Original Article

Halofenate Is a Selective Peroxisome Proliferator–Activated Receptor γ Modulator With Antidiabetic Activity

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Halofenate has been shown previously to lower triglycerides in dyslipidemic subjects. In addition, significant decreases in fasting plasma glucose were observed but only in type 2 diabetic patients. We hypothesized that halofenate might be an insulin sensitizer, and we present data to suggest that halofenate is a selective peroxisome proliferator–activated receptor (PPAR)-γ modulator (SPPARγM). We demonstrate that the circulating form of halofenate, halofenic acid (HA), binds to and selectively modulates PPAR-γ. Reporter assays show that HA is a partial PPAR-γ agonist, which can antagonize the activity of the full agonist rosiglitazone. The data suggest that the partial agonism of HA may be explained in part by effective displacement of corepressors (N-CoR and SMRT) coupled with inefficient recruitment of coactivators (p300, CBP, and TRAP 220). In human preadipocytes, HA displays weak adipogenic activity and antagonizes rosiglitazone-mediated adipogenic differentiation. Moreover, in 3T3-L1 adipocytes, HA selectively modulates the expression of multiple PPAR-γ–responsive genes. Studies in the diabetic *ob/ob* mouse demonstrate halofenate's acute antidiabetic properties. Longer-term studies in the obese Zucker (*fa/fa*) rat demonstrate halofenate's comparable insulin sensitization to rosiglitazone in the absence of body weight increases. Our data establish halofenate as a novel SPPARγM with promising therapeutic utility with the potential for less weight gain. *Diabetes* 55:2523–2533, 2006

Halofenate was tested clinically in the 1970s as a hypolipidemic and hypouricemic agent. In subsequent investigator-led studies, halofenate was shown to lower serum triglycerides and uric acid in patients with a variety of hyperlipidemias (1–4). Treatment of dyslipidemic type 2 diabetic patients also showed triglyceride lowering and, surprisingly, significant reductions in plasma glucose and insulin (3). Subsequent studies in diabetic patients confirmed the glucose- and triglyceride-lowering effects of halofenate in combination with oral hypoglycemic drugs and as monotherapy (4–7). While the precise mechanism of halofenate's potentiation of the glycemic effect of sulfonylureas was not understood, it was originally hypothesized that halofenate, being highly plasma protein bound, might dislodge oral hypoglycemic compounds from serum binding proteins, thus increasing their efficacy (8). However, significant decreases in glucose were also observed with halofenate monotherapy (5) showing that halofenate could function independently of sulfonylureas. In analyzing these historical data, we noted that halofenate lowered glucose levels in diabetic, but not normoglycemic, subjects and that the time course of the beneficial glycemic effects was similar to that of the insulin-sensitizing thiazolidinediones (TZDs), which possesses glucose- and insulin-lowering properties mediated via activation of peroxisome proliferator–activated receptor (PPAR)-γ (9). We hypothesized that the insulin-sensitizing effects of halofenate might similarly involve PPAR-γ; we carried out a series of experiments to test this hypothesis.

PPAR-γ is a member of the NR1C subgroup, which includes PPAR-α and -δ. These receptors form heterodimers with the retinoid X receptor and modulate the transcription of genes. PPAR-γ is predominantly expressed in white and brown adipose tissue, with lower expression in liver, muscle, and other tissues (10). PPAR-γ ligands include a surprisingly diverse set of natural ligands (11) such as linolenic, eicosapentaenoic, docohexaenoic, and arachidonic acid and synthetic ligands such as the TZDs, L-tyrosine–based compounds, several nonsteroidal anti-inflammatory drugs, and a variety of new chemical classes (12,13). Originally identified as a regulator of adipogenesis, PPAR-γ was thought to mediate the actions of TZDs solely through its actions in adipose tissue. However, subsequent studies utilizing tissue-specific *PPAR-γ* gene knockouts have demonstrated a complex role for PPAR-γ in whole-body insulin sensitivity involving multiple tissues, including liver and muscle (14–16).

Two TZDs, rosiglitazone and pioglitazone, are currently approved to treat type 2 diabetes. Despite their proven efficacy, a number of deleterious side effects have been noted, including increased weight gain and edema (17). Weight gain is likely due to both increased adiposity and fluid retention. Edema is particularly a problem in patients

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AUC, area under the curve; HA, halofenic acid; ID, interaction domain; PPAR, peroxisome proliferator–activated receptor; SPPARγM, selective PPAR-γ modulator; TZD, thiazolidinedione.

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who are also taking insulin or sulfonylureas, and TZD treatment has been linked to increased incidence of congestive heart failure (17,18). Accordingly, efforts have been mounted to generate novel PPAR-γ modulators that retain the beneficial clinical effects while avoiding these side effects.

A variety of new PPAR-γ ligands that possess differential pharmacological affinities for PPAR-γ and have been termed selective PPAR-γ modulators (SPPARγMs) have recently been reported (19). SPPARγMs are believed to bind in distinct manners to the ligand binding pocket of PPAR-γ, leading to altered receptor conformational stability and resulting in distinct patterns of gene expression (20–22). The molecular basis of this effect is thought to involve differential cofactor displacement and recruitment that regulates gene expression in a gene- and tissue-specific manner (23). Further characterization of SPPARγMs will ideally yield agents for the treatment of diabetes, which are as effective as current therapies but reduce or eliminate the more deleterious side effects. This study was initiated in an effort to elucidate the underlying mechanism of the antidiabetic effects of halofenate. Our data suggest that halofenate is a novel SPPARγM with robust antidiabetic and insulin-sensitizing activity. Furthermore, halofenate appears to enhance insulin sensitivity in a manner that leads to less weight gain than the currently marketed compounds.

RESEARCH DESIGNS AND METHODS

Plasmids. pCMX-mouse PPAR-γ and -α plasmids were gifts from R. Evans. The ligand-binding domain of mouse PPAR-γ (aa 174–475) and -α (aa 166–463) was generated by PCR and cloned into pGAL0. Full-length mouse PPAR-γ was generated by PCR and cloned into pVP16. The ligand-binding domains for human PPAR-α (aa 166–469), -δ (aa 135–442), and -γ (aa 172–476) were generated by PCR and cloned into pFA-CMV plasmid (Stratagene). pGAL-TRAP220 contained the receptor interaction domain (ID)1 and ID2 fragments, aa 502–667; pGAL-CBP contained the ID1 fragment, aa 1–115; pGAL-p300 contained aa 595–1,240; and pGAL-SMRT contained the COOH-terminal 468 amino acids. pGAL-N-CoR ID I + II ΔI contained two interaction domains from the N-CoR splice variant, RIP13ΔI (24).

Cell-based reporter assays. HEK-293T cells were transfected with Gal4 chimeras and reporter gene plasmids using Lipofectamine 2000 (InVitrogen) and incubated for 4 h before treatment with compound for 20–24 h. Expression was assayed using the Steady-Glo assay system (Promega).

Mammalian two-hybrid assays. JEG-3 human choriocarcinoma cells at 50% confluency were transfected with 400 ng G5E1B-LUC and 80 ng each of pVP16-PPAR-γ full length and pGAL-N-CoR, SMRT, p300/CBP, or TRAP220 in DOTAP/metafectene in HEBS. This mixture was applied to cells in 0.5 ml media supplemented with 5% FCS (and charcoal-stripped serum) and incubated for 16 h. Following this incubation, the media was replaced with fresh media containing the appropriate drug treatment and cultured a further 24 h, after which they were washed and assayed for luciferase activity.

MTT cell viability assay. This assay has been previously described (25). Briefly, JEG3 cells were treated for 24 h with vehicle, rosiglitazone, or halofenic acid (HA) in Dulbecco's modified Eagle's medium supplemented with 5% FCS. Media was aspirated, and 200 μl/well MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (1 mg/ml) was added and incubated at 37°C for 30–60 min. The MTT was removed, and 500 μl/well 100% isopropanol was added and incubated at room temperature for 30 min. After 200 μl from each well was removed, the absorbance was read at 570–600 nmol/l.

Binding assay. The binding between test compounds and the human PPAR-γ ligand-binding domain was measured using the PolarScreen PPAR-γ Competitor Assay, Green (no. PV3355; InVitrogen) using the manufacturer's recommended protocol.

Cell culture and treatment. Differentiated 3T3-L1 adipocytes were treated for 24 h with or without DMSO (0.1%), HA (150 μmol/l), or rosiglitazone (1 μmol/l). After treatment, the cells were harvested with Trizol for total RNA isolation.

Human adipocyte adipogenesis assays. Cultured human preadipocytes were obtained from Zen-Bio. Cells were incubated in Adipocyte Medium (catalog no. AM-1; 100 nmol/l insulin and 1.0 μmol/l dexamethasone) in the presence of 0.25 mmol/l isobutyl-methylxanthine and either rosiglitazone (1 μmol/l) or HA (100 or 200 μmol/l) for 3 days and then fed with adipocyte medium for additional 12 days. PPAR-γ–mediated ligand-induced differentiation was assessed by Oil Red O staining and analysis of *FABP4* gene expression by quantitative PCR.

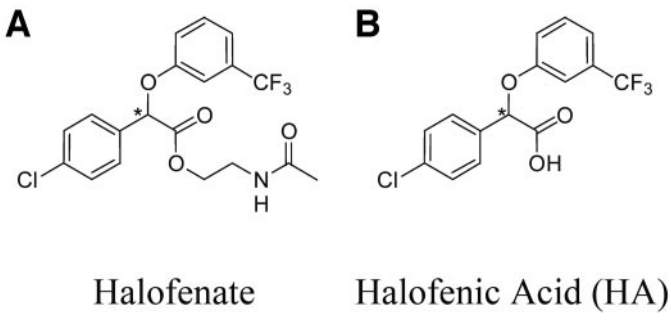

FIG. 1. Chemical structures of compounds. Halofenate (*A*) is a pro-drug ester of HA (*B*). Halofenate is rapidly modified in vivo to HA, the circulating form of the drug.

Quantitative fluorescent real-time PCR analysis. Total RNA was isolated using Trizol (InVitrogen). cDNA was synthesized and used for quantitative fluorescent real-time PCR amplification. Amplification of each target cDNA was then performed with TaqMan PCR Reagent Kits according to the manufacturer's protocol using Assay-On-Demand (Applied Biosystems) assays. 36B4 RNA was used as the control. Data are expressed as the means ± SE. Statistical significance was determined using ANOVA.

***ob/ob* mouse studies.** Male 8-week-old *ob/ob* mice (Jackson Laboratories) were used. All animal procedures were approved by the local institutional animal care and use committee following guidelines issued by the U.S. Department of Agriculture Animal and Plant Health Inspection Services. Halofenate was formulated daily as a suspension by vortexing in a mixture of 2% Tween 80 and 1.0% (wt/vol) methylcellulose. Vehicle and halofenate were administered by oral gavage. Blood samples were collected by tail nipping 3 h after dosing. Plasma glucose levels were measured using the method of Trinder (Glucose Oxidase G7016, Peroxidase P8125; Sigma Chemical, St. Louis, MO) and insulin by radioimmunoassay (Linco). Mice received three treatments of vehicle (5 ml/kg) or halofenate at time 0, 24, and 48 h. The statistical significance between vehicle and treatment groups was analyzed by ANOVA.

Obese Zucker (*fa/fa*) rat studies. Male obese Zucker (*fa/fa*) rats were obtained from Harlan Laboratories (Indianapolis, IN) at 6 weeks of age. They were housed in a temperature-controlled environment with a 12-h light/dark cycle, two rats per cage, and fed Purina 5001 diet. At 7 weeks of age, they were sorted into three groups with similar body weights and plasma insulin values. Rosiglitazone maleate and halofenate were prepared as described above at concentrations of 6 and 40 mg/ml. Each compound was administered daily by oral gavage in a volume of 5 ml/kg to deliver doses of 30 (rosiglitazone) or 200 mg/kg (halofenate). Control rats received a 5-ml/kg dose of the vehicle solution. Animals and food were weighed two times per week until the completion of the study after 28 doses. An oral glucose tolerance test was performed on each rat on day 20 of the study. Rats were fasted for 6 h before being administered a dose of 20% glucose in a volume of 5 ml/kg. Blood samples were collected in EDTA-coated tubes at 0, 15, 30, 60, 90, and 120 min after glucose loading by tail nipping. Statistical significance between vehicle and treatment groups was analyzed by ANOVA. At the end of the study, pharmacokinetic analysis was performed on three animals treated with halofenate over a 24 h period. The averaged data were as follows: C_{max} 618 μg/ml; T_{max} 8 h; area under the curve $(AUC)_{(0-24)}$ 11,242 μg · h · ml^{-1}. Halofenate is highly bioavailable, has a long half-life in most species, and is a highly plasma protein–bound drug (>99%) (data not shown), all of which contribute to high plasma drug levels that are sufficient to modulate PPAR-γ in vivo.

RESULTS

HA is a SPPARγM. Clinical studies have demonstrated significant beneficial glycemic and lipid effects in type 2 diabetic patients treated with halofenate (4–7). The structure of halofenate is shown in Fig. 1*A*. Halofenate is administered as a prodrug ester, which is rapidly and completely modified to its mature circulating free acid

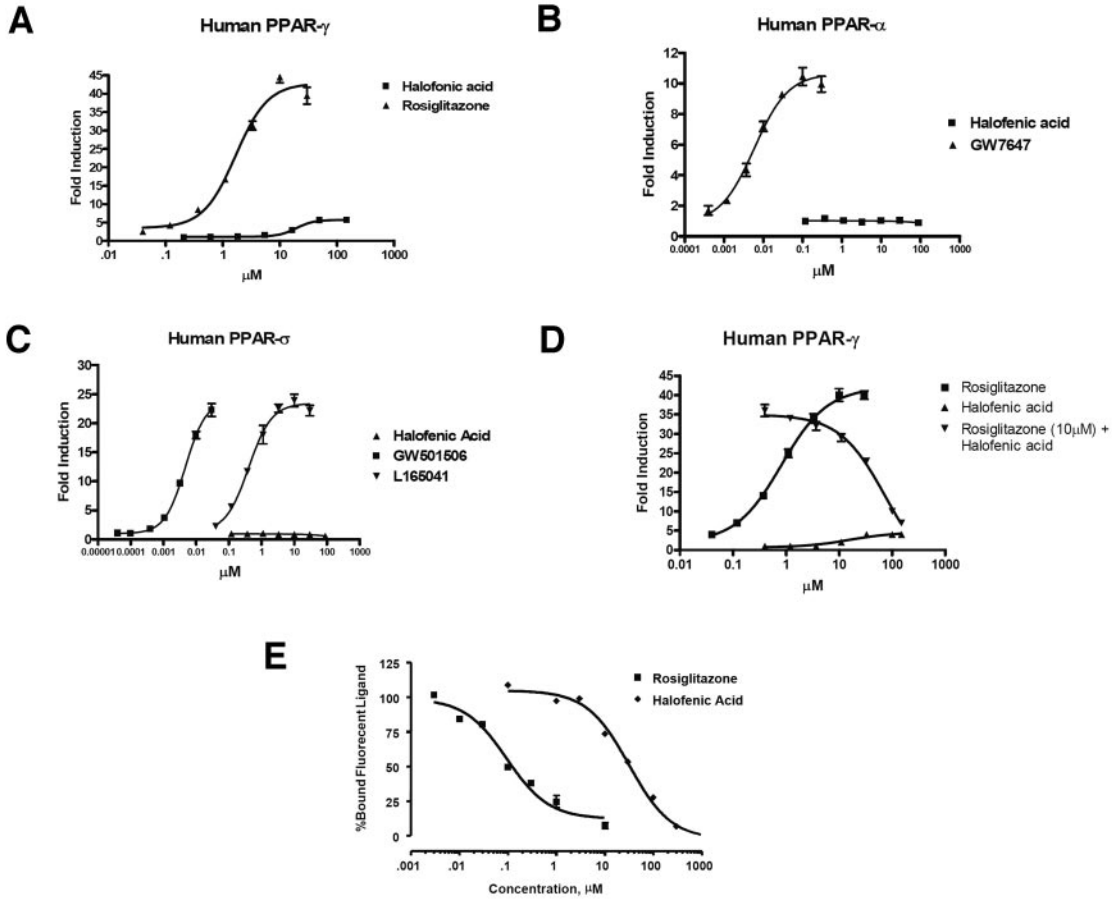

FIG. 2. HA is a PPAR-γ partial agonist/antagonist. The GAL hybrid assay was used to examine the activation of PPARs by HA. *A*: GAL4-human PPAR-γ, -α, or -δ ligand-binding domain expression plasmid was cotransfected with a luciferase reporter plasmid in HEK-293T cells. Human PPAR-γ (*A*), PPAR-α (*B*), PPAR-δ (*C*) activity was monitored in the transfected cells treated with increasing concentrations of the respective PPAR agonists: rosiglitazone (0.033–33 μmol/l), HA (0.1–100 μmol/l), GW7647 (0.33–300 nmol/l), L165041 (33 nmol/l to 33 μmol/l), and GW501516 (0.03 nmol/l to 33 nmol/l). *D*: To evaluate an antagonist effect, PPAR-γ activity was measured in GAL4-human PPAR-γ ligand-binding domain transfected cells treated with increasing concentrations of rosiglitazone (0.033–33 μmol/l), HA (0.4–150 μmol/l), or 10 μmol/l rosiglitazone cotreated with increasing concentrations of HA (0.4–150 μmol/l). Fold induction is expressed relative to vehicle (DMSO). Figures are plotted as means ± SE. Data are representative of minimal three independent experiments of at least triplicates. *E*: HA binds to PPAR-γ. A competitive binding assay was used to assess the ability of HA or rosiglitazone to displace a fluorescent PPAR-γ ligand from a human-derived recombinant PPAR-γ ligand-binding domain. Concentration-dependent displacement of the fluorescent PPAR-γ ligand by HA was observed with an IC_{50} of ~32 μmol/l.

form (Fig. 1*B*). The ester form of halofenate was utilized for in vivo studies, whereas the acid form, HA, was utilized for in vitro studies in order to mimic the active form of the drug. Similarities between the antidiabetic effects of halofenate and those of PPAR-γ agonists prompted us to investigate the ability of halofenate to modulate PPAR-γ activity.

We first used GAL4 hybrid reporter gene assays to examine whether HA was capable of activating human PPAR-α, -δ, or -γ. Treatment of transfected HEK-293T cells with rosiglitazone resulted in activation of GAL-PPAR-γ with a half-maximal effective concentration (EC_{50}) of ~0.5–2 μmol/l (Fig. 2*A*). HA exhibited a dose-dependent activation of human PPAR-γ with an EC_{50} of ~10–20 μmol/l (Fig. 2*A*). The maximal activity achieved by HA was typically ~10–15% of that observed with the full agonist rosiglitazone. In contrast, no activation of the human PPAR-α or -δ by HA was observed at any concentration (Fig. 2*B* and *C*). Similar selective partial activation of human PPAR-γ by HA was observed in reporter gene assays utilizing Chinese hamster ovary and CV-1 cells with EC_{50}s of ~12–24 μmol/l (data not shown). We also used GAL4 hybrid reporter gene assays to examine the ability of HA to activate mouse GAL4-PPAR-γ chimeras in muscle C2C12 cells. Similar to the results obtained from human assays, HA exhibited a weak dose-dependent activation of PPAR-γ with an EC_{50} of ~30 μmol/l, whereas rosiglitazone exhibited an EC_{50} of ~0.5 μmol/l (data not shown). No significant activation of mouse PPAR-α, mouse PPAR-δ, human LXRα, human LXRβ, or mouse retinoid X receptor-γ was observed (data not shown). Together, these in vitro data support the hypothesis that HA is a selective partial PPAR-γ modulator.

We next tested whether HA had the ability to attenuate rosiglitazone-mediated reporter gene activity. This is generally considered to be a measure of the antagonist activity of a compound. As shown in Fig. 2*D*, HA showed a concentration-dependent inhibition of rosiglitazone-dependent human PPAR-γ–mediated reporter activity with a half-maximal inhibitory concentration (IC_{50}) of ~40 μmol/l. These data suggest that HA has the ability to interact with PPAR-γ in such a way as to displace rosiglitazone and inhibit its activity in a cellular context.

HA binds to the ligand-binding domain of human PPAR-γ. To determine whether HA had the ability to directly interact with PPAR-γ, we assessed that ability of

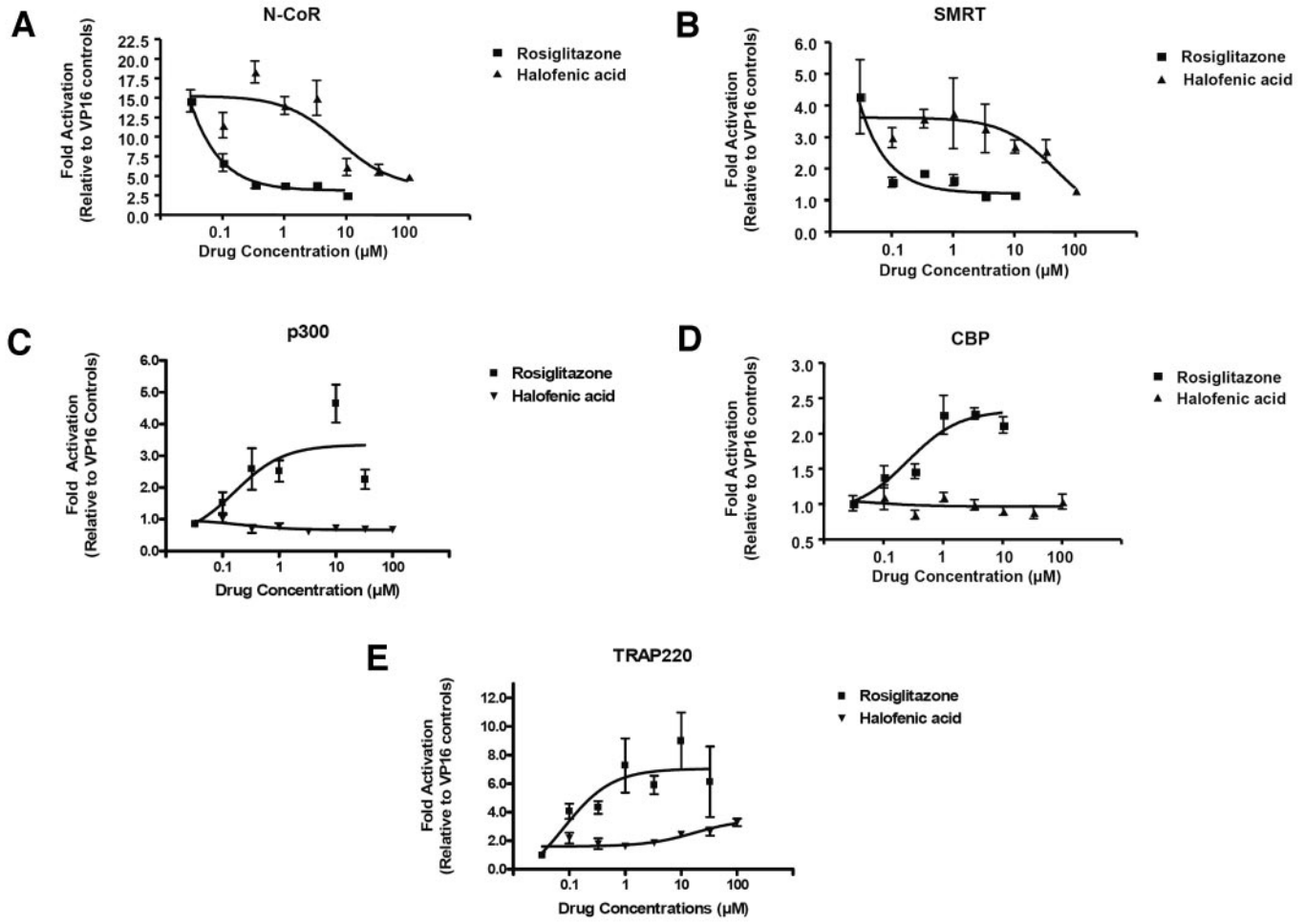

FIG. 3. HA modulation of cofactor displacement and recruitment. Mammalian two hybrid assays were used to establish the interaction of PPAR-γ with N-CoR, SMRT, p300/CBP, and TRAP220 in the presence of HA as described in RESEARCH DESIGN AND METHODS. HA dose dependently displaced N-CoR (*A*) and SMRT (*B*) from PPAR-γ, albeit with less efficiency than rosiglitazone. HA failed to induce the interaction with p300 (*C*), CBP (*D*), or TRAP220 (*E*). Each data point is relative to the equivalent concentration in the presence of VP16 alone. Data are representative of at least three independent experiments of quadruplicates.

HA to displace a selective fluorescent PPAR-γ ligand (fluormone PPAR-γ green) from a human-derived recombinant PPAR-γ ligand-binding domain. As shown in Fig. 2*E*, concentration-dependent displacement of the fluorescent PPAR-γ ligand by HA was observed with an IC_{50} of ~32 μmol/l, while rosiglitazone showed an IC_{50} of ~0.095 μmol/l. These data demonstrate that HA binds directly to PPAR-γ in vitro. Similar data were generated showing concentration-dependent disassociation of labeled rosiglitazone by HA using other assay formats including scintillation proximity assays (IC_{50} ~18 μmol/l) and filter-binding approaches (IC_{50} ~30 μmol/l) (data not shown). Together, these data confirm that HA has the ability to directly interact with the ligand-binding domain of PPAR-γ.

Halofenate causes displacement of corepressors but does not cause efficient recruitment of coactivators. Activation of gene expression by the ligand-dependent nuclear hormone receptors is mediated through the dissociation of complexes containing corepressors (e.g., N-CoR, SMRT) and sequential recruitment of coactivators (e.g., p160s/SRC, p300/CBP, TRAP220/DRIP205). To elucidate the molecular basis of PPAR-γ modulation, we examined the ability of HA, relative to rosiglitazone, to modulate the interaction of corepressors and coactivators with PPAR-γ. The mammalian two hybrid system is a useful assay for the study of ligand-dependent nuclear receptor-cofactor interactions. In these experiments, the expression of the reporter gene reflects the extent of physical interaction with a template- and cell-dependent context. We utilized the GAL4-corepressor chimeras encoding the COOH-terminal receptor IDs I+II from N-CoRΔ1 (24) and SMRT (26), which have been demonstrated to mediate the repression of nuclear receptors in the absence of ligands. The GAL4 chimeras encoding either corepressor were expressed with full-length mouse PPAR-γ fused to the transactivation domain of VP16 in the presence and absence of increasing concentrations of rosiglitazone or HA. Controls for these mammalian two-hybrid studies included treating each GAL-cofactor chimera with increasing doses of rosiglitazone and HA with VP16 only (in the absence of VP16-PPAR-γ) as a normalization control (online appendix Fig. 1 [available at http://diabetes.diabetesjournals.org]). Therefore, all GAL-cofactor:VP16-receptor interactions have been normalized to the control at the same dose of drug with VP16 only. Furthermore, MTT viability assays were conducted with HA and rosiglitazone, demonstrating that these compounds did not compromise the viability of the cells (online appendix Fig. 2).

In the absence of agonist, the wild-type PPAR-γ receptor

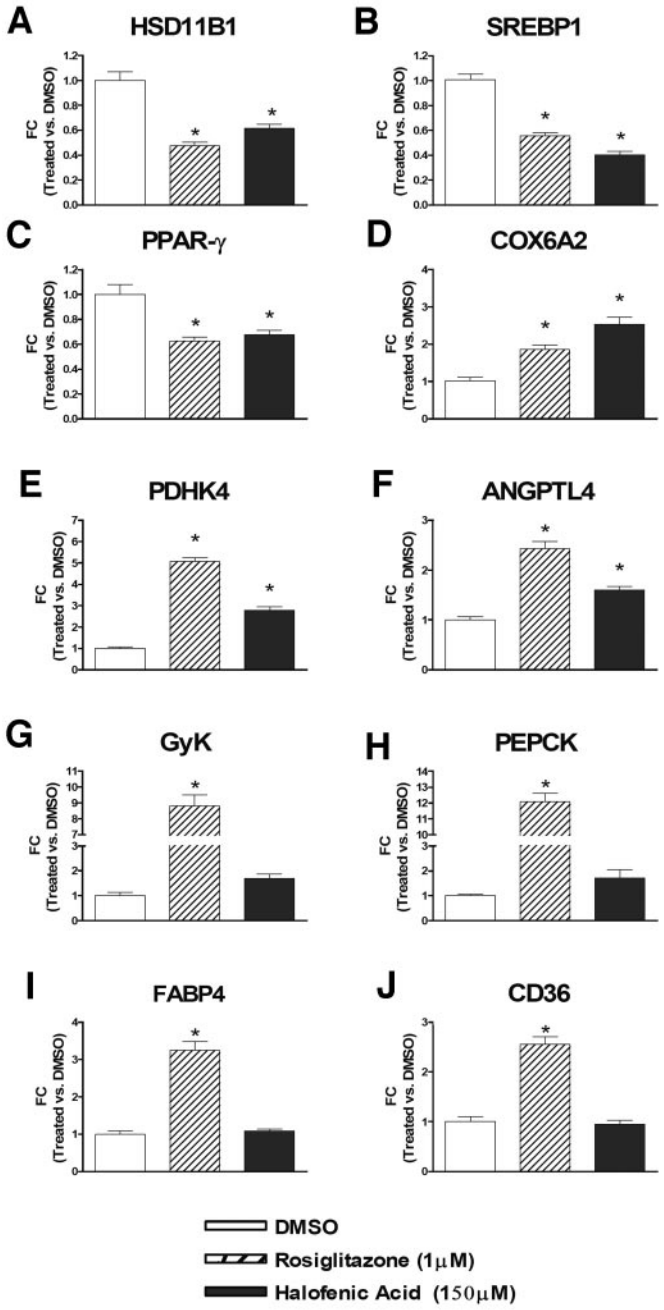

FIG. 4. HA selectively modulates PPAR-γ responsive genes in differentiated 3T3-L1 adipocytes. 3T3-L1 cells were grown to confluence and differentiated into adipocytes. The cells were then treated for 24 h with or without either DMSO (0.1%), HA (150 μmol/l), or rosiglitazone (1 μmol/l). At the end of the treatment period, total RNA was isolated and quantitative fluorescent real-time PCR was performed as described in RESEARCH DESIGN AND METHODS. The expression level of each gene was plotted as relative fold change versus control (DMSO) for the following genes: *HSD11B1*, *SREBP1*, *PPAR-γ*, *COX6A2*, *PDHK4*, *ANGPTL4*, *GyK*, *PEPCK*, *FABP4*, and *CD36*. Ribosomal phosphoprotein P0 (*36B4*) was used as the normalization gene. The figures plot the means ± SE of quintuplicate samples. *$P < 0.05$ vs. control DMSO-treated adipocytes using ANOVA.

efficiently interacted with N-CoR, and this was reflected by a substantial increase (~15-fold [online appendix Fig. 1]) in GAL4-dependent luciferase expression, showing that in an unliganded state, PPAR-γ efficiently recruits and interacts with N-CoR. As expected, treatment with increasing amounts of rosiglitazone resulted in a dose-dependent displacement of N-CoR from PPAR-γ with a K_i of ~0.05 μmol/l (Fig. 3*A*). Similarly, HA was also able to induce a concentration-dependent disassociation of the corepressor N-CoR with a K_i of ~7.6 μmol/l (Fig. 3*A*). Although N-CoR displacement was equally efficient for both compounds, N-CoR displacement by HA was significantly right shifted relative to rosiglitazone.

Similarly, in the absence of agonist, the wild-type PPAR-γ receptor efficiently interacted with SMRT, as reflected by a significant increase in luciferase expression (approximately fourfold [online appendix Fig. 1]). Again, treatment with increasing amounts of rosiglitazone resulted in a concentration-dependent disassociation of the corepressor SMRT from PPAR-γ. HA was also able to induce the displacement of SMRT, with a K_i of ~48 μmol/l, relative to the K_i of ~0.05 μmol/l with rosiglitazone (Fig. 3*B*). In summary, HA induced a concentration-dependent displacement of the corepressors N-CoR and SMRT. HA mediated corepressor disassociation was markedly less potent than that achieved with rosiglitazone, in concordance with the binding and reporter gene data.

We next utilized the GAL4-coactivator chimeras encoding the receptor interaction domains from p300, TRAP220/DRIP205, and CBP, which mediate coactivator interaction with nuclear receptors in an agonist-dependent manner. We examined the ability of the chimeric coactivators to interact with VP16-PPAR-γ and transactivate reporter gene expression in the absence or presence of increasing concentrations of rosiglitazone or HA. In the absence of agonist, the PPAR-γ receptor did not interact with p300, CBP, or TRAP220 (online appendix Fig. 3). Rosiglitazone induced a dose-dependent PPAR-γ–mediated recruitment of the coactivators p300, CBP, and TRAP220 with an EC_{50} between ~0.1 and 0.3 μmol/l (Fig. 3*C–E*). In contrast, HA was unable to induce significant PPAR-γ–mediated coactivator recruitment at any concentration. In our assay, we did not observe any interaction between PPAR-γ and p160 family members (data not shown), and thus, we cannot assess the effects of compounds upon these cofactors. In summary, these data suggest that HA differentially modulates the interaction of PPAR-γ with several coactivators compared with the full agonist, rosiglitazone.

HA modulates some, but not all, PPAR-γ–responsive genes in differentiated 3T3-L1 adipocytes. It has been shown that modulation of PPAR-γ activity alters the expression of numerous genes in adipocytes (27–29). We therefore investigated the effects of HA on mRNA expression of several putative PPAR-γ responsive genes in 3T3-L1 adipocytes. The genes examined included 11-β-hydroxysteroid dehydrogenase type 1 (*HSD11B1*), sterol regulatory element binding transcription factor 1 (*SREBP1*), *PPAR-γ*, cytochrome c oxidase subunit VIa polypeptide 2 (*COX6A2*), pyruvate dehydrogenase kinase isoform 4 (*PDHK4*), angiopoietin-related protein 4 precursor (*ANGPTL4*), glycerol kinase (*GyK*), phosphoenolpyruvate carboxykinase (*PEPCK*), fatty acid binding protein 4 (*FABP4*), and *CD36*, genes that were chosen either based on published literature (21,27–33) or from internal data. Differentiated 3T3-L1 cells were treated for 24 h with or without HA (150 μmol/l), rosiglitazone (1 μmol/l), or DMSO. We chose concentrations of drugs that correspond roughly to five times the IC_{50} in binding studies, which would be predicted to result in a similar degree of receptor occupancy. Rosiglitazone significantly decreased the mRNA levels of *HSD11B1*, *SREBP1*, and *PPAR-γ* and increased the mRNA levels of *COX6A2*, *PDHK4*, *ANGPTL4*, *GyK*, *PEPCK*, *FABP4*, and *CD36* (Fig.

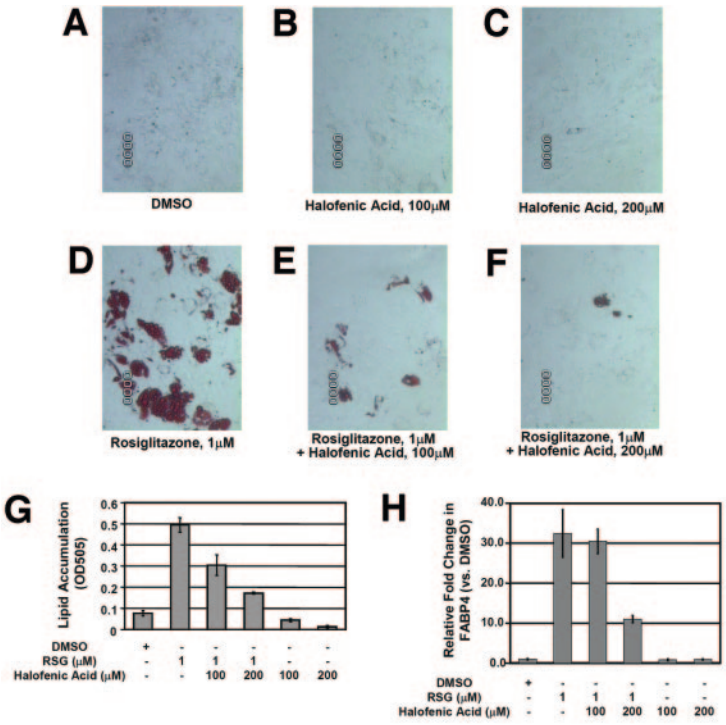

FIG. 5. HA shows weak adipogenic activity and antagonizes rosiglitazone-mediated differentiation in primary human preadipocytes. The effect of HA on primary human preadipocyte differentiation was assessed by Oil Red O staining of accumulated lipids and analysis of transcript levels of the adipogenic marker gene *FABP4*. *A–F*: Differentiation was visualized by Oil Red O staining by microscopy. Rosiglitazone treatment (1 μmol/l) resulted in a significant enhancement of Oil Red O staining, while cotreatment and HA (100 and 200 μmol/l) with rosiglitazone significantly inhibited the enhancement. *G*: Oil Red O staining of accumulated lipids was quantified by measurement of OD_{505}. *H*: Transcript levels of *FABP4* in treated adipocytes were assessed using quantitative real-time PCR. *$P < 0.05$ cotreatment vs. rosiglitazone treatment using ANOVA.

4*A–J*). Similarly, HA significantly decreased the mRNA levels of *HSD11B1*, *SREBP1*, and *PPAR-γ* and increased the mRNA levels of *COX6A2*, *PDHK4*, and *ANGPTL4* (Fig. 4*A–F*). Interestingly, in contrast to rosiglitazone, HA showed only a slight and nonsignificant induction of *GyK* and *PEPCK* (Fig. 4*G* and *H*) and no induction of *FABP4* and *CD36* (Fig. 4*I* and *J*), suggesting that HA is unable to efficiently induce genes involved in fatty acid storage and transport. Overall, these data show that HA is able to selectively modulate PPAR-γ–responsive genes in differentiated 3T3-L1 adipocytes, consistent with its proposed classification as a SPPARγM. The differential gene regulation of HA and rosiglitazone is consistent with the previous observation that both quantitative and qualitative differences exist in the regulation of genes by full and partial agonists (21). The data suggest that HA behaves as a SPPARγM in the differential regulation of gene expression in adipocytes.

HA has weak adipogenic potential and inhibits rosiglitazone-mediated human preadipocyte differentiation. PPAR-γ is a key regulator of adipocyte differentiation, and PPAR-γ modulators have been shown to affect adipocyte differentiation. Full PPAR-γ agonists potently induce adipocyte differentiation, a process that can be antagonized by partial agonists/antagonists (9,34). To further assess the activity of HA as a SPPARγM, we tested its effect on primary human preadipocyte differentiation (Fig. 5). Primary human preadipocytes were treated with a maximal concentration of rosiglitazone (1 μmol/l) or HA (100 or 200 μmol/l) or combinations of the two compounds during differentiation induction. The doses of HA used were roughly three and six times the IC_{50} (~32 μmol/l) in binding studies to assure receptor occupancy. No obvious toxicity was observed during the experiments (data not shown). Ligand-induced differentiation was assessed by both Oil Red O staining and analysis of the transcript levels of the well-known adipogenic marker, FABP4. As expected, treatment with the full PPAR-γ agonist rosiglitazone resulted in a significant enhancement of differentiation in the primary human preadipocytes, as shown by an increase in Oil Red O staining and lipid accumulation (Fig. 5*D* and *G*). HA treatment displayed little adipogenic potential, and cotreatment with rosiglitazone and HA showed a significant inhibition of the rosiglitazone-induced differentiation (Fig. 5*B–G*). Similar observations were seen with *FABP4* mRNA expression levels, an established marker of adipocyte differentiation. Rosiglitazone treatment greatly induced the expression level of *FABP4*, whereas cotreatment with HA (200 μmol/l) significantly decreased rosiglitazone-mediated induction of *FABP4* gene expression levels (Fig. 5*H*). Taken together, the data suggest that HA is able to affect rosiglitazone-induced adipocyte differentiation by acting as a partial PPAR-γ antagonist in human preadipocytes. Because enhancement of preadipocyte differentiation is believed to be partially responsible for increased weight gain after TZD treatment, it is possible that halofenate-based therapeutics will cause less weight gain.

Halofenate displays robust acute antidiabetic properties in *ob/ob* mice. The antidiabetic effects of halofenate were next examined in diabetic mice. Male *ob/ob* mice were treated once daily for 3 days with halofenate at doses of 50, 100, 150, or 200 mg/kg. The body weight of both the vehicle and halofenate-treated animals showed no statistical difference nor was there any difference in food intake between the groups (data not shown). Levels of plasma glucose and insulin were measured 3 h after the third dose. Significant reductions in fasting plasma glucose were seen

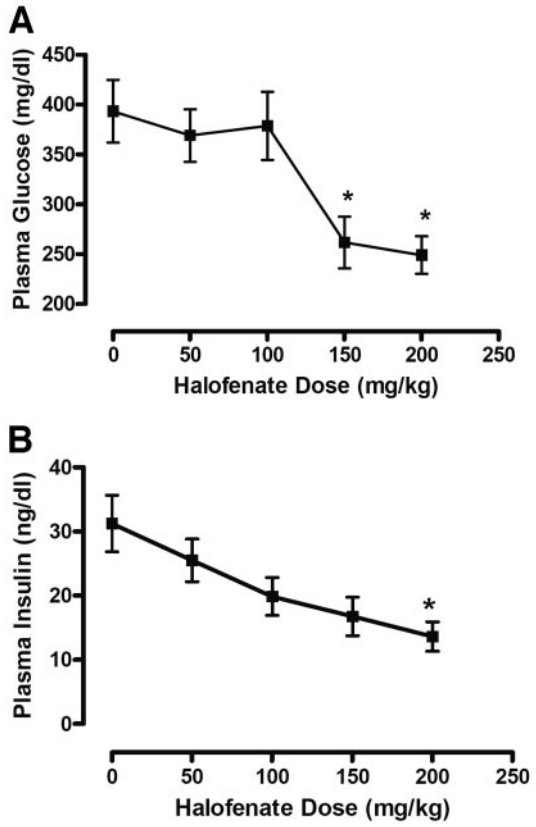

FIG. 6. Halofenate significantly reduces fasting glucose and insulin levels in *ob/ob* mice. Blood samples were taken 3 h after the third dose of vehicle or halofenate for the measurement of fasting plasma glucose and insulin concentrations. Data were derived from eight mice per treatment group (means ± SE). Statistical analysis used ANOVA. *$P < 0.05$ vs. vehicle treatment.

after treatment with halofenate at the two highest doses (Fig. 6*A*) with a calculated ED_{50} of 132 mg/kg. The fasting plasma insulin was also significantly reduced after treatment with halofenate at the dose of 200 mg/kg when compared with vehicle-treated animals (Fig. 6*B*). This acute glucose-lowering and insulin-lowering effect of halofenate observed in *ob/ob* mice is very similar to what has been reported with ciglitazone (35). These data show that halofenate possesses robust antidiabetic activities in vivo.

Halofenate decreases insulin resistance in obese Zucker (*fa/fa*) rats. To examine the insulin sensitization activity of halofenate in longer studies, we utilized the insulin-resistant obese Zucker (*fa/fa*) rat. Obese Zucker rats represent a normoglycemic, hyperinsulinemic, and very insulin-resistant model. Rats were treated with halofenate (200 mg/kg) or rosiglitazone (30 mg/kg) orally, once daily for 28 days. After 20 days of dosing, glucose tolerance tests were performed (Fig. 7). After 20 days of treatment, plasma insulin levels were significantly lower in both halofenate- (1.50 ± 0.17 ng/dl, $P < 0.001$) and rosiglitazone- (1.53 ± 0.15 ng/dl, $P < 0.001$) treated rats compared with vehicle-treated rats (4.17 ± 0.60 ng/dl) (Fig. 7*B*). Because obese Zucker (*fa/fa*) rats have relatively normal glucose levels, no significant decreases in fasting plasma glucose levels were expected (Fig. 7*A*). Nevertheless, fasting plasma glucose levels did show a downward trend for both halofenate- (114.3 ± 6.8 mg/dl) and rosiglitazone- (112.3 ± 6.2 mg/dl) treated rats compared with vehicle-treated rats (137.1 ± 8.7 mg/dl), but these drops were not statistically significant.

In response to an oral glucose load, rosiglitazone significantly lowered plasma glucose and insulin excursions for both glucose and insulin (Fig. 7*A* and *B*). The AUC for plasma glucose in halofenate-treated rats decreased ~30%, and the AUC for plasma insulin decreased ~75% (Fig. 7*C* and *D*). These reductions were very similar and not statistically different from rosiglitazone-treated animals. Together, the oral glucose tolerance experiment showed that halofenate possesses insulin sensitization activity in vivo, which is equivalent to that achieved with rosiglitazone.

In this study, we also evaluated the effect of halofenate on body weight. Rats treated with rosiglitazone gained significantly more body weight than vehicle-treated rats, which was statistically different beginning at day 17 (Fig. 7*E*). In contrast, no significant difference in body weights were seen with halofenate and control animals throughout the 28-day study. In addition, no statistical difference in food intake was observed between the three treatment groups (data not shown). Taken together, these data suggest that halofenate is able to significantly enhance insulin sensitivity without inducing significant body weight gain.

DISCUSSION

Halofenate was tested in the 1970s as a hypolipidemic and hypouricemic drug and was serendipitously found to lower fasting blood glucose in diabetic patients (4–7). In this report, we provide evidence that suggests that halofenate is a SPPARγM. We demonstrate that HA directly binds to human PPAR-γ and selectively activates human PPAR-γ in transactivation assays. Examination of reporter gene activity with human PPAR-γ in three different cell types showed ~10–15% of the activation achieved by rosiglitazone, supporting the designation of HA as a partial agonist. No activation of PPAR-α or -δ was observed. The ability to antagonize a full agonist is a hallmark feature of a partial agonist/antagonist, and we found that HA caused a concentration-dependent displacement of rosiglitazone in competitive binding assays and also demonstrated inhibition of rosiglitazone-mediated gene expression and adipocyte differentiation in human preadipocytes.

Gene expression regulation in 3T3-L1 adipocytes has been described for one PPAR-γ partial agonist (nTZDpa) compared with several full agonists (21). nTZDpa was shown to regulate the expression of numerous genes in a similar manner to the full agonists. However, in contrast to full agonists, nTZDpa modulated the expression of fewer genes and often showed attenuated regulation of genes regulated by full agonists. Similarly, in our study, HA was able to selectively modulate multiple PPAR-γ responsive genes and showed several patterns similar to those reported for nTZDpa (21). Specifically, the patterns observed for *PEPCK*, *CD36*, *HSD11B1*, and *PDHK4* were very similar between nTZDpa and halofenate. Moreover, the lack of ability of HA to induce genes involved in fatty acid storage and transport (*GyK*, *PEPCK*, *FABP4*, and *CD36*) and the inability to promote adipogenesis in human preadipocytes suggests the potential for a reduction in the weight gain associated with full agonists. It is also noteworthy that the degree of suppression of *HSD11B1* appears to be similar between the two compounds and may be part of the mechanism by which these compounds

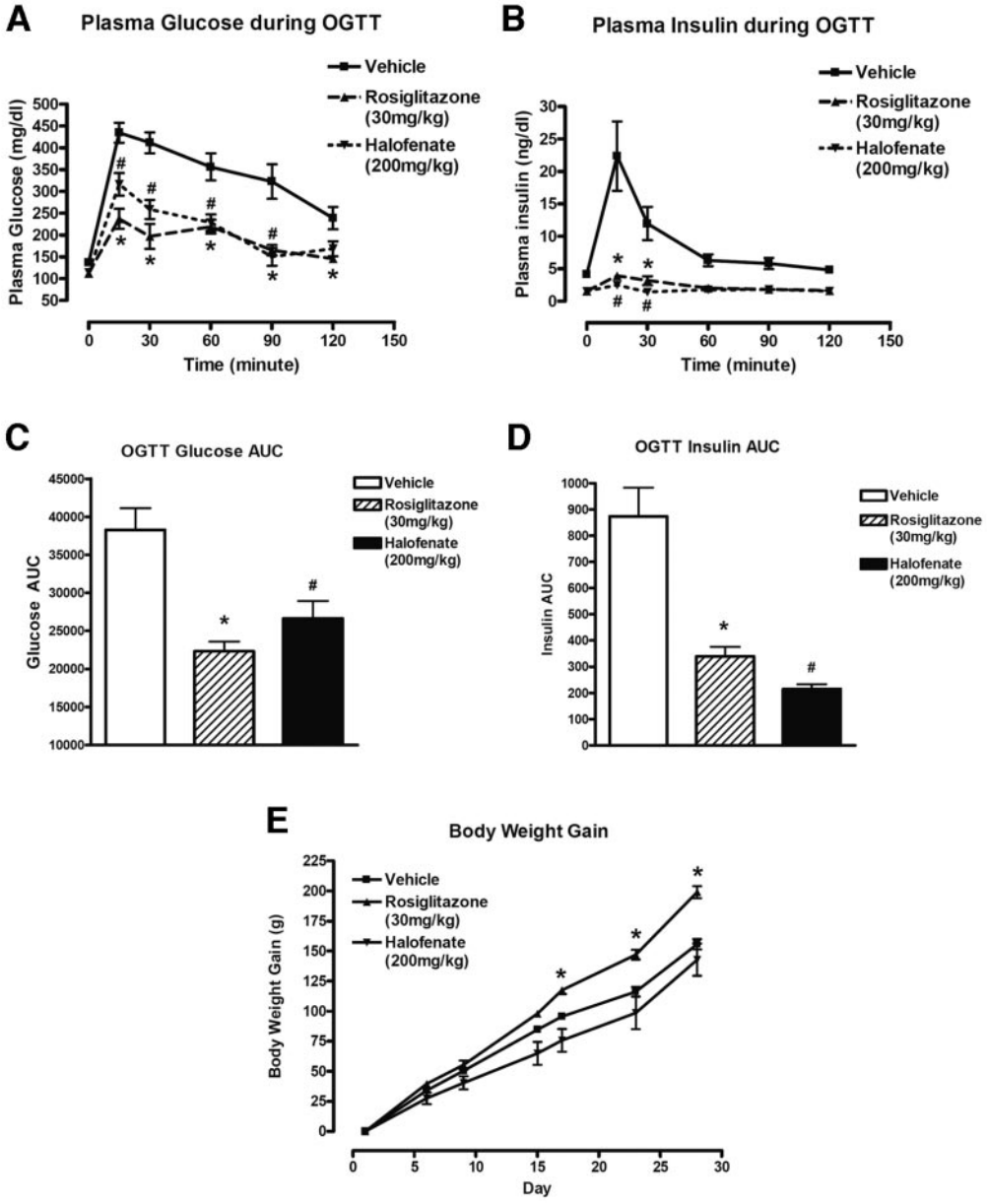

FIG. 7. Halofenate decreases insulin resistance without inducing weight gain in obese Zucker (*fa/fa*) rats. Effect of halofenate treatment on plasma glucose (*A*), plasma insulin (*B*), AUC for plasma glucose (*C*), and AUC for plasma insulin (*D*) during the oral glucose tolerance test in obese Zucker (*fa/fa*) rats. Oral glucose tolerance test were performed after 18 daily doses of vehicle, halofenate (200 mg/kg), or rosiglitazone (30 mg/kg). *E*: Effect of halofenate on body weight gain during the 28 days of treatment. Data were derived from eight rats per treatment group (means ± SE). Statistical analysis used ANOVA. **P* < 0.05 rosiglitazone treatment vs. vehicle treatment. #*P* < 0.05 halofenate treatment vs. vehicle treatment.

sensitize peripheral tissues. Another gene which has been reported to be downregulated by PPAR-γ ligands is *PPAR-γ* itself, and our studies indicate that this effect is observed with both full and partial agonists. The partial induction of *ANGPTL4* (also called *PGAR*) and *PDHK4* is also interesting, although the functional role of these gene products in the adipocyte remains unclear. The repression of *SREBP1* by both rosiglitazone and halofenate is also intriguing, although the physiological significance of these data are also unclear, particularly since at least one study has shown this gene to be upregulated by a tyrosine-based PPAR-γ ligand in white adipose tissue (29). Lastly, we assessed the regulation of *COX6A2*, a mitochondrial protein. HA and rosiglitazone both induced this gene. This is intriguing, given recent reports (36,37) suggesting that modulation of mitochondrial number and function may play a role in the insulin-sensitizing effects of PPAR-γ ligands.

To further elucidate the molecular basis of the modulation of PPAR-γ by HA, we investigated the ligand-dependent modulation of receptor-cofactor interactions in a mammalian cell two-hybrid assay. Previous studies have reported PPAR-γ interaction with a variety of corepressors and coactivators (38). Recent characterization of novel PPAR-γ partial agonists has highlighted the differential coactivator recruitment in comparison to full agonists (20,22,34,39–42). These data have supported the hypothesis that the mechanism underlying differential PPAR-γ modulation is via differential promoter- and tissue-specific cofactor interactions. HA, consistent with its ability to

modulate PPAR-γ, induced disassociation of both N-CoR and SMRT from the receptor, albeit less potently than rosiglitazone. Conversely, HA was unable to recruit the three coactivators tested in this study. These data raise a question as to how a weak partial agonist regulates gene expression despite inefficient coactivator recruitment. One explanation may be that other coactivators not assessed in this study may be recruited to PPAR-γ in the presence of HA. Another explanation may be that the action of HA may reflect PPAR-γ–mediated derepression rather than activation. Recent work (43) supports the concept that derepression, through the displacement of corepressors, rather than activation may play a role in the differential gene regulation. Clearly, our initial findings will require further investigation (e.g., ChIP [chromatin immunoprecipitation] analysis) (44) to better characterize cofactor interactions and their role in modulating gene expression.

The full PPAR-γ agonist rosiglitazone is known to augment preadipocyte differentiation (9), an effect considered to be partially responsible for the deleterious side effects of increased adipogenesis and ultimately in weight gain. Partial PPAR-γ agonists/antagonists that induce little or mild augmentation and/or antagonize full agonist-induced adipocyte differentiation may therefore represent candidates retaining insulin sensitization without causing weight gain (34,45). We observed that HA induced significantly less adipocyte differentiation and lipid accumulation compared with the full agonist and, in addition, suppressed rosiglitazone-mediated adipogenesis. These findings support the conclusion that HA acts as a partial PPAR-γ agonist/antagonist in this human preadipocyte model. The partial PPAR-γ agonism of HA and the lack of ability to recruit adipogenic coactivators (p300, CBP, and TRAP220) may contribute to its weaker adipogenic potential. In addition, the antagonist properties of HA likely contribute to the inhibition of rosiglitazone-mediated adipogenesis.

It is worth noting that current drug discovery approaches would be unlikely to identify compounds such as halofenate. Most modern screens are set up to identify compounds that are potent activators of PPAR-γ in reporter gene assays. As a strong signal in this assay is likely dependent upon efficient recruitment of coactivators, partial agonists such as halofenate would be overlooked. It is our hypothesis that SPPARγMs, which have partial agonist properties and thus modulate gene expression patterns differently than full agonists, will be necessary to maintain efficacy but reduce the side effects of this class of drugs. Since PPAR-γ is known to modulate the expression levels of many genes (up and down) in multiple tissues, it has been very difficult, if not impossible, to identify the exact subset of genes in each tissue that is responsible for the positive and negative effects of PPAR-γ. Indeed, it is serendipitous that halofenate was originally shown to have antidiabetic activity in human subjects. It will be important to generate new approaches with which to identify and optimize SPPARγMs.

There are several areas of investigation that will need to be addressed to fully understand the exact mechanism by which halofenate functions. For example, since this compound is a SPPARγM with no apparent PPAR-α activity, the mechanism by which it lowered triglycerides in past clinical trials needs to be addressed. Other purported PPAR-γ selective agents such as pioglitazone have been shown to have triglyceride-lowering effects, but the mechanism has not been fully elucidated. Another area of investigation will be to understand how such a weak agonist can mediate such profound antidiabetic effects. Although derepression of a set of metabolically important genes may be responsible, it is also possible that it is actually the antagonist properties of halofenate that are important for its activity. In this regard, it is noteworthy that the heterozygote PPAR-γ knockout mouse is paradoxically more insulin sensitive than the wild-type animal, suggesting that an antagonist that mimics this hemizygotic state may itself enhance insulin sensitivity (46,47). The idea that a partial agonist of PPAR-γ could lead to sensitization is supported by the partial loss-of-function mutation of PPAR-γ found in humans in which the Pro12Ala mutation has been associated with a decrease in body mass and an improvement in insulin sensitivity and lipid profiles (48,49).

Here, we provide data that strongly suggest that halofenate is a selective SPPARγM. In vitro, halofenate acts as a partial agonist/antagonist, showing a distinct mechanism of action from that of rosiglitazone that has differing interactions with cofactors and resultant differential effects on gene expression. In the diabetic *ob/ob* model, halofenate acutely lowered glucose and insulin levels in a dose-dependent manner. While acute changes in metabolic parameters are not typically examined with PPAR-γ ligands, it is our experience that rapid effects can be observed with these compounds. These rapid effects have been also reported with other compounds (35). In more typical longer-term studies in the insulin-resistant obese Zucker (*fa/fa*) model, halofenate enhanced insulin sensitivity to a similar degree as rosiglitazone but, unlike rosiglitazone, did not cause any increase in body weight. Taken together, halofenate displays the characteristics of an optimized SPPARγM, retaining insulin sensitization potential with minimal adipogenic activity in vitro and with less weight gain in vivo. Recently reported clinical trial data (50) also strongly support the concept that a compound derived from halofenate did not cause significant increases in body weight in insulin-treated diabetic patients. As these patients are particularly sensitive to the TZD-induced side effects of weight gain and edema, these clinical data are in stark contrast to that observed with the currently marketed compounds. Thus, halofenate and compounds derived from it hold promising therapeutic potential in the treatment of type 2 diabetes, without the side effects of the currently utilized pharmacological agents.

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