatory effects on gene expression. These results suggest that further efforts to develop novel selective PPARγ modulators as therapies for type 2 diabetes, obesity, and other aspects of metabolic syndrome are indeed warranted.

MATERIALS AND METHODS

Materials

The TZDs, rosiglitazone ((+/−)-5-(4-(2-(methyl-2-pyridinylamino)ethoxy)phenyl)methyl)-2,4-thiazolidinedione) (9), TZDfa (5-[4-[2-(5-methyl-2-phenyl-4-oxazoly)-2-hydroxyethoxy]benzyl]-2,4-thiazolidinedione) (20), pioglitazone (5-{4-[2-(5-ethyl-2-pyridyl)ethoxy]benzyl}-2,4-thiazolidinedione) (9), and the non-TZDs, nTZD1 (3-chloro-4-(3-(2-propyl-3-hydroxy-4-(1 hydroxyiminopropyl))phenoxypropyl)thiophenylacetic acid), nTZD2 (3-chloro-4-(3-(3-phenyl-7-propyl-benzofuran-6-yl)oxypropyl)thiophenylacetic acid) (20), nTZD3 (3-(4-(3-phenyl-7-propyl-benzisoxazole-6-yl)oxy)butyloxyphenylacetic acid), nTZD4 (3-chloro-4-(3-(3-trifluoromethyl-7-propyl-benzisoxazole-6-yl)oxypropyl)thiophenylacetic acid) (20), nTZD5 α-(2-phenyloxomethyl)phenylamino)-β-(4-(2-(2-phenyl-5-methyl-oxazole-4-yl)ethoxy))phenylpropionic acid (46), and nTZDpa (1-(p-chlorobenzyl)-5-chloro-3-phenylthiobenzyl-2-yl carboxylic acid) were used in these studies. The structure of nTZDpa is shown in Fig. 1; those of all the other compounds described here are displayed in Table 1. Cell culture reagents were obtained from Life Technologies, Inc. (Gaithersburg, MD). Unless otherwise noted, all other reagents were obtained from Sigma (St. Louis, MO).

Preparation of Recombinant PPAR and Binding Assay

PPARs were expressed as GST-fusion proteins in *Escherichia coli.* The full-length human cDNAs for PPARγ, PPARδ (provided by Dr. Azriel Schmidt, Merck Research Laboratories, West Point, PA) and PPARα (provided by Dr. Tom Rushmore, Merck Research Laboratories) were subcloned into pGEX-KT expression vectors (Pharmacia, Piscataway, NJ), followed by production of purified recombinant proteins in *E. coli* as previously described (25). Using the purified GST-hPPAR receptors, SPA-based receptor binding assays were established. By performing several saturation binding studies using increasing amounts of [$^3H_2$]nTZD3 and an excess of unlabeled nTZD3 followed by nonlinear regression analyses we determined that the dissociation constant values of nTZD3 for PPARγ and PPARα were 2.5 and 5 nM, respectively. Similar analyses of nTZD4 binding to PPARδ determined its dissociation constant for this receptor to be 1 nM. Once the assay conditions had been established, competitive binding experiments were performed in Packard OptiPlate-96 well polystyrene microplates (Packard BioScience, Meriden, CT). In these assays, a minimal amount of receptor (<5 nM) was used to achieve an acceptable signal. For each assay, human GST-PPAR receptor was combined with SPA buffer (10 mM Tris, pH 7.2; 1 mM EDTA; 10% glycerol; 10 mM Na molybdate; 1 mM dithiothreitol; and 2 μg/ml benzamidine), 0.1% nonfat dry milk, 8.3 μg/ml anti-GST antibody (Amersham Biosciences, Piscataway, NJ), and radioligand in a final volume of 74 μl. For both PPARα and PPARγ, 5 nM of [$^3H_2$]nTZD3 (specific activity of 34.3 Ci/mmol) was added, and for PPARδ 2.5 nM of [$^3H_2$]nTZD4 (specific activity of 13.4 Ci/mmol) was added. Yttrium silicate protein A-coated SPA beads (Amersham Pharmacia Biotech), suspended in SPA buffer, were added to a final concentration of 1.25 mg/ml. Test compound (1 μl) in dimethylsulfoxide was then added to the assay. Nonspecific binding was determined by the addition of a 100-fold excess of the respective unlabeled ligand. After incubation for approximately 16 h at 15 C, with shaking, the assay plates were counted in a TopCount Scintillation Counter (Packard Bioscience) to determine the displacement of radioligand from the receptor by test compounds. Results are expressed as percent inhibition and inflection points calculated by a four-parameter logistic equation. K_i values were calculated by the equation of Cheng and Prusoff (47).

Cell Culture and Transactivation Assay

COS-1 cells were cultured and transactivation assays were performed as previously described (20). Briefly, cells were transfected with a pcDNA3-hPPAR/GAL4 expression vector, pUAS(5X)-tk-luciferase reporter vector and pCMV-*lacz* as an internal control for transactivation efficiency using Lipofectamine (Invitrogen, Carlsbad, CA). After a 48-h exposure to compounds, cell lysates were produced, and luciferase and β-galactosidase activity in cell extracts was determined as previously described (20). Inflection points of normalized luciferase activity were calculated by a four-parameter logistic equation.

Measurement of Effects in 3T3-L1 Preadipocytes and Adipocytes

For the assessment of preadipocyte differentiation, murine 3T3-L1 cells (ATCC, Manassas, VA; passages 3–9) were grown to confluence in medium A (DMEM with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin) at 37 C in 5% CO_2 (20). Confluent cells were incubated in medium A containing 1 μM dexamethasone and 150 nM insulin with various concentrations of nTZDpa alone or nTZDpa in the presence of 100 nM rosiglitazone for 4 d with one medium change. The concentration of rosiglitazone used (100 nM) fell within the linear range of its adipogenic effect in preliminary experiments. For measurement of aP2 mRNA, RNA was prepared using the Ultraspec RNA isolation kit (Biotecx, Houston, TX). RNA (20 μg) was denatured in formamide/formaldehyde and slot-blotted onto Hybond-N membrane (Amersham Pharmacia Biotech, Arlington Heights, IL). Prehybridization was performed at 42 C for 1–3 h in 50% formamide and Thomas solution A containing 25 mM sodium phosphate, pH 7.4; 0.9 M sodium chloride; 50 mM sodium citrate; 0.1% each of gelatin, Ficoll, and polyvinylpyrrolidone; 0.5% sodium dodecyl sulfate; and 100 μg/ml denatured salmon sperm DNA. Hybridization was carried out at the same temperature for 20 h in the same solution with a ^{32}P-labeled aP2 cDNA probe (2×10^6 cpm/ml). After washing, the membranes under appropriately stringent conditions, the hybridization signals were analyzed with a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). Inflection points of normalized aP2 mRNA levels were calculated by a four-parameter logistic equation. The probe for aP2 was obtained from Dr. David Bernlohr (University of Minnesota, Minneapolis, MN). To examine lipid accumulation during adipogenesis, confluent 3T3-L1 preadipocytes were differentiated by incubating the cells with medium A supplemented with methylisobutylxanthine, dexamethasone, and insulin as previously described (48). The cells were fixed for 2 h with 10% formalin in isotonic phosphate buffer followed by washing with water. The cells were then stained by complete immersion in a working solution of Oil red O for 2 h. Excess dye was removed by exhaustive washing with water. Oil red O staining was also used to assess lipid content in fully differentiated cells after 48 h exposure to compounds.

Assessment of Receptor Conformation by Partial Protease Digestion

The pSG5-hPPARγ2 plasmid was used to synthesize [^{35}S]-radiolabeled hPPARγ2 in a coupled transcription/translation system according to the protocol of the manufacturer (Promega Corp., Madison, WI). Transcription/translation reactions were subsequently aliquoted into 22.5 μl vol and 2.5 μl of PBS ± compound was added. These mixtures were incubated for 20 min at 25 C, separated into 4.5 μl aliquots and 0.5 μl of deionized (d) H_2O or dH_2O-solubilized trypsin was added. Protease digestions were allowed to proceed for 10 min at 25 C, then terminated by the addition of 95 μl of denaturing gel loading buffer and boiling for 5 min. The products of the digestion were separated by electrophoresis through a 1.5 mm, 4–20% SDS-PAGE (Invitrogen). After electrophoresis, the gels were fixed in 10% acetic acid (vol/vol): 40% methanol (vol/vol) for 30 min, treated in EN^{3}HANCE (NEN Life Science Products, Boston, MA) for a further 30 min and dried under vacuum for 2 h at 80 C. Autoradiography was then performed to visualize the radiolabeled digestion products.

NMR Spectroscopy

For the production of ^{15}N, ^{13}C, and D_2O-labeled GST-hPPARγ LBD, BL21 *E. coli* cells (Stratagene, La Jolla, CA) hosting pGEX-hPPARγ LBD were grown in modified M9 minimal medium with 1 g/liter ^{15}*N*-ammonium sulfate, 5 g/liter [^{13}C]glucose, and 60% D_2O. Isopropyl-1-thio-β-D-galactopyranoside-induced culture was used for GST fusion protein purification. Following cleavage with thrombin, hPPARγ LBD was further purified via gel filtration chromatography. Samples for NMR spectroscopy were at a concentration of 0.3 mM in a buffer consisting of 25 mM sodium phosphate at pH 7.0 in 90% H_2O, 10% ^2H_2O. NMR data were collected at 23 C on a Varian Inova 600 NMR system equipped with a triple-resonance 5-mm probe with a z-axis gradient coil. The sample volume was 250 μl, and the NMR tubes were fitted with Ultem susceptibility plugs (Wilmad Glass, Buena, NJ). Because most three-dimensional HNCO spectra cross-peaks in

Table 3. Primer/Probe Sets Used for the Amplification Step of Quantitative Fluorescent Real-Time PCR

Gene	5′ Primer (5′–3′)	3′ Primer (5′–3′)	Probe (5′–3′)
Acrp30	TGTTGGAATGACAGGAGCTGAA	CACACTGAACGCTGAGCGATAC	CATAAGCGGCTTCTCCAGGCTCTCC
ACS	CGGCAGTACGTGCGCA	CGCTGCAAAAGCCCCA	CCTTCCAACCAACACCCTCATGGG
aP2	GAATTCGATGAAATCACCGCA	CTCTTTATTGTGGTCGACTTTCC	ACGACAGGAAGGTGAAGAAGCAT-CATAAC
CAP	TGGAATATGGAGAAGCTATTGCTAAGT	TGCCTTGTCGGGATGTCC	TGAGAGGATCACACTGCTCCGGCA
CD36	TGGAGATTACTTTTTCAGTGCAGAA	TCCAGCCAATGCCTTTGC	TCACCCCTCCAGAATCCAGACAACCA
FATP	TTCTGCGTATCGTCTGCAAGA	CGCAGCTCTAGCCGAACAC	CGAGACCTCTTTGGCCTCTCTGT-TCTGA
Leptin	CCAAAACCCTCATCAAGACCA	AGTCCAAGCCAGTGACCCTCT	ATTTCACACACGCAGTCGGTATCCGC
LPL	CCCTACAAAGTGTTCCATTACCAA	CTCGCTCTCGGCCACTGT	CAAGCAACACAACCAGGCCTTCGAAA
mCPT1	AATATGTCTACCTCCGAAGCAGGA	CGTGAACGGCATTGCCTAG	CAACTATTATGCCATGGATTTTGTGCT-TATTAAGAACACA
PEPCK	AAATCCGGCAAGGCGC	TCTGGTGCCACCTTTCTTCC	CAGCGATCTCTGATCCAGACCTTC-CAA
TNFα	CCAGGCGGTGCCTATGTCT	GAAGAGCGTGGTGGCCC	AGCCTCTTCTCATTCCTGCTTGTGGCA

the PPARγ LBD-rosiglitazone complex have been assigned, missing peaks in the apo and nTZDpa forms are presumed to be missing due to slow conformational dynamics of the protein (26). The figure shown was made with the MolMol molecular graphics program (49).

Quantitation of 3T3-L1 Adipocyte Gene Expression Using Affymetrix Arrays

3T3-L1 cells (ATCC; passages 3–9) were grown to confluence and differentiated into adipocytes as described above. At d 8 after the initiation of differentiation, adipocytes were incubated in medium A ± compounds for 24 h. The dose used for each compound was 20-fold above the EC_{50} value for that compound in the 3T3-L1 cell differentiation assay described above (data not shown); this strategy was used to saturate and maximally activate PPARγ expressed in the cell. The compounds and concentrations used were as follows: TZDfa (20 nM), rosiglitazone (360 nM), pioglitazone (5000 nM), nTZD5 (6 nM), nTZDpa (5000 nM). After treatment, total RNA was prepared from the adipocytes as described above. Hybridization samples from the 3T3-L1 adipocytes were prepared and hybridization to Affymetrix (Santa Clara, CA) MG-U74Av1 oligonucleotide microarrays, was performed according to Affymetrix instructions as described by Lockhart *et al*. (50). Data from each microarray were normalized to data from a single vehicle microarray using global scaling based on overall hybridization intensities. Normalization, assessments of replicates, and calculations of gene expression levels as average difference values were performed using GeneChip version 3.1 and Data Mining version 1.2 software (Affymetrix). Each treatment was represented by two replicate samples using two microarrays. Two-dimensional hierarchical clustering of the compounds *vs.* gene expression was performed using the Agglomerative clustering program within Resolver (Rosetta, Kirkland, WA). Genes included in the clustergram were selected as the intersection of all four full agonists (fold change $1.5\times$ and $P < 0.1$; 129 genes). Two-dimensional clustering of data derived from 5 ligand treatments was then performed on these 129 genes. This clustergram is presented in *Results*. See the supplemental data at The Endocrine Society's Journals Online web site at http://mend-endojournals.org.

Quantitation of Gene Expression by Quantitative Fluorescent Real-Time PCR

Total RNA was prepared from 3T3-L1 adipocytes or C57BL/6J mouse EWAT using the methods described above. Specific mRNAs were quantitated using quantitative fluorescent real-time PCR. RNA was first reverse transcribed using random hexamers in a protocol provided by the manufacturer (PE Applied Biosystems, Foster City, CA). Amplification of each target cDNA was then performed with TaqMan PCR Reagent Kits in the ABI Prism 7700 Sequence Detection System according to the protocols provided by the manufacturer (PE Applied Biosystems). Primer/probe sets were selected using the Primer Express program (PE Applied Biosystems) and were synthesized by the same company. The levels of mRNA were normalized to the amount of 18S ribosomal RNA (primers and probes commercially available from PE Biosystems) detected in each sample. The primer/probe sets used for the amplification step are shown in Table 3.

In Vivo Methods

Male C57BL/6J mice at age 4 wk were purchased from The Jackson Laboratory (Bar Harbor, ME). They were housed three per cage in a temperature-controlled room (22–23 C) with a 12-h light, 12-h dark cycle. The mice were randomly assigned to a control or treatment groups (n = 12). One group (LF) was fed a LF diet (11% kcal fat from fat). A second group (HF) was fed a HF diet (58% kcal from fat) (40). The treated groups (HF + TZDfa and HF + nTZDpa) were fed the HF diet with the PPARγ ligands TZDfa or nTZDpa incorporated into the diet at the time of manufacture (0.0353 and 0.5883 mg/g diet, respectively). Based on historic food consumption data, calculated daily doses of TZDfa and nTZDpa were approximately 3 and 50 mg/kg·d, respectively. The mice were treated for 13 wk. Body weights and food intake were determined throughout the experiment until its termination at 91 d. Blood samples were collected from nonanesthetized animals. Food was removed 8 h before sample collection. Plasma was collected and frozen at −70 C until analyzed. Plasma glucose concentration was measured using the glucose oxidase method (Beckman Coulter, Inc., Brea, CA; Glucose Analyzer II). Insulin and leptin concentrations were assessed by RIA (Linco Research, Inc., St. Louis, MO). Triglycerides and free fatty acids were assayed with Wako diagnostic kits according to the directions of the manufacturer (Wako Chemicals USA, Richmond, VA). The intrascapular brown, mesenteric, inguinal, and epididymal fat pads were carefully dissected according to defined anatomical landmarks and weighed. Hearts were also removed then weighed and returned to the carcass.

To determine body composition of the animals, carcasses were completely digested by incubation in acidic ethanol for 48 h at 60 C as previously described (51). The glycerol content of the digests was determined using the Glycerol Enzymatic

Analysis kit according to the manufacturer's directions (Roche Molecular Biochemicals, Indianapolis, IN). This data was used to calculate the percent body lipid. Protein content in the digest was determined using the DC Protein Assay kit according to the directions of the manufacturer (Bio-Rad Laboratories, Inc., Hercules, CA) and was used to calculate percent body protein.

Statistical Analysis

Data are expressed as the mean ± SEM. Statistical significance was determined using the least significant difference test for data in Fig. 8 and Table 2 and the Student's *t* test for data in Figs. 7B and 9.

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