N-Acetyl farnesylcysteine is a novel class of peroxisome proliferator-activated receptor \(\gamma\) ligand with partial and full agonist activity in vitro and in vivo* **

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The thiazolidinedione (TZD) class of drugs is clinically approved for the treatment of type 2 diabetes. The therapeutic actions of TZDs are mediated via activation of peroxisome proliferator-activated receptor \(\gamma\) (PPAR\(\gamma\)). Despite their widespread use, concern exists regarding the safety of currently used TZDs. This has prompted the development of selective PPAR\(\gamma\) modulators (SPPARMs), compounds that promote glucose homeostasis but with reduced side effects due to partial PPAR\(\gamma\) agonism. However, this also results in partial agonism with respect to PPAR\(\gamma\) target genes promoting glucose homeostasis. Using a gene expression-based screening approach we identified N-acetyl farnesylcysteine (AFC) as both a full and partial agonist depending on the PPAR\(\gamma\) target gene (differential SPPARM). AFC activated PPAR\(\gamma\) as effectively as rosiglitazone with regard to Adrp, Angptl4, and AdipoQ, but was a partial agonist of aP2, a PPAR\(\gamma\) target gene associated with increased adiposity. Induction of adipogenesis by AFC was also attenuated compared with rosiglitazone. Reporter, ligand binding assays, and dynamic modeling demonstrate that AFC binds and activates PPAR\(\gamma\) in a unique manner compared with other PPAR\(\gamma\) ligands. Importantly, treatment of mice with AFC improved glucose tolerance similar to rosiglitazone, but AFC did not promote weight gain to the same extent. Finally, AFC had effects on adipose tissue remodeling similar to those of rosiglitazone and had enhanced antiinflammatory effects. In conclusion, we describe a new approach for the identification of differential SPPARMs and have identified AFC as a novel class of PPAR\(\gamma\) ligand with both full and partial agonist activity in vitro and in vivo.

More than 20 million people in the United States have type 2 diabetes mellitus (T2DM). T2DM is a chronic metabolic dis-

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2 The abbreviations used are: T2DM, type 2 diabetes mellitus; AFC, N-acetyl farnesylcysteine; LBD, ligand binding domain; MEF, mouse embryonic fibroblast; PPAR\(\gamma\), peroxisome proliferator-activated receptor \(\gamma\); SPPARM, selective PPAR\(\gamma\) modulator; TZD, thiazolidinedione; WAT, white adipose tissue; DIO, diet-induced obesity.
flammary effects and reduced adipogenic activity compared with classic TZDs.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Chemicals**—3T3-L1 preadipocytes were obtained from ATCC. PPARγ knock-out mouse embryonic fibroblasts (MFPAPAR\(\gamma\)\(\text{KO}\)) were obtained from Dr. Evan Rosen (Beth Israel Deaconess Medical Center, Boston MA) (11). MFPAPAR\(\gamma\)\(\text{KO}\) were transformed with retrovirus-expressing empty vector or PPARγ and selected using puromycin as described previously (20). Cells were then treated with compounds as indicated, RNA was isolated, and RT-PCR was performed. All chemicals including the LOPAC library were obtained from Sigma or Alexis Biopharmaceuticals. Tnf-\(\alpha\) was purchased from R&D Systems. Cell culture media and supplements came from Cellgro (Mediatech), and FBS was from HyClone.

**Drug Screening**—3T3-L1 cells were seeded in a 96-well plate and grown overnight. The following day cells were treated with a 20 \(\mu\) M concentration of each compound for 24 h and RNA isolated using 96-well Turbo RNA isolation kit (Qiagen) according to the manufacturer’s directions. RT-PCR was performed using SYBR Green reagent (Applied BioSystems).

**RNA Isolation and Measurement of Gene Expression**—RNA was isolated from cells or tissues using TRIzol reagent (Invitrogen) and RNA reverse transcribed as described previously (20). RT-PCR was performed using the following primers: actin forward, 5'-CCA GTT GGT AAC AAT GCC ATG T-3' and reverse, 5'-GCC TGT ATT CCC CTC CAT CG-3'; aP2 forward, 5'-GTC ACC ATC CGG TCA GAG AG-3' and reverse, 5'-CTT GTG GAA GTC AGC CC-3'; Adip forward, 5'-ATC ACC AGG CTC TCA GCA GG-3' and reverse, 5'-ACT GTG CAT ATT CCT CCT GG-3'; Angptl4 forward, 5'-TCC AAC GCC ACC CAC TTA C-3' and reverse, 5'-AGA CAT CTC ACA GTT GAC CA-3'; AdipoQ forward, 5'-TAT CCC CCA CAA CAT CCG TCT CG-3' and reverse, 5'-TGG TAG GCA AAG TAG TAC AGC C-3'; Tnf-\(\alpha\) forward, CCC CCA CAC TCA GAT CAT CCT CT and reverse, GCC AGC AGC TGG GTC ACA G; Saa2 forward, TGG CTG GAA GAA TGG AGA CAA and reverse, AAA GCT CCT TCT GTC ACT ACT G; Saa3 forward, CTG TCC AGA AGT TCA CGG GAC and reverse, AGG AGC TGG GCA GTG CTG GTT G; Il6 forward, TAG TCC TTC CTA CCC CAA TTT CC and reverse, TTG GTC CTT AGC CAC CCC TCC TTT TC; Arg1 forward, 5'-CAG AAG AAT GGA AGA GTG AGC AG-3' and reverse, 5'-CAG ATA TGA AGG GAG TCA CC-3'; Clec7a forward, 5'-ATT TTG GCG ACA CAA TCG AGA G-3' and reverse, 5'-GCA AGA CTA GAG AAA ACC TCC T-3'; Itgax forward, 5'-CTG GAT GAC CTT TCT GCT G-3' and reverse, 5'-GCA CAC TGT GTC CGA ACT CA-3'. Expression was normalized to \(\beta\)-actin.

**Adipocyte Differentiation**—3T3-L1 cells were subjected to a differentiation protocol as described previously in the absence or presence of AFC or rosiglitazone (20). The level differentiation was by monitoring lipid accumulation as detected by Oil Red staining.

**Western Blot Analysis**—Western blotting for phosphorylated and nonphosphorylated PPARγ was performed as described previously by Hauser et al. (21). In brief, protein lysates from differentiated 3T3-L1 cells (described above) were separated on a 10% polyacrylamide gel containing 8 M urea and transferred to nitrocellulose. Immunoblotting for PPARγ was performed using a rabbit polyclonal antibody (H100; Santa Cruz Biotechnology). Phosphorylation of the CDK5 site on PPARγ at serine 273 was performed as previously described (22). Briefly, 3T3-L1 fibroblasts expressing PPARγ were pretreated with rosiglitazone, MRL24, or AFC with the indicated doses and then incubated with TNT-\(\alpha\). Cells were homogenized in lysis buffer, and phosphorylation of PPARγ at Ser-273 was analyzed with phospho-specific antibody against PPARγ Ser-273. Total PPARγ was analyzed with anti-PPARγ antibody (Santa Cruz Biotechnology). Western blotting for serine 112 (or 82 of PPAR\(\gamma\)) phosphorylated and nonphosphorylated PPARγ was performed as described previously by Hauser et al. (21). Immunoblotting for PPARγ was performed using a rabbit polyclonal antibody (H100).

**Transfections**—3T3-L1 cells were transfected with plasmids containing the PPARγ ligand binding domain fused to Gal4-DNA binding domain (Gal4-PPAR\(\gamma\)-LBD, Gal4-PPAR\(\gamma\)-LBD\(D^{\alpha}\)) and UAS-TATA-luciferase (23). Following transfection the cells were treated overnight with either 250 \(n\) M rosiglitazone, 100 \(\mu\) M AFC or dimethyl sulfoxide as a control. Cell extracts were prepared, and luciferase activity was measured as described previously (20).

**Ligand Binding Assay**—Histidine-tagged PPAR\(\gamma\)-LBD fusion construct (a gift from Dr. John Schwabe, Medical Research Council, UK) was expressed in *E. coli* as described previously (24). In brief, protein expression was initiated by the addition of isopropyl \(\beta\)-D-galactopyranoside and grown for an additional 3 h. Cells were harvested, and pellets were isolated and lysed. The lysate was sonicated, and supernatant was added to nickel-nitriilotriacetic acid slurry (Qiagen) for purification according to the manufacturer’s directions. The presence of Histagged PPAR\(\gamma\)-LBD protein was confirmed by SDS-PAGE. For the ligand binding assay, 100 \(\mu\) g of protein was incubated with 10 \(n\) M \([\text{\textit{\textsuperscript{3}}H}]\)rosiglitazone (PerkinElmer Life Sciences) in the presence of increasing concentrations of unlabeled AFC for 3 h at 4 °C in ligand binding buffer (23, 25). After incubation the beads were washed to remove the unbound ligand and binding quantitated using liquid scintillation counting. We also performed a ligand binding assay using labeled AFC. Unlabeled rosiglitazone was used at various concentrations in the presence of 35S-labeled AFC (Sigma Biosciences). The binding isotherms were analyzed for both ligands, and \(K_D\) values were determined using the one-site binding competition model as implemented in the SigmaPlot (Systat Software, San Jose, CA).

**Animal Experiments**—All animal work was conducted in accordance with the Animal Care Facility at the University of Maryland, Baltimore. Male C57BL/6J (4 weeks old) mice were obtained from Jackson Laboratory and fed a high fat diet (60 kcal% fat, D12492, Research diet) to develop diet-induced obesity (DIO) and hyperglycemia. The blood glucose levels were checked before the start of drug treatment. Animals were administered dimethyl sulfoxide, rosiglitazone, and AFC by intraperitoneal injection daily. After 2 weeks, fasting glucose and glucose tolerance tests were performed. In brief, mice were fasted overnight and tail vein blood used to measure blood glu-
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cose levels. For the glucose tolerance test, blood glucose was measured as indicated after intraperitoneal glucose administration (1 g/kg body weight). White adipose tissue was harvested at the terminal point of the experiment for histology or frozen in liquid nitrogen for further analysis. For histological analysis paraformaldehyde-fixed paraffin-embedded sections of white adipose tissue were stained with hematoxylin & eosin to observe changes in adipose tissue morphology. Counting of adipocytes was performed blinded by a pathologist (Dr. Tweddle).

RESULTS

AFC Regulates PPARγ Activity—We used a small library of pharmacologically active compounds (LOPAC, Sigma) to screen for molecules with differential PPARγ activating abilities. The advantage of this library is that it contains characterized pharmacologically active compounds for which structure and mechanisms have been annotated (see Sigma-Aldrich Web site). Additional compounds that alter signal transduction pathways known to regulate PPARγ were also included (27–29). We performed real-time PCR analysis of well characterized PPARγ target genes Angptl4, Adrp, and aP2 as readouts of PPARγ activation (30–33). Although the specific targets associated with effects of PPARγ are not fully understood, Angptl4 and Adrp are associated with antidiabetic effects of PPARγ (32, 34). In contrast, the fatty acid-binding protein aP2 plays an important role in adiposity associated with PPARγ activation (31). We compared changes in gene expression to rosiglitazone with a focus on similar induction of Angptl4 and Adrp and partial induction of aP2. A number of compounds induced Angptl4 and Adrp but not aP2. Although aP2 was induced by cAMP and glucocorticoid-related compounds in the library (which are known to promote adipogenesis) (35), they did not induce Angptl4 or Adrp as effectively as rosiglitazone (data not shown).

However, one compound, AFC, appeared to have differential selectivity toward activation of PPARγ (Fig. 1A). Angptl4 and Adrp were induced to a similar level by rosiglitazone and AFC (supplemental Fig. S1, A and B). In contrast, aP2 was induced more robustly by rosiglitazone than AFC (supplemental Fig. S1C).

Next, we confirmed the results of the screen and performed a dose response for AFC and rosiglitazone to evaluate the apparent selective partial agonism by AFC. There was a dose-dependent increase in PPARγ target genes Angptl4 and Adrp by both AFC and rosiglitazone (Fig. 1, B and C). In contrast, at all the doses that had a similar effect on Adrp and Angptl4, rosiglitazone was much more effective at inducing aP2 than AFC (Fig. 1D). For example, at the highest dose of each AFC and rosiglitazone (150 μM and 250 nm, respectively), Angptl4 and Adrp were induced similarly. However, rosiglitazone treatment led to a 7-fold greater induction of aP2 than AFC, demonstrating a partial agonism of AFC to activate PPARγ with respect to aP2. Recent studies have also shown that the insulin-sensitizing effects of PPARγ are also mediated in part by induction of the adipokine adiponectin (AdipoQ) (30). AdipoQ was induced to a similar level by rosiglitazone and AFC (supplemental Fig. S1D). This further supports that AFC is a selective partial ligand for aP2 but is a full agonist on other PPARγ target genes.

![FIGURE 1. AFC differentially induces PPARγ target genes.](image)

The ability of AFC to induce several PPARγ target genes suggests that AFC functions by activating PPARγ. To confirm this, we treated PPARγ knock-out MEFs (MEFPPARγKO) and MEFPPARγKO ectopically expressing PPARγ (MEFPPARγKO-PPARγ) with AFC. AFC treatment did not induce Adrp or AdipoQ expression in MEFPPARγKO (Fig. 2, A and B). In the absence of PPARγ, Angptl4 and aP2 were not detected irrespective of treatment (Fig. 2, C and D). However, in the MEFPPARγKO-PPARγ cells, AFC treatment led to an induction of all four genes (Fig. 2, A–D), demonstrating that AFC is inducing these genes in a PPARγ-dependent mechanism.

Next, we subjected 3T3-L1 preadipocytes to a differentiation protocol using a hormonal mixture in the presence and absence
demonstrating a direct effect of AFC on the LBD of PPAR. Although AFC activated the Gal4-PPAR
/H9253 line mutation at amino acid 286 as a negative control. This indicates that AFC interacts directly with PPAR. We also examined the expression of PPAR
ligands (36, 37). The ability of AFC to interact with and activate PPAR as a ligand was tested using a chimeric reporter plasmid containing a Gal4-DNA binding domain fused to the LBD of PPAR. We also used a PPAR
LBD construct (Fig. 3A) without the PPAR
ligand. This further demonstrates that AFC differentially activates PPAR.

AFC Is a PPAR\(\gamma\) Ligand—AFC has a structure similar to several lipid-derived molecules that are known to be PPAR\(\gamma\) ligands (36, 37). The ability of AFC to interact with and activate PPAR\(\gamma\) as a ligand was tested using a chimeric reporter plasmid containing a Gal4-DNA binding domain fused to the LBD of PPAR\(\gamma\). We also used a PPAR\(\gamma\)-LBD with a glutamine to proline mutation at amino acid 286 as a negative control. This mutation renders PPAR\(\gamma\) unable to bind ligands and activate gene expression (23). AFC induced the activity of the Gal4-PPAR\(\gamma\) fusion construct ~3-fold (Fig. 3A). In contrast, neither rosiglitazone nor AFC was able to activate the Gal4-PPAR\(\gamma\)-LBD mutant construct (Fig. 3A and supplemental Fig. S3A), demonstrating a direct effect of AFC on the LBD of PPAR\(\gamma\). Although AFC activated the Gal4-PPAR\(\gamma\)-LBD less than rosiglitazone (supplemental Fig. S3A), these data strongly suggest that AFC is interacting with the LBD of PPAR\(\gamma\).

Next, we performed ligand binding assays to confirm that AFC interacts directly with PPAR\(\gamma\). As shown in Fig. 3B, AFC was able to displace rosiglitazone from the LBD of PPAR\(\gamma\) with a \(K_d\) of 11 ± 3 \(\mu\)M. We also determined whether unlabeled rosiglitazone could displace radiolabeled\(^{35}\)S-labeled AFC from PPAR\(\gamma\) (supplemental Fig. S3B). Increasing concentrations of rosiglitazone effectively displaced AFC from PPAR\(\gamma\) with a \(K_d\) for rosiglitazone of 20 nM. This is in line with the reported \(K_d\) for rosiglitazone (23, 38, 39). Next, we performed molecular dynamics simulation of AFC bound to PPAR\(\gamma\). The modeling predicts that the carboxylate moiety of AFC molecule makes hydrogen bonds to Tyr-473 and His-449. These two residues interact similarly with a majority of the known PPAR\(\gamma\) ligands. In addition, the model predicts that the acetyl group of AFC forms a novel interaction with the backbone nitrogen of His-466 (Fig. 3C). This is a unique interaction that has not been observed for other PPAR\(\gamma\) ligands.

AFC is most well known for its ability to inhibit Ras/Rho-GTPase signaling by competing for farnesyl binding domains and inhibiting carboxymethylation of GTPases because it mimics the C-terminal farnesylcysteine group of Ras-GTPase (40, 41). Because GTPase signaling inhibits PPAR\(\gamma\) activity we wanted to determine whether AFC was activating PPAR\(\gamma\) by virtue of its ability to inhibit GTPase signaling. We treated 3T3-L1 cells with different compounds known to inhibit GTPase processing. Farnesyl transferase and geranylgeranyl transferase inhibitors work upstream of isoprenylcysteine carboxyl methyltransferase by inhibiting the farnesylation or geranylgeranylation of C-terminal CAAX-containing proteins (41). Neither farnesyl transferase inhibitors nor geranylgeranyl transferase inhibitors induced Angptl4 or aP2 in the 3T3-L1 cells (supplemental Fig. S4A). We also used 5-adenosylhomocysteine, an inhibitor of isoprenylcysteine carboxyl methyltransferase that is structurally distinct from AFC. We did not observe a change in the expression of PPAR\(\gamma\) target genes (supplemental Fig. S4A). Additionally, two compounds in the LOAP library mevastatin and pravastatin, known to inhibit isoprenylation of GTPases, did not alter PPAR\(\gamma\) target gene expression (data not shown). Therefore, it does not appear that inhibition of GTPase processing by AFC is mediating its effects on PPAR\(\gamma\).

We then wanted to determine whether AFC alters other signal transduction pathways regulating PPAR\(\gamma\) function. Phosphorylation of PPAR\(\gamma\) in its N terminus by several MAPKs reduces the activity of PPAR\(\gamma\) (21, 42, 43). Previous studies show that AFC decreases MAPK signaling (40, 41). Therefore, we wanted to determine whether the effect of AFC on PPAR\(\gamma\) was a result of decreasing the phosphorylation of PPAR\(\gamma\). Fully differentiated 3T3-L1 cells were treated with rosiglitazone, or AFC and the phosphorylation status of PPAR\(\gamma\) was determined as described under “Experimental Procedures.” Phosphorylation of PPAR\(\gamma\) in differentiation adipocytes was not altered following treatment with AFC (supplemental Fig. S4B, upper band). Therefore, it is unlikely that AFC is increasing PPAR\(\gamma\) activity by reducing the phosphorylation of PPAR\(\gamma\). Interestingly, AFC decreased nonphosphorylated PPAR\(\gamma\), in a fashion similar to rosiglitazone. The reduction in unphosphorylated PPAR\(\gamma\) protein by PPAR\(\gamma\) ligands is a general property of PPAR\(\gamma\) ligands (21). This further supports our findings showing that AFC is a PPAR\(\gamma\) ligand.

Recently phosphorylation of Ser-273 of PPAR\(\gamma\) by CDK5 was shown to alter the ability of PPAR\(\gamma\) to activate a subset of
PPARγ target genes (22). Treatment of 3T3-L1 cells with rosiglitazone or MRL24 reduced the TNF-α-induced phosphorylation of Ser-273 of PPARγ as reported previously (22). In contrast, AFC did not alter the phosphorylation of Ser-273 (supplemental Fig. S4C). Therefore, the ability of AFC to regulate PPARγ differentially does not appear to be mediated via altering phosphorylation of Ser-273 of PPARγ.

**AFC Improves Glucose Homeostasis in Diabetic Mice**—Next, we wanted to determine whether AFC could restore glucose homeostasis in a rodent model of diabetes. Experiments were also performed with rosiglitazone for comparison. After 20 weeks on a high fat diet, fasting glucose levels of the mice were increased, indicating the presence of T2DM (>140 mg/dl). Mice were then treated with AFC (7.5 mg/kg per day) or rosiglitazone (10 mg/kg per day) for 4 weeks at which point fasting glucose in control mice was still >140 mg/dl. However, fasting glucose was reduced significantly in the rosiglitazone- and AFC-treated mice (Fig. 4A). Mice were then subjected to a glucose tolerance test. Rosiglitazone- and AFC-treated mice had similar efficacy in their ability to reduce blood glucose compared with control mice (Fig. 4B). Body weight of control and rosiglitazone-treated mice increased ~12 and 14%, respectively (Table 1). In contrast, the weight of AFC-treated mice only increased 5.8%. The weight of AFC-treated mice was not significantly different from control mice. However, AFC-treated mice gained significantly less weight than the rosiglitazone-treated mice (p < 0.05). The reduced weight gain/resistance to weight gain did not appear due to food intake, which appeared similar (data not shown). There was a trend toward a decrease in total epididymal white adipose tissue (WAT) weight in the AFC-treated mice compared with control or rosiglitazone-treated mice, but this was not statistically significant (Table 1). We also examined the expression of liver enzymes, aspartate aminotransferase and alanine transaminase, to determine whether the decreased weight gain of AFC-treated mice was due to toxicity. Expression of these markers was unaltered in livers from AFC-treated mice compared with mice in the control group (data not shown). These studies demonstrate that AFC is as effective as rosiglitazone at reducing plasma glucose levels without the weight gain typically associated with TZD treatment.

Histological analysis of epididymal WAT of rosiglitazone- and AFC-treated mice showed that adipocytes of treated mice

**FIGURE 3. AFC is an endogenous PPARγ ligand.** A, 3T3-L1 cells were transfected overnight with GAL4-PPARγ-LBD and GAL4-PPARγ-QP and UAS TATA reporter constructs and treated with 100 μM AFC. Luciferase activity was measured after 24 h. n = 3 ± S.D. (error bars). B, PPARγ directly binds AFC. Ligand binding assays were performed with a His-tagged-PPARγ-LBD using [3H]rosiglitazone in the presence of vehicle or the indicated concentrations of unlabeled AFC as competitor. C, molecular modeling of PPARγ/AFC interaction is shown. Yellow, carbon; blue, sulfur; red, oxygen; green, carbons from PPARγ amino acid.
were smaller than the adipocytes of the control-treated mice (Fig. 4C). This is noteworthy because previous studies have shown that smaller adipocytes are associated with glucose tolerance (44). In addition, a decrease in adipocyte size is reported to be in part responsible for the effects of PPARs (44). In addition, a decrease in adipocyte size is reported to be in part responsible for the effects of PPARs (44). We also examined the effect of AFC on the expression of PPARγ target genes in WAT. D–F, mRNA fold changes in Adrp (D), AdipoQ (E), and aP2 (F) are shown. n = 4–6 ± S.D., *p < 0.05; **p < 0.005 versus control. NS, not statistically significant.

TABLE 1
Percentage weight change and percentage WAT weight in respective groups of DIO-treated mice

| Treatment | Weight changea | WAT weightb |
|-----------|----------------|-------------|
| Control   | +12 ± 0.39     | 4.5 ± 0.3   |
| Rosiglitazone | +14 ± 0.01    | 5.6 ± 0.3   |
| AFC       | +5.8 ± 0.22    | 4.5 ± 0.2   |

a % Weight change = (final weight – initial weight)/initial × 100%

b % WAT weight = grams of WAT weight/total grams of body weight × 100%

AFC Is a Differential SPPARM

Reducing the production of proinflammatory cytokines is one of the mechanisms mediating the antidiabetic effects of PPARγ. In addition, AFC has been shown to inhibit inflammation (50). However, examination of WAT from AFC-treated mice showed increased infiltration of macrophages. This would imply that AFC is promoting inflammation in the WAT, despite improved glucose homeostasis. Therefore we investigated whether AFC could alter cytokine production associated with diabetes. 3T3-L1 preadipocytes were treated with Tnf-α in the presence or absence of AFC or rosiglitazone, and the gene expression of proinflammatory cytokines was measured. Saa2 expression was reduced by both rosiglitazone and AFC (Fig. 5A). Interestingly, whereas AFC reduced Saa3 mRNA levels ~50%, rosiglitazone did not alter its expression (Fig. 5B). In addition, AFC reduced Il6 expression almost 80%, whereas rosiglitazone reduced it by ~20% (Fig. 5C). Next, we wanted to confirm the antiinflammatory effects of AFC in vivo. The expression of Il6, Tnf-α, Saa2, and Saa3 was reduced in the WAT of AFC-treated mice (Fig. 5, D–G). Surprisingly, we did not observe a significant effect of rosiglitazone on cytokine gene expression in WAT (supplemental Fig. S5). These data demonstrate that AFC represses proinflammatory cytokines associated with insulin resistance to a greater degree than rosiglitazone despite the increase in macrophage infiltration.

The reduction in inflammatory cytokines in WAT from AFC-treated mice, yet increased macrophage infiltration appears paradoxical. Adipose tissue macrophages are typically
made of two populations, type I (M1) and type II (M2) macrophages (51) (52). Insulin resistance is associated with an increase in M1/M2 macrophages, whereas insulin sensitivity is associated with an increase in M2, which is thought to promote tissue repair and glucose homeostasis. This is supported by data showing that recruitment of M2 macrophages to fat is reduced in diabetic mice (53). Furthermore, loss of PPARγ results in reduced M2 macrophage activation (54). Therefore, we investigated the effect of AFC on macrophage activation. AFC induced the mRNA expression of arginase I (ArgI) and Clec7a, two markers of M2 macrophage activation (Fig. 5, H and I) (54–57). In contrast, AFC decreased the expression of Itgax (which encodes CD11c), a marker of M1 macrophages that is elevated in the WAT of diabetic mice and humans (Fig. 5 J) (56, 58, 59). These data suggest that the antiinflammatory effects of AFC may be partially mediated by alternative macrophage activation.

**DISCUSSION**

TZDs are a class of PPARγ agonists used in the treatment of T2DM. However, PPARγ agonists are associated with numerous side effects, which have prompted the development of SPPARMs. Most of these molecules do not show differential activation of PPARγ target genes associated with beneficial versus deleterious effects. Therefore, to identify differential SPPARMs, we developed a gene expression-based approach based on a method described previously (60). This approach enables the identification of both full and partial agonist effects on PPARγ depending on the genes of interest. Using this approach we have identified AFC as a novel class of PPARγ ligand.

AFC was initially described as a synthetic compound that inhibits GTPase processing by blocking farnesyl binding to target proteins and/or carboxymethylation of Ras/Rho-GTPases. However, the ability of AFC to activate PPARγ is independent of its ability to inhibit carboxymethylation. A unique aspect of AFC function is its specificity with regard to different PPARγ target genes. AFC acts as both a partial agonist for certain PPARγ target genes and a full agonist for others, both in vitro and in vivo. Although the target genes mediating both antidiabetic and deleterious effects of PPARγ ligands are not well understood, AFC induced PPARγ target genes associated with insulin sensitivity such as Angptl4, Aday, and AdipoQ to a similar extent as rosiglitazone. In contrast, induction of aP2, a PPARγ target gene promoting adiposity, was attenuated compared with rosiglitazone. Although we do not know the mechanisms of differential specificity by AFC, other SPPARMs have been shown to alter the interaction of PPARγ with transcriptional coactivators. Therefore, it is likely that AFC also alters the ability of PPARγ to interact with its coactivators and hence promote differential recruitment of PPARγ to the promoters of

**FIGURE 5. AFC inhibits induction of inflammatory cytokines.** A–C, 3T3-L1 cells were treated with TNF-α (1 ng/ml) in the presence or absence of rosiglitazone or AFC for 24 h. RNA was isolated, and real-time PCR performed for Saa2 (A), Saa3 (B), and Il6 (C) genes, n = 3 ± S.D. (error bars). D–J, RNA was isolated from WAT of control and treated mice. RT-PCR was performed for Saa2 (D), Saa3 (E), TNFα (F), Il6 (G), ArgI (H), Clec7a (I), and Itgax (J). *, p < 0.05; **, p < 0.005 versus control. NS, not statistically significant.
its target genes. The unique manner in which AFC interacts with PPARγ supports the idea of novel changes in structure that may alter PPARγ function. Subsequent studies should elucidate these mechanisms and the manner in which PPARγ target genes are differentially induced by AFC.

Molecular modeling suggests one potential mechanism of how PPARγ is activated differentially by AFC. AFC is bound by PPARγ in a unique manner compared with TZDs and other SPPARMs. The model predicts that similar to classic TZDs, AFC makes crucial contacts with His-449 in helix 11 (H11) and Tyr473 in helix 12 (H12). The ability of AFC to interact with Tyr-473 represents a novel feature for a partial PPARγ agonist, which usually does not make this interaction (61–65). Moreover, unlike other full agonists and partial agonists described to date, AFC interacts with His-466 of PPARγ. This would result in a substantial shift in the orientation of H12, which is known to be a key element in PPARγ activation. The interaction of AFC with Tyr-473 and His-466 likely determines its specificity for full and partial agonism toward different PPARγ target genes. These are dynamic modeling studies and not actually AFC/PPARγ crystal structures. Therefore, current crystal structure studies are seeking to validate these results, and subsequent studies will determine the role of these residues in mediating the differential effects of AFC on PPARγ activation.

Treatment of mice with AFC was as effective at lowering fasting glucose levels in diabetic mice as rosiglitazone. In addition, rosiglitazone and AFC treatment improved glucose tolerance to a similar degree. However, treatment of mice with AFC was not associated with one of the known side effects of TZDs, increased weight gain. Therefore, although AFC lowers glucose levels to a similar degree as rosiglitazone, mice treated with AFC either did not gain as much weight as rosiglitazone-treated mice or were resistant to weight gain from a high fat diet.

Proinflammatory cytokines Saa2, Saa3, Tnf-α, and Il6 play an important role in insulin resistance (46–49). PPARγ represses the expression of a number of these proinflammatory cytokines (54, 66–68). Although rosiglitazone reduced cytokine mRNA expression in vitro (albeit less effectively than AFC), it remained unchanged in vivo, despite improved glucose tolerance. Although this was somewhat surprising, conflicting results in mRNA expression for inflammatory genes after TZD treatment have been reported previously. TZDs reduce the levels of IL6, TNF-α and SAA in adipose tissue from humans (66, 69, 70). However, other studies, show that in diabetic mice there was no change in inflammatory gene expression from the WAT of mice (71, 72). In addition, it was interesting to note that there appeared to be greater macrophage infiltration into WAT of AFC-treated mice. This prompted us to investigate whether these were type I or type II macrophages. Indeed, we observe a shift from M1 to M2 macrophages in WAT from AFC-treated mice. Although PPARγ is known to be required for M2 macrophage activation, the TZD class of ligands does not appear to increase M2 macrophages considerably. This suggests that some of the antiinflammatory effects of AFC compared with rosiglitazone may be due to differences in macrophage activation. It is tempting to speculate that ligand activation of PPARγ by AFC promotes M2 macrophages to a greater extent than rosiglitazone, although it is unclear at this time whether it is PPARγ-dependent. Regardless, this suggests that AFC is either more effective at promoting the antiinflammatory effects of PPARγ or that it is functioning in a PPARγ-independent manner.

Several cell-based and in vitro molecular approaches have been used in the past to identify SPPARMs (26, 63, 73–75). However, these approaches only identify general PPARγ ligands and do not take into account differential activity of endogenous PPARγ target genes. Our differential gene expression-based approach enables the identification of activators of PPARγ with both full and partial agonist effects on PPARγ depending on the genes of interest. Indeed, as additional targets are identified, it will help refine and develop this methodology. This approach can also be applied to other systems where identification of molecules or drugs with differential effects is being investigated. A potential drawback of this approach, as with any cell-based technique, is that it identifies compounds that alter PPARγ activity through direct and indirect mechanisms. Hence, this approach does not discover compounds that are only PPARγ ligands. We actually view this as an advantage because it identifies not only ligands, but also other molecules that can alter PPARγ activity independent of PPARγ binding. Indeed, a report several years ago identified harmine as an inducible adipose-selective regulator of PPARγ expression that increases PPARγ activity independent of ligand binding (26). In addition, recent work from Spiegelman and co-workers highlights the effect of ligands on differential induction of PPARγ gene expression distinct from ligand binding (22).

The approach described here to identify SPPARMs is rapid and cost-effective, making it amenable to screening large drug libraries. Although the actual PPARγ target genes and mechanisms responsible for some of the side effects of TZDs are not well understood, these studies represent advancement toward identifying SPPARMs with differential activity and mechanisms mediating the effects of PPARγ ligands. In conclusion, AFC represents a novel class of PPARγ ligand with both full and partial agonist activity. Importantly, AFC promotes glucose homeostasis without the adipogenic action of class TZDs and hence supports further studies into the role of AFC and its derivates as antidiabetic therapies.

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