Multi-tissue, selective PPARγ modulation of insulin sensitivity and metabolic pathways in obese rats

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MATERIALS AND METHODS

Animal studies. Male Zucker fatty (fa/fa) and lean (fa/+) rats (Charles River, Wilmington, MA) were received at 6 wk of age, were housed individually under controlled light (12:12-h light-dark) and unknown. Thiazolidinediones (TZDs) are clinically potent insulin-sensitizing drugs. These compounds are ligands of and alter gene transcription via the nuclear receptor peroxisome proliferator-activated receptor-γ (PPARγ). PPARγ and its ligands are transcriptionally active in insulin target tissues (liver, adipose, muscle, macrophages). Studies of tissue-specific PPARγ knockout mice and in insulin resistant, obese Zucker fa/fa rats show that PPARγ regulates metabolism and insulin action in each of these tissues (7). Although hundreds of TZD target genes have been identified, it is unclear which TZD-induced gene expression changes specifically induce insulin sensitization. The coordinated functional and metabolic pathway changes that occur as a consequence of gene expression change during insulin sensitization have also not been characterized.

Insulin target tissues depend on PPARγ for their proper metabolic functions. Ordinarily, this is modulated by tissue-specific endogenous ligands and transcriptional regulators, resulting in tissue-specific PPARγ-regulated expression profiles. Thus, tissue-specific PPARγ target gene regulation is coordinated to maintain whole body insulin sensitivity and normal metabolic homeostasis. PPARγ can bind to and is differentially regulated by many synthetic and natural ligands. Binding of various ligands induces ligand-specific three-dimensional structures to PPARγ, conferring ligand-specific interactions of PPARγ with transcriptional regulators and DNA and imparting ligand-specific transcriptional activity (2, 4, 10, 39). This concept is described by the SPPARM (selective PPAR modulator) model (30). Some insulin-sensitizing PPARγ ligands are full agonists, activating transrepression and transactivation (34); some are primarily partial agonists, e.g., activating transrepression only (11).

We (40) have shown in insulin-resistant human subjects that TZDs improve dysregulated expression of genes involved in specific skeletal muscle and adipose tissue metabolic pathways and, furthermore, that some of these pathway changes correlate with improved insulin sensitivity. We now report similar studies in insulin resistant, obese, nondiabetic Zucker (fa/fa) rats. Additionally, we show that the SPPARM concept can be manifested in vivo, demonstrating for the first time that ligand treatment-selective PPARγ modulation of insulin sensitivity correlates with ligand treatment-selective alteration of inflammatory and branched-chain amino acid (BCAA) metabolic pathway expression in adipose tissue.

INSULIN RESISTANCE is a pathophysiological state in which the intracellular signaling actions of insulin are blunted. It is manifested in multiple insulin target tissues, including adipose tissue, skeletal muscle, and liver. Each tissue must respond to insulin with specific metabolic outcomes to maintain normal glycemia and lipidemia. Altered expression of cellular components that ensure insulin’s tissue-specific metabolic outcomes is known to affect insulin sensitivity; however, the exact changes in gene expression that induce insulin resistance are open to study.
climate conditions, and had free access to food and water throughout all study conditions. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the University of California, San Diego, Animal Subjects Committee. Fatty rats were weight-matched upon arrival and randomly divided into one of five experimental groups. The fatty rat groups varied by the type of chow they were fed: normal chow alone or with a PPARγ ligand admixture (normal chow, fatty control; FC), rosiglitazone-treated (Rosiglitazone; 2 mg kg\(^{-1}\)day\(^{-1}\)), pioglitazone-treated (Pioglitazone; 10 mg kg\(^{-1}\)day\(^{-1}\)), troglitazone-treated (Troglitazone; 200 mg kg\(^{-1}\)day\(^{-1}\)), or AG-035029-treated (AG; 10 mg kg\(^{-1}\)day\(^{-1}\)). Drug doses were chosen for near-maximal insulin-sensitizing effect without nonspecific effects (approximate EC\(_{90}\) doses) (27, 50, 54). AG-035029 (22, 38) is an experimental PPARγ-selective ligand obtained from Pfizer, Inc., La Jolla, CA (see also DISCUSSION). Lean control (LC) rats were all fed normal chow. Rats groups were maintained on the diets for 21 days (LC n = 16, FC n = 17, AG n = 12, Pio n = 13, Rosi n = 12, Tro n = 13 rats per group), after which each of which were subjected to clamp and tissue collection (Table 1). Tissues were excised, flash-frozen in liquid nitrogen, and stored at \(-80^\circ\)C for subsequent analysis. Fatty rats were euthanized as described above) were harvested, frozen, and stored as described above. Tissue RNA was isolated using RNeasy kits (Qiagen, Valencia, CA). Biotinylated-cRNA made from pooled RNA preparations (RNA from two rats per preparation) was used to probe Affymetrix Rat Expression 230A GeneChips as per the Invitrogen protocol (Invitrogen, Carlsbad, CA), three arrays per group. Briefly, cDNA was synthesized with the SuperScript Choice system using a T7(dT)$_{24}$ oligomer (Ambion). Biotin-labeled cRNA probes were in vitro transcribed using the BioArray High Yield RNA Transcription Labeling Kit (Enzo Life Sciences). Gene expression levels, as expressed as average difference scores, were determined using Affymetrix MAS 5.0 software. Data files are deposited in the Gene Expression Omnibus (GEO) database as series GSE21329.

**Statistical analyses.** Biochemical characteristics were analyzed using analysis of variance (ANOVA), Tukey’s post hoc test, and a statistical cutoff of \(P < 0.05\). We applied our Variance Modeled Posterior Inference With Regional Exponentials (VAMPIRE) (21) microarray analysis web suite (20) to the gene expression data to identify statistically significant differences between rat group gene expression profiles. Variance-stabilized ANOVA (using variance estimates computed by VAMPIRE) was used to identify ligand-selective modulation of gene expression. Spearman rank correlation coefficients (\(\rho\)) were calculated to identify genes whose ligand-specific expression was correlated with the ligand’s insulin-sensitizing potency. We identified significantly enriched Gene Ontology (v. 200704) and KEGG pathway annotation terms in gene expression data sets using戈by (20). We corrected for familywise error associated with multiple comparisons (accounting for the number of probe sets or genes examined) using the stringent Bonferroni error rate cutoff of 5%, i.e., significance level cutoff, \(\alpha_{\text{bonf}} = 0.05\).

**RESULTS**

Characterization of lean, obese, and PPARγ ligand-treated obese rats. We analyzed the whole body insulin sensitivity and tissue-specific transcriptional profiles of six age-matched Zucker rat groups: LC and FC rats or FC rats fed chow containing one of four insulin-sensitizing PPARγ ligands, AG, Pio, Rosi, or Tro, for 21 days (see MATERIALS AND METHODS). Physical characteristics of the rat groups are shown in Table 1. The FC rats had significantly higher body weight, hyperinsulinemia, and hypertriglyceridemia than the LC rats. Body weight of the PPARγ ligand-treated fatty rats was not significantly different from that of the FC rats. Both hyperinsulinemia and hypertriglyceridemia were significantly blunted in all the PPARγ ligand-treated groups compared with the FC rats. In fact, fasting insulin and triglyceride (TG) levels in the PPARγ ligand-treated groups were not significantly different from the those in the LC group, although they tended to be higher. Fasting glucose levels in the FC rats tended to be higher than but were not significantly different from the LC group. AG- and Tro-treated rats exhibited significantly decreased fasting glucose levels compared with the FC group. Basal FFA levels were significantly different between the groups, with the Pio- and AG-treated groups having the lowest average levels.

We determined the whole body insulin sensitivity of the rat groups by using hyperinsulinearic-euglycemic clamp studies (Table 1 and Fig. 1). Both insulin-stimulated glucose disposal rate (IS-GDR) and total GDR were markedly impaired in the FC rats, indicating skeletal muscle insulin resistance (vs. the LC group). Skeletal muscle insulin resistance in fatty rats was ameliorated to varying degrees by PPARγ ligand treatment. AG treatment was significantly more potent than the other ligand treatments in improving insulin sensitivity in muscle;
# Table 1. Rat group characteristics and corresponding rankings of PPARγ treatment ligand potencies

<table>
<thead>
<tr>
<th>Ligand Treatment</th>
<th>ANOVA P Value</th>
<th>Body weight, g</th>
<th>Food intake, g/day</th>
<th>Arterial glucose, mg/dl</th>
<th>Plasma insulin, ng/ml</th>
<th>Steady-state clamp</th>
<th>Triglycerides, mg/dl</th>
<th>FFA, μmol/ml</th>
<th>GDR, mg·kg⁻¹·min⁻¹</th>
<th>%Suppression during clamp</th>
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<tr>
<td></td>
<td></td>
<td>LC n=8</td>
<td>FC n=8</td>
<td>H11005</td>
<td>Pio n=7</td>
<td>Rosi n=6</td>
<td>Tro n=7</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>0.000</td>
<td>236.6 ± 4.5†</td>
<td>379.4 ± 15.1</td>
<td>402.7 ± 6.1</td>
<td>415.7 ± 6.6</td>
<td>418.7 ± 6.6</td>
<td>419.7 ± 6.6</td>
<td>130.0 ± 3.6</td>
<td>2.80 ± 0.30</td>
<td>38.6 ± 3.0†</td>
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<tr>
<td>Steady-state clamp</td>
<td>0.002</td>
<td>n.d.</td>
<td>29.1 ± 3.5†</td>
<td>45.7 ± 1.6*</td>
<td>39.6 ± 1.2*</td>
<td>42.3 ± 3.8*</td>
<td>n.d.</td>
<td>3.2 ± 0.3</td>
<td>4.2 ± 0.3</td>
<td>3.8 ± 0.3†</td>
</tr>
<tr>
<td>Basal</td>
<td>0.005</td>
<td>117.8 ± 3.6</td>
<td>136.7 ± 7.2†</td>
<td>105.2 ± 2.7*</td>
<td>124.8 ± 3.7</td>
<td>125.5 ± 9.7</td>
<td>111.2 ± 4.2*</td>
<td>3.7 ± 0.6</td>
<td>3.7 ± 0.6</td>
<td>3.7 ± 0.6†</td>
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<td>22.1 ± 4.9†</td>
<td>2.7 ± 0.20*</td>
<td>4.8 ± 1.26*</td>
<td>9.2 ± 3.31*</td>
<td>3.7 ± 1.06*</td>
<td>AG &gt; Pio &gt; Rosi</td>
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<td>Basal</td>
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<td>42.0 ± 7.64</td>
<td>51.7 ± 4.60</td>
<td>54.4 ± 6.26</td>
<td>58.8 ± 6.61</td>
<td>AG &gt; Pio &gt; Rosi</td>
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<td>46.86 ± 2.00*</td>
<td>30.63 ± 2.72†</td>
<td>24.82 ± 1.70†</td>
<td>36.84 ± 3.04*</td>
<td>AG &gt; Pio &gt; Rosi</td>
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<tr>
<td>Basal</td>
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<td>52.61 ± 1.77*</td>
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<td>43.56 ± 2.27†</td>
<td>36.30 ± 2.32†</td>
<td>44.76 ± 2.15†</td>
<td>AG &gt; Pio &gt; Rosi</td>
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<td>96.2 ± 11.5*</td>
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<td>141.4 ± 24.3†</td>
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<td>29.8 ± 4.4*</td>
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<tr>
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<td>32.80 ± 19.1†</td>
<td>80.20 ± 2.8*</td>
<td>67.70 ± 6.50</td>
<td>64.80 ± 6.70</td>
<td>74.60 ± 2.8*</td>
<td>AG &gt; Pio &gt; Rosi</td>
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</table>

Data are means ± SE. ANOVA P values for each characteristic and post hoc statistics for comparisons to fatty control (FC) and AG-035029-treated (AG) groups are shown. Potency ranking established using average values compared with FC. GDR, glucose disposal rate; IS-GDR, insulin-stimulated GDR during clamp; FFA, free fatty acids; HGO, hepatic glucose output; Basal, fasted preclamp state; LC, lean control group; Pio, pioglitazone-, Rosi, rosiglitazone-, and Tro, troglitazone-treated fatty groups; NS, not significant. *Significant vs. FC group, P < 0.05; †significant vs. AG group, P < 0.05.
Rosiglitazone (Ros) treatment had the weakest effect. Insulin sensitivity in the liver was impaired in the FC rats and was near normal in the PPARγ ligand-treated groups. Although basal and insulin-suppressed HGO was not significantly different in the groups, insulin-induced suppression of TG levels at steady state during the clamp was severely blunted in the FC rats compared with the LC group. Insulin-induced TG suppression was improved and steady-state TG levels significantly reduced in each of the ligand-treated groups. Insulin-induced FFA suppression, a marker of insulin sensitivity in adipose tissue, was significantly different among the groups. Each ligand-treated group exhibited better than average FFA suppression than the FC group, with the AG-treated group exhibiting significant improvement.

Although all the PPARγ ligand treatments improved aspects of insulin resistance in the fatty rats, they did not exhibit uniform potency in all physiological outcomes. We generated ligand treatment potency rankings for each physiological characteristic by comparing the average values for each PPARγ ligand treatment (Table 1). AG treatment was the most potent for every characteristic shown; Rosi treatment was the least potent for most. We sought to determine whether the insulin-sensitizing potency of the ligands was related to their transcription-altering potency in insulin target tissues. We generated genome-wide, microarray gene expression profiles from epididymal white adipose tissue, liver, and gastrocnemius skeletal muscle harvested from unclamped animals from each of the experimental groups. We generated lists of genes that were differentially expressed between the FC, LC, pooled PPARγ ligand treatment (Rx), and individual treatment (AG, Pio, Rosi, Tro) profiles. Next, we identified enriched gene ontology and KEGG pathway annotations associated with the differentially expressed genes. This functional analysis enabled our identification of condition-associated transcriptional alterations in specific biochemical pathways. In addition, we identified ligand treatment-selective modulation of gene expression in the PPARγ ligand-treated groups by using ANOVA. Table 2 shows a list of each analysis’ comprehensive results files. Differential expression, annotation enrichment, and ligand treatment selectivity were all determined using stringent statistical tests of significance (see MATERIALS AND METHODS).

Selective PPARγ ligand modulation of inflammatory markers in adipose tissue correlates with insulin sensitization.

We compared adipose tissue expression profiles from lean and fatty control rats (LC vs. FC) and identified differentially expressed genes (Supplementary 1.AT) (Supplementary materials are found with the online version of this paper at the Journal website). Our functional analysis of this gene set identified significantly enriched biochemical pathway terms, including the immune-related ontology terms “immune system process,” “leukocyte-mediated immunity,” and others (Supplementary 2.AT).

Many (47/78) of the immune-related genes were up-regulated in the FC group, including activated leukocyte cell adhesion molecule (Alcam, 2.6-fold), Cd4 (10.4-fold), Cd40 (7.4-fold), protein tyrosine phosphatase receptor (Ptprc, aka Cd45, 1.9-fold), and sialophorin (Spn, aka Cd43, 2.6-fold). The enrichment of immune system-related genes indicates the presence of antigen-presenting cells in adipose tissue from the insulin-resistant, obese rats. PPARγ ligand treatment of the obese rats induced the differential expression of 732 genes in adipose tissue. Genes downregulated by ligand treatment included immune cell markers (Cd68 and Cd74) and genes involved in macrophage transendothelial infiltration [chemokines Ccl6, Cxcl1, Cxcl12, Cxcl14, and Spp1 (aka Opm, see Ref. 5)], cell adhesion (Cd24, Cd44, Icam1, Lsp1), and matrix remodeling (Mmp2) (examples in Fig. 2). Overexpression of nearly all (43/47) of the immune-related genes in FC rats was decreased in the ligand-treated rats. Of these 43 genes, 36 were normalized to LC group levels (examples in Fig. 2; all data in Supplementary 3).

We compared the individual ligand treatment expression profiles to identify PPARγ ligand treatment-selective gene and pathway regulation. More than 700 genes were selectively modulated by the individual ligand treatments (Supplementary 1.AT and Supplementary 4), meaning that their transcriptional responses were significantly different for the different ligand treatments. Functional analysis of this gene set revealed enrichment of many inflammation-related terms including the KEGG pathway term “cell adhesion molecules” and the ontology term “immune system process” (Supplementary 5), showing that the PPARγ ligand treatments modulated these bio-

![Fig. 1. PPARγ ligand treatments improve parameters of insulin sensitivity.](https://example.com/fig1)

**Table 2. List of supplemental data**

| Supplementary 1 | Differential gene expression in AT, SM, and L |
| Supplementary 2 | GOby reports of the differential gene expression in AT, SM, and L |
| Supplementary 3 | Immune-related genes overexpressed in FC AT that are decreased in ligand-treated rats |
| Supplementary 4 | Ligand-selectively modulated genes in AT, SM, and L |
| Supplementary 5 | GOby reports of ligand-selectively modulated genes in AT, SM, and L |
| Supplementary 6 | Gene expression correlations with GDR, %FFA suppression, and %TG suppression |
| Supplementary 7 | GOby reports from the list of genes from AT whose expression levels correlate with GDR and/or %FFA su |
| Supplementary 8 | Differentially regulated genes in AT involved with mitochondria-associated metabolism |

AT, adipose tissue; SM, skeletal muscle; L, liver.
chemical pathways to varying degrees. Next, we correlated the individual ligand treatment expression profiles with their matched insulin-sensitizing potency rankings with respect to GDR and FFA suppression (Supplementary 6). We subjected the top positively and negatively correlated genes (Spearman, ρ > 0.79) from each correlation to functional analysis. Immune-related ontology and KEGG terms were enriched in the list of genes negatively correlated with GDR and FFA suppression (Supplementary 7); i.e., expression of immune-related genes negatively correlated with ligand treatment insulin-sensitizing potency. Thirty-one percent (29/93) of the immune-related genes overexpressed in FC adipose tissue and selectively modulated by the ligand treatments were most potently downregulated by the AG treatment, the most effective muscle and adipose tissue insulin sensitizer. The Rosi treatment, the least effective muscle and adipose tissue insulin sensitizer, was the weakest ranking transcriptional regulator for 57% (53/93) of these genes. Single gene examples of this pattern are shown in Fig. 2 (Cd74, Cxcl14, Spp1). These results suggest a functional relationship between ligand treatment-specific insulin-sensitizing potency and modulation of inflammation-related biochemical pathways. Furthermore, there seems to be a functional relationship between the degree of adipose tissue inflammation and the degree of insulin resistance in muscle and adipose tissue.

**PPARγ ligands increase adipose tissue expression of mitochondrial metabolic pathways and modulate BCAA metabolism with ligand treatment-specificity.** Whereas expression of immune-related genes was decreased predominantly in the adipose tissue of PPARγ ligand-treated rats, expression of genes involved in mitochondrial metabolic pathways was mostly increased (Supplementary 8). Functional analysis of the genes differentially expressed between FC and PPARγ ligand-treated rats (Supplementary 2.AT) revealed enrichment for the term “oxidative phosphorylation” (OXPHOS), represented by 33 upregulated electron transport chain genes (Fig. 3A). The terms “citrate cycle (TCA cycle),” represented by 25 genes, and “valine, leucine, isoleucine degradation” (BCAA degradation), represented by 22 genes, were also enriched (most of the pathway genes are represented in Fig. 3B). Interestingly, none of the genes annotated with the terms OXPHOS, TCA cycle, and/or BCAA degradation (except for Mel) were differentially expressed between FC and LC rats. These pathway genes were differentially expressed only after PPARγ ligand treatment, resulting in levels exceeding those observed in LC rats (Supplementary 8).

Most PPARγ ligand-regulated genes associated with TCA cycle and OXPHOS pathways (54 of 58) were similarly modulated by the individual ligand treatments. Only five genes were ligand treatment-selectively modulated, one in TCA cycle and four in OXPHOS (Supplementary 8; see also 3 yellow-outlined ovals in Fig. 3A). In contrast, the BCAA degradation pathway was markedly enriched with ligand treatment-selectively modulated genes, 10 of 22 genes (Supplementary 8, including yellow-outlined ovals in Fig. 3B); i.e., the PPARγ ligand treatments modulated this pathway to varying degrees (see also Supplementary 8). Six of the 10 selectively regulated, BCAA-annotated genes (Acadsb, Aldh1a7, Aldh6a1, Ehhadh, Ivd, and Pecq) were most potently regulated by the AG treatment, the most potent insulin sensitizer; the Rosi treatment, the least effective insulin sensitizer, was the least potent regulator for four of the 10 genes (Echs1, Ehhadh, Hmgcs1, and Pecq). Induction of Ehhadh and Pecq expression was perfectly correlated to ligand treatment-specific insulin-sensitizing potency (Spearman, ρ = 1, Fig. 3C, all correlations shown in Supplementary 6). Thus, our data show that ligand treatment-selective induction of the BCAA pathway is correlated with ligand treatment insulin-sensitizing potency and suggests that increased flux through this pathway is related to PPARγ ligand treatment-induced insulin sensitization. Although one-third (148/454) of the ligand treatment-induced adipose tissue genes were annotated with mitochondria-related terms, we think it unlikely that PPARγ ligand treatment simply increases overall mitochondrial density. The TCA cycle and OXPHOS pathways were uniformly induced, not correlated with insulin sensitization, and the BCAA pathway was ligand treatment-selectively induced and correlated with insulin sensitization.

**Elevated expression of adipocyte and type I (slow-twitch) fiber genes in insulin-resistant skeletal muscle is enhanced after PPARγ ligand treatment.** We identified 33 differentially regulated genes in skeletal muscle expression profiles from the FC and LC groups (Supplementary 1.SM). Interestingly, expression of insulin-like growth factor (Igf1) and PPARγ (Pparg) was significantly blunted in FC rats. These two genes are important modulators of insulin sensitivity in skeletal muscle (9, 15, 23). Several overexpressed genes in skeletal muscle of the FC rats are adipocyte markers (Table 3), including Csd3, fatty acid synthase (Fasn), and stearoyl-coenzyme A desaturase (Scd1), which have roles in fatty acid trafficking and synthesis. Eighty-nine genes were differentially expressed in skeletal muscle from PPARγ ligand-treated compared with FC.
rats. We used functional analysis to identify muscle biochemical pathway alterations associated with PPARγ ligand treatment. Several lipid metabolism ontology categories were enriched (Supplementary 2.SM). All the genes annotated with lipid metabolism-related terms, as well as other related genes, were upregulated by ligand treatment (37 genes). Most of these are adipocyte-associated genes (25/37, shown in Table 3) including acetyl-coenzyme A carboxylase-α (Acaca), a fatty acid elongase (Elov6), fatty acid-binding proteins (Fabp4, Fabp5), hormone-sensitive lipase (Lipe), lipoprotein lipase (Lpi), and retinol-binding proteins (Rbp4, Rbp7). Pparg, highly expressed in adipocytes, was also increased (5.6-fold) after ligand treatment. Interestingly, genes overexpressed in FC vs. LC rats (Angptl4, Cd36, Fasn, and Scd1) were further overexpressed in the PPARγ ligand-treated rats. Muscle tissue expression of adipocyte markers was induced by all the PPARγ ligand treatments, and all the genes exhibited ligand treatment-specific modulation, except for Pparg (Supplementary 1.SM).

Fig. 3. PPARγ ligand treatments increase expression of mitochondria-associated metabolic pathways in adipose tissue. Selected genes upregulated in ligand-treated adipose tissue are shown for the oxidative phosphorylation (A) and TCA cycle and BCAA degradation metabolic pathways (B). Red ovals, genes upregulated in PPARγ ligand-treated vs. FC groups. Unfilled ovals, no gene expression difference comparing ligand-treated and FC groups. Yellow-outlined ovals, genes exhibiting ligand-selective expression patterns (ANOVA, $P < 0.05$). Figures adapted from KEGG. C: examples of BCAA degradation pathway genes exhibiting ligand-selective expression that perfectly correlates with ligand treatment potency (Spearman, $p = 1$). Data are averages normalized to LC group ± SE. ANOVA $P < 0.05$ for each. *$P < 0.05$ vs. FC group.
Remarkably, ligand treatment-specific expression of the adipocyte markers did not correlate with ligand treatment-induced insulin sensitization (Supplementary 6). For example, Tro treatment was the most potent inducer of adipocyte marker expression but was significantly less effective than the AG treatment as an insulin sensitizer. These results suggest that PPARγ ligand treatment-selective modulation, which was not correlated with ligand treatment insulin-sensitizing potency. Overall, our observations in skeletal muscle profiles suggest that insulin resistance is associated with moderate remodeling of the skeletal muscle, involving increased adiposity and slow-twitch muscle fibers, and that this remodeling is further amplified after PPARγ ligand treatment.

PPARγ ligand treatment normalizes overexpression of liver lipogenic genes associated with insulin resistance. Comparison of liver expression profiles revealed 126 genes differentially expressed between the FC and LC groups (Supplementary 1.L). This gene set was enriched for annotation terms including the “biosynthesis of fatty acids” and “biosynthesis of steroids” (Supplementary 2.L). All genes annotated with “biosynthesis of fatty acids” were upregulated (Acaca, Acols5, Fasn), as were other related genes (Elov6, G6pdx, Mels, Scd1). Correspondingly, expression of sterol regulatory element-binding transcription factor 1 (Srebf1) was overexpressed 2.3-fold in FC rats (Fig. 4A). Srebf1 encodes Srebp1, a transcription factor that positively regulates fatty acid synthesis (17) and directly
regulates the expression of all the aforementioned genes (18, 33). In contrast, genes involved in cholesterol synthesis were underexpressed in FC rats.

*Srebf2* encodes *Srebp2*, a transcription factor that preferentially regulates cholesterol and steroid biosynthesis genes (17). *Srebf2* expression was slightly blunted in FC rats (fold change 0.879). Although downregulation of *Srebf2* itself did not meet our stringent statistical threshold, expression of several *Srebf2* target genes (*Cyp51, Idi1, Sc4mol, Sqle*) was significantly decreased. Figure 4B shows the aberrant regulation of *Srebf1* and *Srebf2* target genes in insulin-resistant, obese rats and how this would alter fatty acid and cholesterol synthesis.

*Srebf1* expression in PPARγ ligand-treated fatty rats was normalized down to LC levels and was not different between the ligand treatment groups (Fig. 4A). Expression of several *Srebf1* target genes associated with lipogenesis was also decreased by at least one of the ligand treatments (*Elov15, Fasn, G6pdx, Mel, Scd1*; green-outlined ovals, in Fig. 4B and Supplementary 1.L). Of these, *G6pdx* expression was completely normalized to LC levels (Supplementary 1.L). Ligand treatment increased pyruvate dehydrogenase kinase-4 (*Pdk4*) expression. In its active state, PDK4 protein inhibits pyruvate dehydrogenase complex conversion of pyruvate into acetyl-CoA, effectively preventing the accumulation of the fatty acid precursors. Blunted cholesterol synthesis gene expression in FC rats was increased by PPARγ ligand treatment (*Cyp51, Idi1, Sc4mol, Sqle*; red-outlined ovals, in Fig. 4B and Supplementary 1.L) with *Cyp51, Sc4mol*, and *Sqle* expression normalized to LC levels. Improved expression of these genes was selectively modulated by the PPARγ ligand treatments (see asterisks in Fig. 4B, Supplementary 1.L and Supplementary 4).

Using data from each ligand treatment group, we correlated expression of these genes with indexes of ligand treatment insulin-sensitizing potency (i.e., GDR and TG suppression) (Supplementary 6). Expression of *Fasn* exhibited a perfect negative correlation with ligand treatment potency (Spearman, $r = -1$). Expression of *Sc4mol*, a cholesterol synthesis gene, exhibited a perfect positive correlation with ligand treatment potency (Spearman, $r = 1$). Expression of most of these genes was not correlated with ligand treatment potency. The Tro treatment was the most potent transcriptional regulator for the majority of the selectively modulated genes (7 of 11) but was not the most effective insulin sensitizer. Thus, PPARγ ligand treatment-induced reversal of elevated fatty acid and blunted cholesterol synthesis pathway expression are unlikely the primary mechanisms by which PPARγ ligands modulate insulin sensitivity unless mediated by expression changes in the individual genes *Fasn* and *Sc4mol*.

**DISCUSSION**

PPARγ ligands have been used extensively to study physiological and molecular changes affecting insulin sensitivity (53). Nonetheless, little is known about how PPARγ ligand treatment-specific modulation of cellular mechanisms correlates with gradations of insulin sensitization. Our studies have addressed this question and reveal mechanistic actions shared by PPARγ ligands and, importantly, mechanistic actions that
are functionally related to insulin sensitization. Although four different PPARγ ligand treatments improved whole body insulin sensitivity of obese rats in our study, they did so to varying degrees. The AG-035029 treatment ranked the most potent in insulin-sensitizing the rats; the rosiglitazone treatment ranked the least potent. AG-035029 (22, 38) is a PPARγ-activating ligand with in vitro activation profiles similar to that of rosiglitazone: 28-fold higher specificity for PPARγ over PPARα in HepG2/aP2-luciferase assays (PPARγ EC50 = 145 nM) with no detectable affinity for PPARα, full agonist activity with PGC-1, and potent activation of genes in 3T3-L1 adipocytes (EC50 = 33 vs. 20 nM with rosiglitazone; J. Chapman, data not shown). Using genome-wide gene expression profiling of adipose, skeletal muscle, and liver tissue, we identified functional pathway changes that correspond to insulin resistance, PPARγ ligand treatment, and insulin sensitization.

Chronic, low-grade inflammation is associated with and can cause insulin resistance. Macrophages infiltrate adipose tissue of obese animals and humans, where they secrete cytokines that interfere with insulin signaling (19, 30). Our results indicate increased immune cell residence in adipose tissue from obese, insulin-resistant rats. PPARγ ligand-induced repression of inflammatory marker genes in adipose tissue correlated with ligand-specific insulin sensitization potency; i.e., PPARγ ligand-induced repression was greatest in response to the most effective insulin-sensitizing treatment; repression was weakest in response to the least effective insulin-sensitizing treatment. We (40) have also shown that adipose tissue inflammation is inversely proportional to insulin sensitivity in human subjects. These findings suggest that suppressing the inflammatory state is central for reversing insulin resistance and are supported by published studies showing that disruption of inflammatory signaling in immune cells prevents high-fat diet-induced insulin resistance (1, 37, 42).

PPARγ ligand-induced mitochondrial capacity, gene expression, and mass have been observed in adipose tissue from insulin-resistant humans and rodents (3, 6, 35, 46, 52). Our studies extend previous findings and show that oxidative phosphorylation, TCA cycle, and BCAA degradation pathways are upregulated in ligand-treated fatty rats to levels significantly higher than in LC and FC rats. Furthermore, the degree of induction of the BCAA pathway correlated with the degree of PPARγ ligand treatment-induced insulin-sensitizing potency. Together, these results show that PPARγ ligands are not indiscriminately increasing mitochondria-associated metabolic pathways and suggest that insulin sensitivity is mechanistically linked to BCAA pathway flux. We (40) have observed a similar correlation between insulin sensitivity and adipose tissue BCAA degradation pathway expression in human subjects. BCAs are elevated in plasma of obese human and rodents, including Zucker fatty rats (8, 14, 29, 31, 41), and BCAA degradation pathway components are downregulated (41). Reversal of obesity by weight loss ameliorates these defects (26, 41). BCAAs may inhibit insulin sensitivity in peripheral tissues via activation of mTOR and serine phosphorylation of IRS proteins (24, 29, 47, 48). Our rat and human data suggest that PPARγ ligand-induced BCAA degradation in adipose tissue may serve as a compensatory metabolic shift that relieves BCAA accumulation and their insulin-desensitizing effects in the obese, overnutritive state.

Skeletal muscle profiles from insulin-resistant, obese rats exhibited elevated intramuscular adipocyte marker expression, which was further increased after ligand treatment. We observed the same phenotypes in insulin-resistant humans (40), and related observations have been reported (12, 13, 28, 32, 49). Intramuscular adipocyte expression of lipid-processing genes has the potential to improve myocyte sensitivity by alleviating local lipotoxicity and storing excess fatty acids (25, 32). Expression of slow-twitch oxidative muscle fiber genes was also increased after PPARγ ligand treatment. Studies have shown that increasing slow-twitch fibers in skeletal muscle can lead to improved insulin sensitivity (36, 51). In the liver, overexpression of Srebfl and its target genes supports that insulin resistance is characterized by increased hepatic lipogenesis (17). Liver overexpression of Srebfl causes increased expression of fatty acid synthesis genes and causes fatty liver (17). PPARγ ligand treatments in the obese rats uniformly reversed Srebfl overexpression back to LC levels, simultaneously repressing the lipogenesis program. This effect may be partly indirect due to concurrent lowering of plasma insulin levels, as Srebfl is an insulin target gene. However, fasting insulin levels did not correlate with liver expression of Srebfl.

The SPPARM (selective PPAR modulator) model describes the ability of heterogeneous ligands to impart specific three-dimensional structure and transcriptional activity to PPARγ (39, 40). We have demonstrated herein that SPPARM effects on gene expression and insulin sensitivity can be observed in vivo and in multiple tissues, even between ligands with structural similarity (e.g., rosiglitazone and pioglitazone). These effects varied by gene and tissue and, therefore, cannot be explained by drug dose and/or exposure; i.e., in some cases the ligand treatments elicited similar responses; in other cases the ligand treatments elicited significantly distinct responses. In liver, for example, Ghbpdx expression was similarly regulated by all the ligand treatments (no SPPARM; see RESULTS and Supplementary 1.L), but Fasn was differentially expressed and exhibited a perfect negative correlation with ligand treatment insulin-sensitizing potency (SPPARM; see RESULTS, Supplementary 1.L, and Supplementary 6). The rosiglitazone treatment was significantly more potent in activating expression of Mmp12, Lcn2, and G0S2 (and others) in adipose tissue, liver, and muscle, respectively, compared with the other ligand treatments, after which expression of these genes was unchanged (SPPARM; see Supplementary 1.AT, 1.L, and 1.M). We also observed many tissue-specific examples of gene-regulating efficacy; e.g., after rosiglitazone treatment, Lcn2 expression was upregulated 3.5-fold in muscle (Supplementary 1.M) but was unchanged in adipose tissue (Supplementary 1.AT). We observed that some SPPARM effects on gene expression were correlated with insulin sensitization and some were not, again indicating that the PPARγ ligands had variable potency for different outcome measures independent of drug dose or exposure. Importantly, we observed that SPPARM effects on adipose tissue inflammatory and BCAA degradation pathways had inverse and direct correlations with insulin sensitization potency, respectively. We identified SPPARM effects on skeletal muscle adipocyte and slow-twitch fiber marker expression, but these changes did not correlate with ligand insulin-sensitizing potency. In liver, we observed SPPARM effects on improved expression of Srebfl target genes, but this pattern also did not generally correlate with...
ligand-specific insulin-sensitizing potency. These findings in skeletal muscle and liver suggest that these ligand-induced alterations may be necessary, but not sufficient, for PPARγ ligand-induced insulin-sensitization.

In summary, our results reveal functional pathway alterations that correlate with obesity, PPARγ ligand treatment, and insulin sensitization. Interestingly, we observed a strong correlation between differentially modulated biochemical pathways and insulin sensitization potency only in adipose tissue. We have reported recently (44) that adipocyte-specific PPARγ activation is sufficient to mediate whole body insulin sensitivity. Although we have discerned important functional alterations in the skeletal muscle and liver, it is in the adipose tissue where pharmacological intervention seems to play a more significant role in whole body insulin sensitization.

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DISCLOSURES

The terms of the arrangement have been reviewed and approved by the University of California, San Diego in accordance with its conflict of interest policies. J. Chapman is an employee and shareholder of Pfizer and participated as a collaborator in sample and data analysis for the project.

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