Distinct Mechanisms of Glucose Lowering by Specific Agonists for Peroxisomal Proliferator Activated Receptor γ and Retinoic Acid X Receptors

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Agonists for the nuclear receptor peroxisomal proliferator-activated receptor-γ (PPARγ) and its heterodimeric partner, retinoid X receptor (RXR), are effective agents for the treatment of type 2 diabetes. To gain insight into the antidiabetic action of these compounds, we treated female Zucker diabetic rats (ZFF) with AGN194204, which we show to be a homodimer–specific RXR agonist, or the PPARγ agonist, troglitazone. Hyperinsulinemic-euglycemic clamps in ZFF showed that troglitazone and AGN194204 reduced basal endogenous glucose production (EGP) ~30% and doubled the insulin suppression of EGP. AGN194204 had no effect on peripheral glucose utilization, whereas troglitazone increased insulin-stimulated glucose utilization by 50%, glucose uptake into skeletal muscle by 85%, and de novo skeletal muscle glycogen synthesis by 300%. Troglitazone increased skeletal muscle Irs-1 and phospho-Akt levels following in vivo insulin treatment, whereas AGN194204 increased hepatic Irs-2 and insulin stimulated phospho-Akt in liver. Gene profiles of AGN194204-treated mouse liver analyzed by Ingenuity Pathway Analysis identified increases in fatty acid synthetic genes, including Srebp-1 and fatty acid synthase, a pathway previously shown to be induced by RXR agonists. A network of down-regulated genes containing Foxa2, Foxa3, and G-protein subunits was identified, and decreases in these mRNA levels were confirmed by quantitative reverse transcription–PCR. Treatment of HepG2 cells with AGN194204 resulted in inhibition of glucagon-stimulated cAMP accumulation suggesting the G-protein down-regulation may provide an additional mechanism for hepatic insulin sensitization by RXR. These studies demonstrate distinct molecular events lead to insulin sensitization by high affinity RXR and PPARγ agonists.

Thiazolidinediones (TZDs) and other compounds that bind and enhance the transcriptional activity of peroxisomolar proliferator-activated receptor γ (PPARγ) have proven to be effective treatments for insulin-resistant diabetes (1, 2). The increase in insulin sensitivity that occurs after treatment with TZDs likely involves actions in adipose tissue, muscle, and liver (3–6), although the complete set of genes that are modulated to result in improved insulin action by TZDs remains unknown. The heterodimeric partner of PPARγ receptor is the retinoid X receptor (RXR) (7). In overexpression studies, binding of ligand to the RXR receptor has been reported to result in the recruitment of coactivators to the RXR/PPARγ heterodimer (8) and increased transcription from idealized peroxisomal proliferator response elements (PPREs). Some RXR activators can increase transcription of genes in vitro that are also increased by PPARγ ligands (8, 9). Like TZDs, RXR agonists can differentiate 3T3-L1 adipocytes (10), and administration of specific, high affinity RXR agonists to hyperglycemic ob/ob (11) and db/db (12) mice is effective in lowering glucose levels. These results have led to the hypothesis that the antihyperglycemic effect of RXR agonists is due to transactivation of the PPARγ/RXR heterodimer.

In contrast, other studies suggest that in vivo, specific RXR and PPARγ ligands regulate the expression of different genes in adipose tissue and liver, suggesting distinct mechanisms for glucose lowering by these ligands (13). In addition, a recent report showed a different effect of the rexinoid LG268 and the PPARγ activator rosiglitazone on signal pathways in skeletal muscle of diabetic (db/db) mice (14). Although both result in a significant increases in insulin-stimulated glucose transport activity in skeletal muscle, LG268 increased Irs-1 and Akt phosphorylation while rosiglitazone increased the levels of CAP expression and insulin-stimulated c-Cbl phosphorylation without having an effect on the Irs-1/Akt pathway, suggesting distinct sensitizing pathways.

In preliminary studies, we used microarrays to evaluate gene expression in insulin-responsive tissue of diabetic ZFF rats following treatment with the high affinity RXR ligand AGN194204 or troglitazone, a prototypical PPARγ agonist. We found distinct populations of genes were regulated by each compound. In light of this finding, we sought to systematically evaluate the ability of these compounds to increase transcription in vitro and determine their pharmacological activities in vivo. We also utilized a new gene array analysis tool to gain insight into the pharmacological effects of AGN194204. We found that the AGN194204 does not transactivate PPARγ in vitro and lowers glucose in vivo only by suppressing hepatic glucose production without affecting peripheral insulin sensitivity. We also utilized a novel gene expression analysis tool to identify the potential pathways in the liver that contribute to the ability of RXR agonists to lower liver glucose production.

MATERIALS AND METHODS

Transient Transfection Studies—CV-1 cells were grown in 6-well plates with minimal essential medium containing 10% fetal bovine...
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serum and L-glutamine (2 mM). Cells were transfected with plasmids pGL3Luc containing the PPRE fragment of the fatty acid transport protein (FATP) or the acyl-CoA oxidase (ACO) PPRE (0.5 μg) with or without expression constructs pcDNA3 containing RXRα and/or PPARγ (0.5 μg) using FuGene 6 transfection reagent (Roche Applied Science). CMV-β-galactosidase DNA (5 ng) was co-transfected for normalization of transfection efficiency. Twenty-four hours after transfection, cells were treated with different concentrations of troglitazone, AGN194204, AGN195203, or LG100268. After 24 h, cells were extracted and the luciferase activity and β-galactosidase activity were measured using the Dual-light assay system (Tropix, Inc., Foster City, CA). Data are expressed as the ratio of Luc/β-galactosidase activity.

Animals and Biochemical Measurements—Female Zucker diabetic fatty rats (ZDF/Gmi-fa/fa; “ZFF” rats) were purchased from Genetic Models, Inc. (Indianapolis, IN) at 5–6 weeks of age and were fed with a semipurified high fat diet (48% fat, 16% protein, diet 13004) prepared by Research Diets Inc. (New Brunswick, NJ). All animals were approved by the Animal Care and Use Committees at Park-Davis, Inc. and at the University of Michigan, Ann Arbor. After the animals became hyperglycemic (fasting blood glucose > 250 mg/dl, 3–4 weeks on the diet) they were treated orally with vehicle (carboxymethylcellulose), troglitazone as described above. The clamp procedure was initiated in the morning of day 8, following overnight food restriction (6–7 g after 6:00 p.m.). One hour before starting the clamp, glucose and insulin infusions (t = –60 min), a prime-continuous infusion (15 μCi of bolus, 0.15 μCi/min) of [3H]glucose (PerkinElmer Life Sciences) was initiated. At t = –30 min, four arterial plasma samples were obtained at 10-min intervals for determination of plasma glucose specific activity in the basal state. At t = 0 min, a prime-continuous infusion of porcine insulin (72 milliunits·kg−1·min−1; Eli Lilly & Co., Indianapolis, IN) was initiated. Plasma glucose concentration, determined every 5–10 min, was maintained at 100–110 mg/dl by a variable infusion of 45% dextrose. Steady state was generally achieved within 120–150 min, at which time four arterial plasma samples were obtained at 10-min intervals for determination of plasma glucose specific activity. Plasma [3H]glucose specific activity was measured after barium hydroxide-zinc sulfate precipitation. Aliquots of the supernatant were evaporated to dryness to eliminate tritiated water prior to counting.

In a subset of clamp animals, in vivo glucose uptake in skeletal muscle and adipose tissue was determined using the 2-deoxyglucose bolus technique (16) at ~150 min after initiation of the insulin infusion. Tissue accumulation of 2-deoxyglucose was assessed by counting neutralized perchloric acid extracts before (free and phosphorylated) and after (free) barium hydroxide-zinc sulfate precipitation.

Assessment of Liver Insulin Signaling following in Vivo Insulin Administration—Following an overnight fast, three ZFF rats from each treatment group were anesthetized with sodium pentobarbital. A bolus of saline (1.0 ml) was administered via a catheter placed in the jugular vein. The lateral head of the right gastrocnemius muscle was rapidly excised and clamp frozen (basal). A bolus of insulin (10 units/kg body weight in 1.0 ml of saline) was injected via the catheter. The liver and the lateral head of the left gastrocnemius were clamp frozen at 60 and 120 s, respectively, following insulin administration. Liver and muscle lysates were prepared according to the method of Saad et al. (17). Lysates were subjected to Western blot analysis with commercially available antibodies against the Irs-1 or Irs-2 proteins, Akt (purchased from Upstate Biotechnology, Waltham, MA), p85 subunit of phosphoinositide 3-kinase (BD Transduction Laboratories, San Diego, CA), and/or phospho-Akt (Ser-473) (New England Biolabs, Beverly, MA) and developed by enhanced chemiluminescence (ECL).

GeneChip Analysis—GeneChip analysis was performed according to the standard Affymetrix (Santa Clara, CA) protocol using liver RNA from four C57BL/6 mice treated for 7 days with AGN194204. Following probe preparation according to the manufacturer’s instructions, the fragmented cRNAs were hybridized on Affymetrix GeneChips using quantitative controls, processed, and scanned according to the manufacturer.

A total of 1,466 genes prefiltered to have an average difference score of >100 and showed a mean change (both decrease and increase) in expression level by at least 1.5-fold between treated and control samples were used for the network generation and pathway analysis. GenBank™ accession numbers were imported into the Ingenuity Pathway Analysis Application Tool (Ingenuity Systems). Of these, 741 genes were mapped to the Ingenuity Knowledge Base, which were assembled into biological networks and were ranked by score. The score is the likelihood of a set of genes being found in the networks due to random chance (supplemental Table S1). A score of 3 indicates that there is a 1/1000 chance that the focus genes are in a network due to random chance. Therefore, scores of 3 or higher have a 99.9% confidence of not being generated by random chance alone. This score was used as the cut-off for identifying gene networks significantly affected by treatment with AGN194204.

Northern Blot Analysis—Total RNA from liver was subjected to Northern analysis with the indicated cDNA probes, obtained either from the American Type Tissue Collection (Manassas, VA) or generated by PCR amplification and confirmed by direct sequencing. The blots were washed and subjected to autoradiography and quantified by phosphorimaging.

Western Blotting—Membrane fractions for sterol regulatory element-binding protein-1 (SREBP-1) determination from mouse or rat liver were prepared as previously described (18). Twenty micrograms of protein was separated on 4–20% gradient SDS-PAGE gel and transferred to nitrocellulose membranes. Blots were incubated with polyclonal antibody SREBP-1 (Santa Cruz Biotechnology, Santa Cruz, CA) and developed with ECL reagent using goat anti-rabbit antisera. For FAS protein determination, liver tissues were homogenized in extraction buffer containing 1% Triton X-100, 1% Nonidet P-40, 10% glycerol, 50 mM HEPES (pH 7.9), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 5 mM sodium vanadate, and 0.5 mM phenylmethylsulfonyl fluoride and centrifuged at 55,000 × g for 1 h. 25 μg of protein from the supernatant fraction was subjected to Western blot analysis with a monoclonal antibody against FAS (BD Transduction Laboratories, San Diego, CA).
Real-time Reverse-transcription PCR—Total RNA from rat livers (1 μg) was used for reverse transcription reaction in accordance to the manufacturer’s instructions (Promega, Madison WI). Real-time PCR was performed on a DNA Engine Opticon PCR cycler (MJ Research, Waltham, MA), by using the following protocol: one cycle of 15 min at 95°C, 40 cycles of 15 s at 95°C, 30 s at 60°C, 30 s at 72°C, and plate read, 1 cycle of 10 min at 72°C, and 1 cycle of melting curve from 65°C to 95°C. For FoxA2, the anneal temperature is 56°C. The following synthetic oligonucleotide primers were used in real-time PCR: rGnas (forward, 5'-ACGCTTTCCCCAGACGTGCGC-3'; reverse, 5’-GGACGGAGGTCAACCCATTAGT-3'), rGna2 (forward, 5’-AGCCCCCTGACTCTGTTTC-3'; reverse, 5’-CTGGGCTTCGAGGAGGGGCCAC-3'), rGnb2 (forward, 5’-ATCAGATGATGCCACATGTCG-3'; reverse, 5’-CAAGGATCATCCATCATCTTG-3'), rFoxA3 (forward, 5’-CACCCTATTTCTACCCGGCTG-3'; reverse, 5’-GAAAGAGTCATGTCACAGC-3'), rGlut2 (forward, 5’-ACATCTACTGGCCTATCTG-3'; reverse, 5’-CAACCCGCGGATGGTGACATAC-3'), rGcc (forward, 5’-AGGGTCTGCTGGTGAGCCG-3'; reverse, 5’-CTGGTGGCAGGAGGGCTCAAG-3'), and universal 18 S (forward, 5’-ACTCAACAGGGGAAACTCTACC-3'; reverse, 5’-CCAGAACATGCTGCACCACAC-3'). Results of the real-time PCR data were calculated from Ct values where Ct was defined as the threshold cycle of PCR that the amplified product was first detected. The PCR reaction and protocol for the 18 S ribosome were the same as described above. ΔCt was the difference in the Ct values derived from the specific gene being assayed and the 18 S control, whereas ΔCt represented the difference between the paired tissue samples, as calculated by the formula ΔCt = ΔCt of wild type tissue − ΔCt of other tissue. The n-fold differential expression in a specific gene of a sample compared with the wild type counterpart is expressed as 2^ΔCt. Relative RNA equivalents for each sample were obtained by normalizing to 18 S levels. Each of the 3 samples per group was run in duplicate to determine sample reproducibility, and the average relative RNA equivalents per sample pair was used for further analysis.

cAMP Assay in HepG2 Cells—HepG2 cells were grown in 6-well dishes in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and treated with 10 μM troglitazone or 500 nM AGN194204 for 48 h. Cells were starved for 2 h and then incubated with 100 nM glucagon for 30 min, extracted, and assayed for cAMP according to the manufacture’s instructions (R&D Systems, Minneapolis, MN).

Statistical Analysis—Data were analyzed by Student’s t test or analysis of variance with Turkey’s post-hoc testing as appropriate using SAS. Differences were determined to be significant at p < 0.05.

RESULTS

RXR Agonists Do Not Transactivate PPARγ—The RXR agonists used in this study, AGN194204 and AGN195203, were selected as potent activators of RXR subtype homodimers (15). The compounds show no activity toward retinoic acid receptor subtypes (not shown). We determined the ability of these RXR compounds to drive transcription in the context of PPARγ/RXR heterodimers. CV-1 cells, which contain endogenous PPARγ and RXR receptors, were transfected with luciferase reporter constructs containing the PPRE from either the ACO or FATP promoter. The PPARγ agonist troglitazone increased transcription nearly 3-fold, whereas treatment with AGN194204 and AGN195203 caused non-significant increases in transcription from the ACO promoter (Fig. 1A). When AGN194204 and troglitazone were added together, the TZD-induced transcriptional activity was consistently blunted (Fig. 1B). AGN194204 treatment also significantly blunted transcription induced by the PPARγ ligands rosiglitazone and non-thiazolidinedione G62570 (not shown). Overexpression of RXRα, or PPARγ, into CV-1 cells resulted in an increase of ACO-luc activity in response to RXR agonist treatments (Fig. 1B). Because the ACO promoter can be activated in the absence of PPARγ (19), these results suggest that the RXR homodimer is binding to the PPRE of the ACO promoter and activating transcription. We also evaluated transcriptional activity using the FATP PPRE, which binds the PPARγ/RXR heterodimer in preference to RXR or PPARγ homodimers (20). In this context, treatment with troglitazone increased luciferase expression to nearly 3-fold,
whereas neither AGN194204 nor LG100268 (another RXR agonist (21)) induced luciferase reporter expression above baseline (Fig. 1C). Thus, in contrast to previous reports (8, 9, 11), the agents used in the present study show no ability to transactivate PPARγ/H9253/RXR. We also found that rats treated with AGN194204 showed no induction of PPARγ/H9253 target genes in adipocytes following treatment with the RXR agonist (data not shown) suggesting minimal transactivation of PPARγ in vivo.

Metabolic Effects of AGN194204 and Troglitazone in ZFF Rats—Previous studies have suggested a peripheral insulin-sensitizing effect of RXR agonists. To investigate the effect of AGN194204 and AGN195203, we studied the physiological effect of these agents in ZFF. ZFF rats become diabetic after ~3 weeks feeding of high fat diet (20). Treatment of 9- to 10-week-old diabetic ZFF rats (ZFF-diabetic) with AGN194204 (1 mg/kg), AGN195203 (not shown), or troglitazone (400 mg/kg) orally for 7 days lowered blood glucose and insulin to control levels (Fig. 2A). In contrast to troglitazone, AGN194204 treatment resulted in markedly elevated triglyceride (Fig. 2A) and free fatty acid levels (not shown). ZFF rats treated with AGN195203 showed the
same glucose lowering and triglyceride elevating effects (data not shown). Thus, like other RXR agonists, AGN194204 and AGN195203 are effective antihyperglycemic agents but also increase of serum lipid levels.

Site of Action of AGN194204 and Troglitazone by Hyperinsulinemic-Euglycemic Clamp—We next performed hyperinsulinemic-euglycemic clamps with tracer $[^3]$H]glucose infusion in a separate group of ZFF rats to determine the degree of insulin sensitization in the liver and peripheral tissue afforded by each agent. ZFF rats fed high fat diet (ZFF-diabetic) for 5 weeks were ~20 g heavier, and had significantly higher fasting serum glucose and insulin concentrations than those rats fed a standard chow diet (TABLE ONE). Treatment of the diabetic rats with AGN194204 or with troglitazone resulted in an additional increase in body weight (5.8% and 11.3%, respectively).

Steady-state plasma glucose concentrations during the insulin clamp were not significantly different among groups (TABLE TWO). Steady-state plasma insulin concentrations, however, were 2-fold higher in the untreated diabetic rats compared with the obese controls, despite identical insulin infusion rates (72 milliunits kg$^{-1}$ min$^{-1}$), suggesting that insulin clearance was impaired in the diabetic rats. Plasma insulin concentrations in the diabetics treated with RXR agonists or troglitazone were decreased 25.5% and 21.4%, respectively, but still slightly higher compared with control group.

Basal endogenous glucose production (EGP), likely reflecting primarily hepatic glucose production, was increased ~25% in ZFF-diabetic rats compared with obese controls (Fig. 2B). Suppression of EGP by insulin during the hyperinsulinemic phase of the clamp was impaired in the diabetic rats, such that glucose production during steady-state in the diabetics was 2-fold higher than that in obese controls, even in the face of significantly higher steady-state insulin concentrations. Treatment of ZFF-diabetic with AGN194204 or troglitazone restored basal EGP to

**TABLE ONE**

| Fasting plasma glucose and insulin concentrations in ZFF rats following 7 days of treatment with RXR or PPARγ agonists |
|--------------------------------------------------|
| Body weight | Plasma glucose | Plasma insulin |
| g           | mg/dl      | ng/ml      |
|----------------|
| Obese (9)  | 252.9 ± 6.6 | 138.3 ± 3.7 | 6.3 ± 0.6 |
| Diabetic (9) | 273.7 ± 5.6 | 179.3 ± 9.7 | 17.3 ± 9.7 |
| 4204 (5)    | 290.2 ± 7.0 | 165.0 ± 5.2 | 5.4 ± 0.6 |
| Troglitazone (6) | 305.4 ± 9.0 | 134.9 ± 5.1 | 5.6 ± 0.6 |

**TABLE TWO**

| Steady-state glucose infusion rates and plasma glucose and insulin concentrations during the hyperinsulinemic-euglycemic clamp in ZFF rats |
|--------------------------------------------------|
| Glucose infusion rate | Plasma glucose | Plasma insulin |
| mg kg$^{-1}$ min$^{-1}$ | mg/dl      | ng/ml      |
|----------------|
| Obese (8)  | 14.1 ± 0.7 | 102.0 ± 2.2 | 509 ± 59 |
| Diabetic (8) | 8.5 ± 1.8 | 110.0 ± 2.4 | 1030 ± 126 |
| 4204 (5)    | 11.8 ± 1.4 | 108.9 ± 1.7 | 767 ± 38 |
| Troglitazone (5) | 21.7 ± 1.1 | 112.4 ± 0.9 | 809 ± 75 |

**FIGURE 3.** De novo glycogen synthesis and glycogen levels in skeletal muscle and liver. A and B, $[^3]$H]glucose incorporated into plantaris (A) and liver (B) glycogen during hyperinsulinemic-euglycemic clamp. C and D, glycogen levels in plantaris (C) and liver (D) at termination of clamp. *, significantly different ($p < 0.05$) from diabetic.
control levels and completely normalized insulin suppression of EGP during the clamp.

The rate of glucose infusion for maintaining steady-state plasma glucose levels in ZFF-diabetic rats during the insulin clamp was 40% less than that required in obese controls (TABLE TWO). Insulin-stimulated whole body glucose disposal (R\text{w}g), however, was only slightly lower in the diabetics than in the obese controls (Fig. 2C). These findings indicate that the primary effect of the high fat diet in the ZFF female rat is on glucose production by the liver, with little change in insulin action in the already highly insulin-resistant peripheral tissues. Troglitazone treatment increased the rate of insulin-stimulated whole body glucose disposal by ~50% (versus ZFF-diabetic). Interestingly, ZFF-diabetic rats treated with AGN194204 showed no improvement and even a slight decline in R\text{w}g. Thus, although the PPAR\textgamma agonist troglitazone improved both peripheral and hepatic insulin actions, the RXR agonists selectively improved glucose metabolism in the liver, with no significant improvements in insulin sensitivity in peripheral tissues.

Insulin-stimulated glucose uptake (R\text{s}g) into skeletal muscle (gastrocnemius) was decreased somewhat in the ZFF-diabetic compared with the obese control rats, although this difference was not statistically significant (Fig. 2E). Glucose uptake in skeletal muscle was not improved following 7 days of treatment with RXR agonists (4.5 ± 0.9 \mu mol/g of wet wt/min, and 4.9 ± 0.9 \mu mol/g of wet wt/min in vehicle-treated and AGN194204-treated ZFF-diabetics, respectively), a finding that is consistent with the lack of improvement in whole body glucose disposal following RXR treatment. Glucose uptake in white adipose tissue in the ZFF-diabetic was not different from obese controls, and was not significantly changed following RXR treatment (Fig. 2F). Glucose uptake was assessed in only two troglitazone-treated animals, the R\text{s}g in skeletal muscle was 7.2 and 10.1, R\text{s}g in white adipose tissue was 0.9 and 1.0. These values were higher than any corresponding value in the diabetic or AGN194204 groups. In addition, clamp studies in two diabetic ZFF rats treated with AGN195203 showed identical results to those seen in the AGN194204-treated rats (not shown).

In agreement with the uptake data, the amount of \textit{de novo} glycogen synthesis (as assessed by \[^{3}H\]glucose incorporated into glycogen) was increased in the ZFF-diabetic rats following treatment with troglitazone to levels above that of the obese, non-diabetic controls (Fig. 3A). In parallel with R\text{w}g measurement AGN194204 treatment had no effect on \textit{de novo} glycogen synthesis in muscle. Neither compound had an effect on glycogen incorporation into glucose in the liver during the clamp (Fig. 3B). On the other hand, glycogen levels were slightly, but significantly higher at the end of the clamp period in the AGN194204 muscles compared with diabetic animals (Fig. 3C) and significantly lower in the liver of troglitazone treated rats (Fig. 3D). The combination of lower glycogen synthesis and higher glycogen levels in the RXR agonist-treated skeletal muscle suggests that there is a lower glycogen turnover. In contrast there may be an elevated glycogen turnover in the liver of troglitazone-treated animals.

**Insulin Signaling Molecule Expression in AGN194204 and Troglitazone-treated ZFF-diabetic Animals**—We examined the expression levels of proteins thought to be important in the early signaling cascade of insulin in skeletal muscle and liver. In this study, ZFF rats were rendered diabetic by 3 weeks of high fat diet and then treated for 7 days with either AGN194204 or troglitazone. A small piece of gastrocnemius muscle was snap frozen \textit{in situ} in anesthetized rats and insulin injected into the inferior vena cava. After 60 s, the left lobe of the liver was snap frozen followed by the contralateral gastrocnemius after 2 min. In liver, AGN194204 treatment resulted in a marked increase in the levels of Irs-2 protein without changes in Irs-1, p85 subunit of phosphoinositide 3-kinase, or Akt (Fig. 4A). Following intravenous insulin bolus, there was a reduction in phosphorylated Akt in the ZFF-diabetic rat liver compared with obese, non-diabetic animals. Following AGN194204, but not troglitazone treatment, there was an increase in the amount of phosphorylated Akt following insulin administration (Fig. 4A). In skeletal muscle, there was a small but consistent increase in total Irs-1 levels in troglitazone treated ZFF-diabetic rats without changes in the other signaling molecules (Fig. 4B). Insulin treatment stimulated Akt phosphorylation in each group, but the increase was far greater in the troglitazone-treated ZFF-diabetic rats. There was no significant change in the phospho-Akt levels in AGN194204 treated rats compared with the untreated diabetic rats. These results are consistent with a primary effect of the RXR agonist in the liver with the primary effect of troglitazone to increase peripheral insulin sensitivity.
Expression Profiling of Liver following RXR Treatment—To gain insight into the mechanism underlying the improvement in insulin response in the liver by RXR treatment, we examined expression levels of mRNA in the liver of mice using Affymetrix GeneChips. The data were analyzed using a Web-based expression analysis program from Ingenuity that uses a genome-scale biological knowledge base and gen-
erates multiple biological networks with associated functional analysis (www.ingenium.com). Mice were treated for 3 days with AGN194204 or vehicle and total liver mRNA from four mice in each group hybridized to an Affymetrix 11K Mouse Array. Hierarchical maps were generated to find metabolic pathways populated by genes that changed an average of 2-fold following treatment with the RXR activator compared with controls. A total of 23 (of 71) defined pathways were populated by at least 4 transcripts with 8 having ≥15 members (supplemental Table S1). Not unexpectedly, a highly populated pathway centered on Srebp-1c (Network 4), which was up-regulated 12-fold following RXR treatment (Fig. 5B). The genes that populated this include a number of other genes that were not originally identified as being part of the family or did not have changes that would be normally assessed because of low-fold change results.

To confirm alterations in the expression of a subset of genes in this cluster, we performed a time-course experiment. RXR agonist treatment in diabetic db/db mice resulted in a decrease in blood glucose after 7 days and an increased levels of serum triglyceride and liver triglyceride levels after 1, 3, or 7 days of administration (TABLE THREE). In parallel, SREBP-1c protein and mRNA levels increased after only 1 dose of RXR and continued elevated for the 7 days of treatment (Fig. 5, B and C). Interestingly, the levels of fatty acid synthase mRNA also increased after 1 day, but we did not detect a significant increase FAS protein until 3 days of treatment (Fig. 5C).

We also found that the expression levels of genes in this network in the livers of ZFF rats were affected by AGN194204 treatment. FAS, ACO, 3-keto-CoA thiolase, and fatty acid-binding protein were markedly up-regulated by RXR agonist treatment, whereas the mRNA levels of these genes were down-regulated or not changed following troglitazone treatment (Fig. 5D). In parallel, we found that the protein levels of SREBP-1 and FAS were increased following AGN194204 treatment (Fig. 5E). Interestingly, while the gene profiling in normal mouse liver suggested an increase in PEPCK levels, the mRNA levels were decreased in the livers of diabetic rats treated with both RXR and troglitazone. This is likely due to the profiling in non-diabetic mouse liver versus diabetic rat liver.

A second gene expression network was also significantly changed in the normal mouse liver following AGN194204 treatment (Fig. 6A). This network consisted of genes found in a cluster containing G-protein subunits coupled to the glucagon receptor as well as Foxa, which suggested an additional potential mechanism for the improvement in hepatic insulin resistance seen following treatment with the RXR agonist.

When we determined the expression levels of a subset of these genes in the livers of diabetic ZFF rats following treatment with AGN194204 and troglitazone, we found that as predicted by Affymetrix profiling, FoxA3 was significantly lower in the AGN194204-treated livers compared with vehicle treated and again, we found no significant alteration following troglitazone treatment.

We detected no change in the levels of the glucagon receptor mRNA in livers of ZFF rats following treatment with either troglitazone or AGN194204 (Fig. 6B). A statistically non-significant decrease was found in Glut2 mRNA levels following RXR treatment. Both RXR and troglitazone treatment resulted in a significant decline in the mRNA levels of the G-protein subunit Gαi3, whereas only treatment with AGN194204 resulted in decreased expression of Gαi3 and GB3 (Fig. 6B).

To determine if the changes in G-protein subunit levels have a functional consequence, we incubated HepG2 cells with either troglitazone or AGN194204 for 24 h and assessed the generation of cAMP in response to glucagon stimulation. Glucagon treatment caused a significant increase in cAMP levels over baseline in both the vehicle and troglitazone-treated cultures. An increase in baseline cAMP levels was found after RXR, but glucagon did not stimulate a further increase, suggesting desensitization of the receptor.

**DISCUSSION**

The TZDs are an important therapeutic option in the treatment of type 2 diabetes. By activating the PPARγ receptor, TZDs enhance peripheral insulin sensitivity in both humans and animal models of insulin resistance and type 2 diabetes (2). Prototypic RXR ligands that show glucose lowering in rodents have been suggested to do so by transactivation of the PPARγ/RXR heterodimer (11, 12). In this study, we demonstrate that ligands for RXR that do not activate transcription through the PPARγ heterodimer, as assessed by transcription from the PPRE of two different PPARγ regulated gene promoters, can be effective agents in lowering blood glucose in an animal model of insulin resistant diabetes. The absence of activation of ACO and FATP PPRE-driven reporter gene expression by the RXR agonist AGN194204 or AGN195035 in CV-1 cells without over-expression of RXRa receptors suggests that the PPARγ/RXR heterodimer is not consistently activated by this RXR agonist. In fact, in the context of endogenous PPARγ and RXR, AGN194204 diminished TZD-induced transcriptional activity. The increase in reporter gene activity after overexpression of RXR gene in CV-1 cells may be due to homodimer formation. Indeed, a recent publication demonstrated using in vivo chromatin immunoprecipitation that overexpressed RXR homodimers can selectively bind to functional PPREs and induce transactivation in vivo (22). These data suggest that the relative abundance of receptors that form heterodimers play a role in the relative ability of a ligand to activate or repress gene transcription.

In determining the mechanism whereby RXR agonists lower glucose levels we used the ZFF rat, which is hyperphagic, obese and transition to diabetes only when given a high fat, high carbohydrate diet (20). The mechanisms underlying the significant insulin resistance at baseline in these female rats is presumably due to significant obesity and elevated levels of free fatty acids. However, a recent paper suggested that free fatty acids have a minimal effect on the insulin resistance of female rats (23). Interestingly, the transition to diabetes appears to involve primarily a decline in β-cell function, as there is no change in the significant...
peripheral insulin resistance and only a small increase in basal endogenous glucose production (Fig. 2). Previously in rodents, TZD treatment results in both increased peripheral insulin sensitivity and decreased hepatic glucose production (24, 25) and these effects are confirmed in the present study. In addition, we found that the primary site of increased glucose uptake is in skeletal muscle (Fig. 3) with an accumulation of \textit{de novo} synthesized glycogen. In contrast, the effect of the RXR agonist is confined to the liver, with a significant reduction in hepatic glucose production at baseline and following insulin infusion. We found no effect of the RXR agonist to enhance peripheral glucose disposal in
skeletal muscle. This is in contrast to an earlier study which showed a similar improvement in the suppressibility of hepatic glucose production but also a small (~20%) increase in peripheral glucose disposal (13). Any peripheral insulin sensitizing effect following AGN194204 treatment may be counteracted by the increased triglyceride levels found following treatment.

Hypertriglyceridemia is a recognized side effect of RXR agonist therapy and can be a limitation in the development of these compounds (26, 27). In our hands, both ZFF rats and male db/db mice respond to short term RXR agonist treatment with an elevation in serum triglyceride levels. In other reports, db/db mice respond to a variety of RXR agonist with lowering of serum triglyceride level while rats routinely respond with elevations (12, 28–30). Interestingly, in Haffner et al. (30), male ZDF rat showed elevations in triglyceride levels at the lower RXR agonist doses with decreasing effects at higher doses while glycemia was improved in a dose–response manner. In our study, db/db mice showed a small, but consistent elevation in serum and liver triglyceride. However, the elevations in the serum were not as pronounced by 7 days of treatment. The differences in mice and rats may be due to adaptation of the periphery in mice, with an increase in the ability to clear triglycerides produced in the liver, perhaps by modulation of lipoprotein lipase activity (28).

One mechanism proposed for the increase in triglyceride synthesis is the “transactivation” of the RXR/LXR heterodimer leading to increases in SREBP-1 transcription (31). However, our gene profiling analysis suggests an additional potential mechanism for elevations in hepatic triglyceride synthesis. Gene expression and quantitative RT–PCR demonstrated a significant decrease in Foxa2 mRNA levels. Foxa2, one of a forkhead family of transcription factors, appears to be important in regulating fatty acid oxidation as well as SREBP-1 levels in rodents (32). Foxa2 increases the expression of fatty acid–oxidizing enzymes in the liver, and Foxa2 activity can be inhibited by insulin via both Irs-1 and Irs-2 signaling pathways, which sequester Foxa2 in the cytoplasm. AGN194204 treatment restores Irs-2 activity in the liver, which, along with suppressed Foxa2 levels, would result in a decrease in the expression of fatty acid-oxidizing genes and an increase in SREBP-1c levels (32) and other genes associated with de novo triglyceride synthesis. Thus, a high fat diet combined with the inhibition of Foxa2 expression could explain the marked elevations in peripheral triglyceride levels in these ZFF rats treated with RXR agonists.

The production of glucose by the liver is regulated primarily by the relative action of glucagon and insulin to activate or suppress both glycolysis and gluconeogenesis. Irs-2 is critical for proper suppression of hepatic glucose production by insulin and disruption of Irs-2, but not Irs-1, results in increased hepatic glucose output (33). The decrease in Irs-2 has been proposed to decrease phosphorylation of FoxO1 leading to increased nuclear FoxO1 and activation of PEPCK expression, the rate-limiting enzyme for gluconeogenesis. The restoration of Irs-2 following RXR treatment is likely one factor in the improvement in glucose tolerance. The decrease in PEPCK levels in animals treated with AGN194204 may reflect this enhanced signaling. The mechanism by which troglitazone improves insulin signaling in the liver is not entirely clear; however, the increased adiponectin levels following TZD treatment (3) may result in improvement in hepatic insulin sensitivity.

Gluconag is also important for the regulation of hepatic glucose production. Glucagon increases glycolysis by activation of the classic protein kinase cascade involving binding to its G-protein coupled receptor and activating the GTP-dependent dissociation of the βγ-subunit from the GoS subunit. This triggers a series of cascades, including activation of cAMP-dependent protein kinase, which results in an increased breakdown of hepatic glycogen (34). Glucagon also increases gluconeogenesis by increasing the transcription of PEPCK via the cAMP response element-binding protein (35). Small down-regulation of G-protein subunits are associated with an impairment in glucagon action (36), which would result in inhibition of glucagon-mediated hepatic glucose output by decreasing cAMP signaling via the FoxA protein (37–39), which itself is down-regulated following RXR treatment. Significantly, this cluster of genes was not altered by treatment with PPARγ activators, again suggesting a different mechanism for insulin sensitization by the PPARγ and RXR agonists.

In summary, we have shown that RXR agonists can be effective in lowering blood glucose by a mechanism that is likely independent of PPARγ/RXR heterodimer transactivation. RXR insulin-mediated insulin sensitization appears to be confined to the liver and may have multiple mechanisms, including changes in insulin signaling protein expression and changes in glucagon responsiveness. The hypertriglyceridemic effects may be due to alteration in FoxA transcription factor expression as well as a potential transactivation of RXR/LXR receptors. Further dissection of the hepatic effects of novel RXR agonists could lead to identification of new targets for the treatment of type 2 diabetes. Finally, these studies confirm the power of a novel analytical tool to enhance the interpretation of gene expression data sets which may lead to the identification of new in vivo metabolic pathways that may be targets for antidabetic agents.

REFERENCES

1. Vasudevan, A. R., and Balasubramanyam, A. (2004) Diabetes Technol. Ther. 6, 850–863
2. Alarcon de la Lstra, C., Sanchez-Fidalgo, S., Villegas, I., and Motilva, V. (2004) Curr. Pharm. Des. 10, 3505–3524
3. Tonelli, J., Li, W., Kishore, P., Pajvani, U. B., Kwon, E., Weaver, C., Scherer, P. E., and Hawkins, M. (2004) Diabetes 53, 1621–1629
4. Miyazaki, Y., Mahankali, A., Wajjeb, E., Bajaj, M., Mandarino, I. J., and DeFronzo, R. A. (2004) J. Clin. Endocrinol. Metab. 89, 4312–4319
5. Sturnovoll, M. (2003) Expert Opin. Investig. Drugs 12, 1179–1187
6. Burant, C. F., Sreenan, S., Hiranbo, K., Tai, T. A., Lohnmiller, J., Lukens, J., Davidson, N. C., Ross, S., and Graves, R. A. (1997) J. Clin. Invest. 100, 2980–2986
7. Mangelsdorf, D. J., and Evans, R. M. (1995) Cell 83, 841–850
8. Martin, G., Schoonjans, K., Leffebre, A. M., Staels, B., and Auwerx, J. (1997) J. Biol. Chem. 272, 28210–28217
9. Schulman, I. G., Shao, G., and Heyman, R. A. (1998) Mol. Cell. Biol. 18, 3483–3494
10. Canan Koch, S. S., Dardashti, L. J., Cesarro, R. M., Croston, G. E., Boehm, M. F., Heyman, R. A., and Nadzan, A. M. (1999) J. Med. Chem. 42, 742–750
11. Leonard, J. M., Lancaster, M. E., Paili, M. A., Weiss, J. E., Binz, J. G., Sundsseth, S. S., Gaskill, A. R., Lightfoot, R. M., and Brown, H. R. (1999) Diabetes 42, 545–554
12. Mukherjee, R., Davies, P. J., Cumbibie, D. L., Bischoff, E. D., Cesarro, R. M., Jow, L., Hamann, L. G., Boehm, M. F., Mondon, C. E., Nadzan, A. M., Paterniti, J. R., Jr., and Heyman, R. A. (1997) Nutrition 36, 407–410
13. Davies, P. J., Berry, S. A., Shipley, G. L., Eckel, R. H., Hennuyer, N., Cumbibie, D. L., Ogivie, K. M., Peinado-Osuribe, J., Pfeett, C., Leibowitz, M. D., Heyman, R. A., and Auwerx, J. (2001) Mol. Pharmacol. 59, 170–176
14. Sheng, Q., Cline, G. W., Shulman, G. I., Davies, P. J., Corsets, J. P., Sparks, J. D., Peterson, R. G., Smith, R. L., and Sparks, C. E. (2000) J. Med. Chem. 43, 3452–3459
15. Beard, R. L., Colon, D. F., Song, T. K., Davies, P. J., Kochhar, D. M., and Chandraratna, M. A. (2000) Mol. Endocrinol. 14, 219–231
16. James, D. E., Jenkins, A. B., and Kraegen, E. W. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 935–938
17. Kreig, M., Mahouf, A., and Wahl, W. (1995) Mol. Endocrinol. 9, 219–231
18. Corsetti, J. P., Sparks, J. D., Peterson, R. G., Smith, R. L., and Sparks, C. E. (2000) Atherosclerosis 148, 238–241
19. Liu, Y. L., Seminit, M. V., Hsiaop, D. C., Cumbibie, D. L., Heyman, R. A., and Cawthorne, M. A. (2000) J. Obes. Relat. Metab. Disord. 24, 997–1004
20. A. J. F., Tan, N. S., Gelman, L., Kersten, S., Seydloux, J., Xu, J., Metzger, D., Canable, L., Chambon, P., Wahl, W., and Desvergne, B. (2004) EMBO J. 23, 2083–2091
21. Hevener, A., Reichart, D., Janez, A., and Ofelsky, J. (2002) Diabetes 51, 1907–1912
22. Sugiyama, Y., Shimura, Y., and Ikeda, H. (1990) Arzneimittelforschung 40, 436–440
25. Ye, J. M., Dzamko, N., Cleasby, M. E., Hegarty, B. D., Furler, S. M., Cooney, G. J., and Kraegen, E. W. (2004) Diabetologia 47, 1506–1513
26. Duvic, M., Martin, A. G., Kim, Y., Olsen, E., Wood, G. S., Crowley, C. A., and Yocum, R. C. (2001) Arch. Dermatol. 137, 581–593
27. Rigas, J. R., and Dragnev, K. H. (2005) Oncologist 10, 22–33
28. Standeven, A. M., Thacher, S. M., Yuan, Y. D., Escobar, M., Vuligonda, V., Beard, R. L., and Chandraratna, R. A. (2001) Biochem. Pharmacol. 62, 1501–1509
29. Standeven, A. M., Escobar, M., Beard, R. L., Yuan, Y. D., and Chandraratna, R. A. (1997) Biochem. Pharmacol. 54, 517–524
30. Haffner, C. D., Lenhard, J. M., Miller, A. B., McDougald, D. L., Dwornik, K., Ittoop, O. R., Gampe, R. T., Jr., Xu, H. E., Blanchard, S., Montana, V. G., Consler, T. G., Bledsoe, R. K., Ayscue, A., and Croom, D. (2004) J. Med. Chem. 47, 2010–2029
31. Repa, J. J., Liang, G., Ou, J., Bashmakov, Y., Lobaccaro, J. M., Shimomura, I., Shan, B., Brown, M. S., Goldstein, J. L., and Mangelsdorf, D. J. (2000) Genes Dev. 14, 2819–2830
32. Wolfrum, C., Asilmaz, E., Luca, E., Friedman, J. M., and Stoffel, M. (2004) Nature 432, 1027–1032
33. Shimomura, I., Matsuda, M., Hammer, R. E., Bashmakov, Y., Brown, M. S., and Goldstein, J. L. (2000) Mol. Cell 6, 77–86
34. Jiang, G., and Zhang, B. B. (2003) Am. J. Physiol. 284, E671–E678
35. Yeagley, D., Agati, J. M., and Quinn, P. G. (1998) J. Biol. Chem. 273, 18743–18750
36. Bouchardel, R., Matsuzaki, Y., Le, M., Gettys, T. W., and Fromm, H. (1998) Am. J. Physiol. 274, G1151–G1159
37. Kaestner, K. H. (2000) Trends Endocrinol. Metab. 11, 281–285
38. Lin, B., Morris, D. W., and Chou, J. Y. (1997) Biochemistry 36, 14096–14106
39. Wang, J. C., Stafford, J. M., Scott, D. K., Sutherland, C., and Granner, D. K. (2000) J. Biol. Chem. 275, 14717–14721