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Differential Effects of Rexinoids and Thiazolidinediones on Metabolic Gene Expression in Diabetic Rodents

HARLEEN SINGH AHUJA, SHA LIU, DIANE L. CROMBIE, MARCUS BOEHM, MARK D. LEIBOWITZ, RICHARD A. HEYMAN, CHRISTOPHE DEPRE, LASZLO NAGY, PETER TONTONOZ, and PETER J. A. DAVIES

Departments of Integrative Biology and Pharmacology (H.S.A., P.J.A.D.) and Cardiology (C.D.), University of Texas Medical School, Houston, Texas; Ligand Pharmaceuticals, San Diego, California (S.L., D.L.C., M.B., M.D.L., R.A.H.); and The Salk Institute for Biological Studies, La Jolla, California (L.N., P.T.)

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ABSTRACT

Both retinoid X receptor (RXR)-selective agonists (rexinoids) and thiazolidinediones (TZDs), PPAR (peroxisome proliferator-activated receptor)-γ–specific ligands, produce insulin sensitization in diabetic rodents. In vitro studies have demonstrated that TZDs mediate their effects via the RXR/PPAR-γ complex. To determine whether rexinoids lower hyperglycemia by activating the RXR/PPAR-γ heterodimer in vivo, we compared the effects of a rexinoid (LG100268) and a TZD (rosiglitazone) on gene expression in white adipose tissue, skeletal muscle, and liver of Zucker diabetic fatty rats (ZDFs). In adipose tissue, rosiglitazone decreased tumor necrosis factor-α (TNF-α) mRNA and induced glucose transporter 4 (GLUT4), muscle carnitine palmitoyl-transferase (MCPT), stearoyl CoA desaturase (SCD1), and fatty acid translocase (CD36). In contrast, LG100268 increased TNF-α and had no effect or suppressed the expression of GLUT4, MCPT, SCD1, and CD36. In liver, the rexinoid increased MCPT, SCD1, and CD36 mRNAs, whereas rosiglitazone induced only a small increase in CD36. In skeletal muscle, rosiglitazone and LG100268 have similar effects; both increased SCD1 and CD36 mRNAs. The differences in the pattern of genes induced by the rexinoids and the TZDs in diabetic animals found in these studies suggests that these compounds may have independent and tissue-specific effects on metabolic control in vivo.

Two classes of nuclear receptor ligands, thiazolidinediones (TZDs) and rexinoids, have been shown to lower hyperglycemia and hyperinsulinemia in diabetic rodents (Mukherjee et al., 1997). TZDs are ligands for peroxisome proliferator-activated receptor-γ (PPAR-γ), a nuclear hormone receptor that has been demonstrated to be critically involved in regulating both the differentiation and metabolism of adipocytes (Tontonoz et al., 1994; Rocchi and Auwerx, 1999). Studies in diabetic rodents and in man have demonstrated that TZDs increase insulin sensitivity in adipose tissue and muscle (Oakes et al., 1994; Komers and Vrana, 1998). Although the precise molecular basis for this insulin sensitizing effect is not fully understood, it is clear that PPAR-γ is a key regulator of the expression of a number of genes [such as tumor necrosis factor-α (TNF-α) and glucose transporter 4 (GLUT4)] that are involved in the control of glucose homeostasis and insulin signaling pathways (Young et al., 1995; Okuno et al., 1998).

Recently, we have reported that rexinoids [retinoid X receptor (RXR) ligands] also have antidiabetic effects in vivo (Mukherjee et al., 1997). RXRs are nuclear receptors that serve as obligate heterodimeric partners for a number of nuclear receptors, including PPARs (Mangelsdorf and Evans, 1995). In cotransfection studies, RXR ligands have been shown to be as effective as PPAR-γ ligands in activating RXR/PPAR-γ heterodimers and, in cultured preadipocytes, both rexinoids and TZDs induce adipose differentiation (Mukherjee et al., 1997; Tontonoz et al., 1997). Given these in vitro results, one explanation for the insulin-sensitizing effects of rexinoids is that they might be "TZD-mimetics", activating RXR/PPAR-γ heterodimers in vivo and producing the same metabolic effects as TZDs. However, there are important differences between the pharmacologic activities of rexinoids and TZDs. First, whereas PPAR-γ ligands can only activate RXR/PPAR-γ heterodimers, RXR ligands can activate a number of different heterodimers that may play important roles in metabolic regulation. RXRs can also form

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ABBREVIATIONS: TZD, thiazolidinedione; PPAR, peroxisome proliferator-activated receptor; TNF-α, tumor necrosis factor-α; GLUT4, glucose transporter 4; RXR, retinoid X receptor; OGTT, oral glucose tolerance test; AUC, area under the curve; ZDF, Zucker diabetic fatty rat; PCR, polymerase chain reaction; dNTP, deoxynucleotide triphosphates; RT, reverse transcriptase; MCPT, muscle carnitine palmitoyl transferase; SCD1, stearoyl coenzyme A desaturase 1; CD36, fatty acid translocase; CoA, coenzyme A; ANOVA, analysis of variance.

homodimers, and ligand activation of homodimers may have its own unique profile of pharmacologic activity. Second, there are major differences in the tissue-specific pattern of expression of RXRs and PPAR-γ. In adipose tissue, PPAR-γ expression levels are very abundant compared with that of RXRs. In contrast, RXR transcript levels are significantly higher than PPAR-γ in both muscle and liver. Although the role of thiazolidinediones in insulin resistance has been the subject of many studies, almost nothing is known about rexinoids. We are therefore interested in determining whether rexinoids are TZD-mimetic or have their own selective profile of effects on metabolic gene expression. As a first step in delineating the mechanisms by which these compounds act, we first establish rexinoids as insulin sensitizers and then identify molecular targets that these compounds regulate under conditions where they reduce serum glucose.

For these reasons, we have undertaken a comparison of the changes in gene expression induced in diabetic rodents in response to rexinoid and TZD administration. We have treated diabetic rats with either a prototypic TZD, rosiglitazone (BRL49653), or a prototypic RXR-specific retinoid, LG100268, at concentrations sufficient to produce normalization of serum glucose levels. The results we have obtained clearly demonstrate that in adipose tissue and liver, rexinoids and TZDs have very different effects on the expression of key PPAR-γ regulated genes that are involved in glucose or lipid metabolism (Young et al., 1995; Miller and Ntambi, 1996; Okuno et al., 1998; Mascaro et al., 1998; Tontonoz et al., 1998). In contrast, both classes of compounds have common molecular targets in skeletal muscle. It seems that even though TZDs and rexinoids induce insulin sensitization in diabetic rodents, they do so by distinct alterations in tissue specific patterns of metabolic gene expression.

Materials and Methods

Oral Glucose Tolerance Test (OGTT). Male Zucker fatty rats were treated with either vehicle or LG100268 (30 mg/kg/day) starting at 8 weeks of age. A group of Zucker lean rats was also dosed with vehicle as healthy control animals. After 2 weeks of treatment, animals were fasted overnight before the OGTT (2 g of glucose/kg of body weight). Whole blood was obtained through tail vein before and up to 2 h after the glucose challenge. Plasma was prepared for determination of glucose and insulin levels. The Insulin Resistance Index for each animal was calculated using the glucose area under the curve (AUC) × the insulin AUC.

Euglycemic-Hyperinsulinemic Clamp. Euglycemic-hyperinsulinemic clamps were performed using methods similar to those published previously (Lee et al., 1994; Barzilai et al., 1995). Briefly, male Zucker fatty rats were treated with vehicle or LG100268 (10 mg/kg) for 9 to 11 days and indwelling arterial (carotid) and venous (jugular) cannulae were implanted 4 to 6 days before the clamp procedure. On the day of the clamp, animals were dosed and the food was removed. Cannulae were connected via polyethylene tubing to syringe-pumps and the animal was permitted to habituate to the testing chamber. Infusion, via the venous cannula, of [^{3}H]glucose (5 μCi bolus followed by 0.1 μCi/min; PerkinElmer Life Sciences, Boston, MA) was begun 4 h after dosing and followed 1 h later by insulin (10 mU/kg/min; Humulin, Eli Lilly & Co., Indianapolis, IN). Blood was obtained via the arterial cannula to determine glucose levels at 5-min intervals. Euglycemia (100 ± 10 mg/dl) was maintained by a variable rate infusion of unlabeled glucose. Hepatic glucose production and glucose disposal were calculated as described elsewhere (Lee et al., 1994).

Serum Glucose Levels. Male Zucker diabetic fatty rats were obtained from Genetic Models Inc. (Indianapolis, IN) at 6 weeks of age and allowed to acclimate for 2 weeks before initiation of treatment. ZDFs were randomized and dosed orally by either rosiglitazone (10 mg/kg/day) or LG100268 (30 mg/kg/day) for 2 weeks. Plasma samples were obtained under isoflurane anesthesia in the fed state 3 h after dosing via the tail. Glucose concentrations were measured on days 0, 3, 7, and 14. Animals were euthanized after 2 weeks and samples of epididymal white adipose tissue, muscle, and liver were dissected and snap frozen in liquid nitrogen for RNA analysis.

RNA Isolation. Tissues were homogenized in Trireagent (Molecular Research Center, Inc., Cincinnati, OH) with a Dounce homogenizer, precipitated with isopropanol and the homogenates were applied to RNeasy spin columns (Qiagen). RNA was eluted and treated with RNase-free DNase for 30 min at 37°C, followed by heat inactivation at 75°C, and stored at −70°C.

Development of Synthetic RNA. Synthetic RNAs were made using either the cDNA product of total RNA or plasmid DNA. Templates were first amplified with 1× PCR buffer, 4 mM $MgCl_2$, 500 μM dNTPs, 300 nM T7 partial sequence attached to the specific forward primer, 300 nM reverse primer, and 1 U/50 μl *Taq* polymerase (Roche Molecular Biochemicals, Indianapolis, IN) at 95°C for 1 min; 95°C for 12 s, 60°C for 30 s; and 72°C for 1 min, for 30 cycles. An aliquot of the PCR product was further amplified in the presence of 1× PCR buffer, 4 mM $MgCl_2$, 500 μM dNTPs, 300 nM extra long T7 primer, 300 nM reverse primer, and 1 U/50 μl *Taq* Polymerase at 95°C for 1 min; 95°C for 12 s, 55°C for 30 s; and 72°C for 1 min, for 30 cycles, followed by an elongation at 72°C for 5 min. Seven microliters of the extra long T7 PCR product was added to 2 μl each of 10× buffer, ATP, CTP, GTP, UTP, T7 Mega enzyme mix (all provided in the Ambion MEGAshortscript Kit; Ambion, Austin, TX) and 0.5 μl of [^{32}P]UTP and incubated at 37°C overnight. The sRNA was DNase-treated at 37°C for 15 min, and 1 μl of the reaction was used for quantification. The remaining was precipitated with glycogen, ammonium acetate, and isopropanol. The pellet was resuspended in water and 1 μl was trichloroacetic acid-precipitated to quantitate percent incorporation. Synthetic RNAs were serially diluted from 20 pg to 2 fg to generate a standard curve.

Reverse Transcription and Quantitative Polymerase Chain Reaction. Aliquots (100 ng) of each RNA from multiple rodents [control (n = 10), LG100268-treated (n = 5), and rosiglitazone treated (n = 6)] to be analyzed were reverse transcribed in quadruplicate [including an RT(−)] for each sample with 1× PCR buffer, 300 nM reverse primer, 4 mM $MgCl_2$, 500 μM dNTPs, and Superscript II (Life Technologies, Gaithersburg, MD) at 42°C for 30 min, followed by 72°C for 5 min. The RT reaction (20 μl) was added to a 30-μl PCR mix containing 1× PCR buffer, 300 nM forward primer, 4 mM $MgCl_2$, *Taq* polymerase, and 100 nM fluorogenic probe. Amplification was performed by use of the ABI Prism 7700 (Applied Biosystems, Norwalk, CT) at 95°C for 1 min, followed by 40 cycles of 95°C for 12 s and 60°C for 1 min. Data were analyzed by the use of the Sequence Detection Application and absolute values of RNAs were generated by normalizing copy number values of the gene of interest to the copy number values of 36B4 ribosomal protein (Laborda, 1991).

Statistical Analysis. Statistical calculations were performed using Jandel's SigmaStat software (High Text Interactive, San Diego, CA). Statistical significance between groups was evaluated using one-way analysis of variance. Differences between groups were determined by the Tukey test. Results are presented as mean ± S.D., with $P < 0.05$ considered statistically significant.

Results

Rexinoids Reduce Hyperinsulinemia and Hyperglycemia in Diabetic Rats. Vehicle-treated Zucker fatty obese rats had higher glucose levels than lean animals after an

overnight fast, as indicated by the time 0 glucose levels (162 ± 6.4 versus 117 ± 3.8 for vehicle-treated obese and lean rats, respectively, $n = 6$, $P < 0.05$, Fig. 1A). LG100268 treatment (30 mg/kg/day) for 2 weeks produced a minor but statistically significant decrease in fasting plasma glucose levels (137 ± 5.5, $n = 6$, $P < 0.05$ versus vehicle-treated obese animals). After an oral glucose challenge, plasma glucose levels increased dramatically in vehicle-treated obese animals to more than 300 mg/dl at 30 and 60 min, compared with ~200 mg/dl at the same time points in lean and LG100268-treated obese rats. At all time points after the glucose challenge, the glucose levels in LG100268-treated obese animals were significantly lower than those of vehicle-treated obese animals ($P < 0.05$). Fasting insulin levels were also significantly reduced in the LG100268-treated Zucker fatty rats (9.14 ± 0.87, 3.82 ± 0.49, and 0.35 ± 0.02 for vehicle-treated obese, LG100268-treated obese, and vehicle-treated lean rats, respectively; Fig. 1B, time 0). After the glucose challenge, plasma insulin levels were increased considerably in vehicle-treated Zucker fatty rats. In comparison, there was only a moderate increase in insulin levels for the LG100268-treated animals. The calculated insulin resistance indices (glucose AUC × insulin AUC) were 808 ± 173, 203 ± 11, and 26 ± 1.8 for vehicle-treated obese, LG100268-treated obese, and vehicle-treated lean rats, respectively (Fig. 1C). The significantly lowered insulin resistance index indicates that LG100268 treatment resulted in a significant improvement in insulin sensitivity in the obese animals.

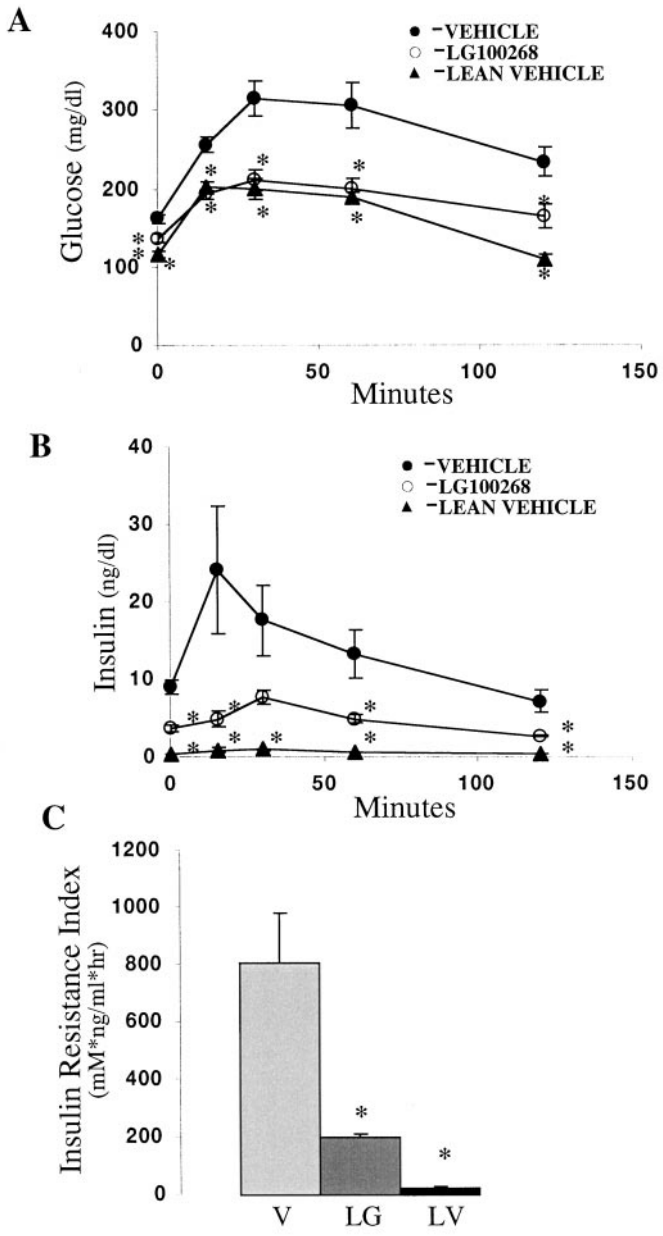

Fig. 1. Oral glucose tolerance test and serum glucose levels in Zucker rats. Male Zucker fatty rats were dosed with either vehicle or LG100268 (30 mg/kg/day). A group of Zucker lean rats was also dosed with vehicle as healthy control animals. After 2 weeks of treatment, animals were fasted overnight before the OGTT (2 g/kg). Plasma levels of glucose (A) and insulin (B) were determined before and up to 2 h after the glucose dosing. Insulin resistance index for each animal was calculated using the glucose AUC × the insulin AUC (C). *$P < 0.05$ versus vehicle-treated Zucker fatty rats using Student's t test.

To elucidate the site of action for the insulin-sensitizing effect of LG100268, euglycemic-hyperinsulinemic clamp studies were performed in 5 h-fasted Zucker fatty rats that had been treated either with vehicle ($n = 7$) or LG100268 ($n = 8$; 10 mg/kg/day) for 9 to 11 days (Table 1). The rate of glucose infusion needed to maintain euglycemia under insulin infusion was increased by 120% in LG100268-treated animals compared with vehicle-treated animals. The glucose disposal rate was increased significantly by LG100268 treatment, indicating an increase in insulin-stimulated glucose uptake in peripheral tissues. Simultaneously, hepatic glucose production was decreased by 41% in LG100268-treated animals, suggesting that there was an increase in liver insulin sensitivity as well.

Comparison of the Effects of Rosiglitazone and LG100268 on Plasma Glucose. Given the insulin sensitizing effects of LG100268, we asked whether this RXR ligand targets a molecular pathway similar to that of TZDs. To this end, we determined and compared the effects of LG100268 and rosiglitazone on a set of transcripts that have shown to be regulated by TZDs and/or contain PPAR response elements (PPREs). To ensure that the effects observed were carried out under comparable circumstances, we chose conditions (maximal doses) in which both compounds induced an equivalent lowering of plasma glucose of diabetic rats (Fig. 2A). In a time course study, control ZDF rats ($n = 10$), rosiglitazone-treated ZDF rats ($n = 6$), and LG100268-treated ZDF rats ($n = 5$) received vehicle, or maximal doses

TABLE 1
Euglycemic-hyperinsulinemic clamp

Treatment	n	Glucose Infusion Rate	Glucose Disposal Rate	Hepatic Glucose Production
			mg/kg/min	
Vehicle	7	5.29 ± 0.94	13.18 ± 0.78	7.50 ± 0.91
LG100268 (10 mg/kg)	8	11.67 ± 0.63*	16.06 ± 0.63*	4.43 ± 0.53*

* $p < 0.05$ vs. vehicle treatment (student's ± test).

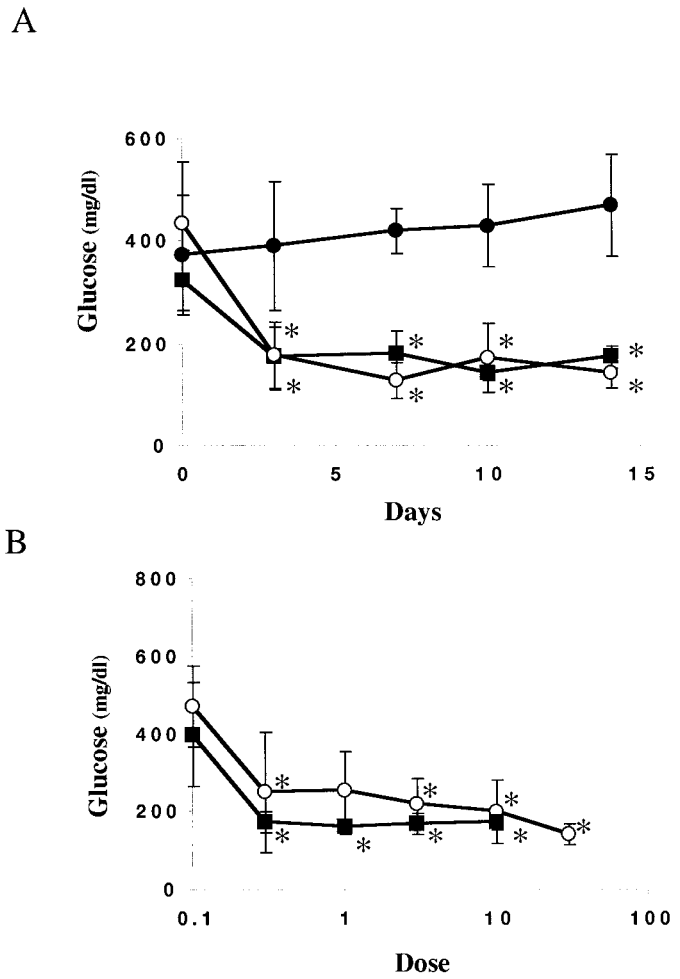

Fig. 2. Comparison of the effects of rosiglitazone and LG100268 on plasma glucose levels in ZDF rats. A, time course of Zucker diabetic fatty rats treated with vehicle (●), rosiglitazone (10 mg/kg, ■), or LG100268 (30 mg/kg, ○) over a course of 14 days. Average plasma glucose levels were measured on days 0, 3, 7, 10, and 14. B, dose response on serum glucose levels of Zucker diabetic fatty rats treated rosiglitazone (■) or LG100268 (○) at doses of 0.1, 0.3, 1, 3, 10, or 30 mg/kg) for 14 days. Significant within-group difference comparing treatment and control values (ANOVA, $*P < 0.001$).

of rosiglitazone (10 mg/kg) or LG100268 (30 mg/kg), respectively, by oral gavage daily for 2 weeks. Plasma glucose was determined on days 0, 3, 7, 10, and 14 (Fig. 2A). In the control animals, plasma glucose increased from 380 mg/dl to greater than 470 mg/dl over the 2-week period. Administration of either rosiglitazone or LG100268 resulted in a prompt decrease in plasma glucose as early as 3 days after initiating treatment. By 2 weeks, plasma glucose levels had dropped to approximately 170 and 145 mg/dl in rosiglitazone and LG100268-treated ZDF rats, respectively. Animals were euthanized and tissues were collected for transcript analysis on day 14. In a dose response, rosiglitazone significantly lowered glucose levels with a dose of 0.3 mg/kg (Fig. 2B). Similar effects were observed with 1.0, 3, and 10 mg/kg doses. Higher doses (10 and 30 mg/kg) were required for LG100268 to produce similar effects to rosiglitazone.

Development of Quantitative PCR Assays. We have used "real time" Q-RT-PCR to quantify the level of transcripts for nuclear receptors, cytokines, transporters, and enzymes in RNA prepared from tissues of control, rexinoid-, and TZD-treated ZDF rats (Depre et al., 1998; Depre et al., 1999). For each transcript, specific PCR primer pairs and a dual fluorochrome-tagged hybridization probe (Taqman probe) were designed using ABI's Primer Express software package (Table 2). When possible (regions were not too GC rich), assays were designed on intron-exon junctions to avoid signals from genomic DNA. No amplification controls (without reverse transcriptase) were performed on all samples to rule out the possibility that the signal was derived from DNA contamination. No template controls were also used. Blast searches were performed with each amplicon to establish specificity with the desired target. Assays for each transcript were optimized for $MgCl_2$, primer, and probe concentrations. Under optimal conditions, each assay was calibrated against serial dilutions of a cognate synthetic RNA (sRNA) template. A standard curve of the Ct (the number of the PCR cycles required to reach a threshold level of probe dequenching) versus the log of the input sRNA template molecules was developed for each assay.

Total RNA from ZDF rat tissues was assayed for each transcript in parallel with sRNA template standards and the

TABLE 2
Primers and probes used for real-time quantitative PCR assays
For assays that were originally developed for mouse sequences, accession numbers are listed for both mouse (M) and rat (R) sequences.

Assay	Primers (5′-3′)	Taqman Probe (5′-3′)	Accession No.*
36b4	(189+)AGATGCAGCAGATCCGCAT (269−)GGATGGCCTTGCGCA	(222+) F-AGGCTGTGGTGCTGATGGGCAAGAACT	X15096
RXR-α[a]	(979+)CTGAGCTGCCCCTAGACGA (1074−)CTTTCACAGCTATGGAGCGG	(1014+) F-CGGGCAGGCTGGAACGAGCT-T	M: M84817 R: L06482
PPAR-γ	(1213+)ATGGAGCCTAAGTTTGAGTTTGC (1348−)TGTCCTCGATGGGCTTCA	(1322−) F-AAGCCTGGGCGGTCTCCACTGA-T	M: NM_011146 R: AF156665 (γ1) R: AF156666 (γ1)
TNF-α	(243+)ATCATCTTCTCAAAACTCGAGTGA (368−)TTGAGATCCATGCCATTGG	(276+) F-AGCCCACGTCGTAGCAAACCACC-T	X66539
GLUT4	(783+)CCCCCGATACCTCTACATCATC (872−)GCATCAGACACATCAGCCCAG	(821+) F-CTGCCCGAAAGAGTCTAAAGCGCCT-T	M25482
mCPT	(870+)ATCATGTATCGCCGCAAACT (952−)ATCTGGTAGGAGCACATGGGT	(925−) F-CCCAGTGCCATTACCGGCTTGA-T	D43623
SCD1[a]	(3012+)TAGCTCCAGTGAGGTGGTGTG (3127−)GTGGGTTTGTTACAAGAGAAAGGATA	(3035+) F-AGGTTAGCAAAGCCACCATCTGCTGG-T	M: M21285 R: J02585
CD36	(762+)TAATGGCACAGATGCAGCCT (857−)ACAGCATAGATGGACCTGCAA	(802+) F-AAGTCTCAAACACTGAGGTTCTTTTCCTCTGACA-T	AF072411

[a] Although there are mismatches between mouse and rat sequences (nucleotides underlined), these mismatches do not effect the assays.
F, Fam; T, Tamra.

number of transcript molecules was calculated by interpolation of the Ct value with the sRNA standard curve. To correct for variations in RNA input into the assays, all samples were normalized to the number of 36B4 transcripts (mRNA for ribosomal protein PO, a housekeeping gene). To confirm that the levels of 36B4 were not being altered themselves, we compared these values to a second normalizer, cyclophilin (data not shown). Linear regression analysis of 36B4 and cyclophilin transcript levels showed a significant relation between them ($R^2 = 0.83$, $P < 0.05$). Therefore, the values of transcript abundance were expressed as a percent of 36B4 molecules.

Nuclear Receptor Expression Levels. We first used real time quantitative PCR assays to quantify the transcript levels of PPAR-γ (measuring both PPAR-γ1 and PPAR-γ2), RXR-α, and RXR-γ in whole adipose, muscle, and liver tissues collected from control ZDF rats (Table 4). PPAR-γ was most abundant in adipose tissue, the level in adipose tissue was 20-fold higher than in skeletal muscle. The level of PPAR-γ in liver was very low, approximately 2% of the level in adipose tissue. RXR-α transcripts in the three tissues were more consistent than PPAR-γ. The liver had the most abundant levels of RXR-α. However, because of the heterogeneity of cell types that make up the liver, we were not able to determine distribution of receptor levels among different the different cell types. The levels of RXR-α transcripts in muscle and adipose tissue were comparable, approximately 25% of that found in liver. RXR-γ transcripts on the other hand were most abundant in muscle, followed by liver and adipose. It is noteworthy that in adipose tissue, the level of PPAR-γ transcript exceeds that of RXR-α whereas the situation in muscle and liver is reversed with RXR-α and RXR-γ transcripts present at significantly higher levels than PPAR-γ.

Comparison of Rosiglitazone and LG100268 Effects on Adipose Tissue Gene Expression. Treatment of ZDF rats with rosiglitazone resulted in marked changes in the level of expression of transcripts for key regulatory enzymes in adipose tissue (Figs. 3–4). TNF-α transcripts were suppressed by rosiglitazone to 37% of control (Fig. 3A) and there was a reciprocal increase in GLUT4 mRNA (Fig. 3B). In contrast, LG100268 increased TNF-α and decreased GLUT4 modestly. Rosiglitazone also induced a dramatic increase in the levels of MCPT1 and SCD1 in adipose tissue (Fig. 4, A and B). CD36 was also increased in rosiglitazone-treated animals although the -fold increase was less than that of MCPT or SCD1 (Fig. 4C). Again, the effects of LG100268 on gene expression were quite different from those of rosiglitazone. MCPT and CD36 mRNA levels were lowered by LG100268 and no effect was observed on SCD1 transcripts. Given the differences in the effects we observed on transcript levels with maximal doses of rosiglitazone (10 mg/kg) and LG100268 (30 mg/kg) and that rosiglitazone effectively lowered serum glucose levels with a minimal dose of 0.3 mg/kg (see Fig. 2B), we also measured the effects of rosiglitazone on transcript levels at a dose of 0.3 mg/kg. We found that, similar to its effects on serum glucose, rosiglitazone displayed no effect at 0.1 mg/kg but significantly induced SCD1 transcripts by 2- to 3-fold at 0.3 mg/kg (data not shown). Thus in adipose tissue, the effect of LG100268 on gene expression is completely different from the effects of rosiglitazone (at minimal or maximal doses). Under conditions in which both compounds produce an equivalent lowering of plasma glucose, LG100268 suppressed the expression of genes that rosiglitazone induced and, in the case of TNF-α, induced a gene that rosiglitazone suppressed.

Comparison of Rosiglitazone and LG100268 Effects on Skeletal Muscle Gene Expression. It has been suggested that the insulin-sensitizing effects of TZDs are caused by a direct effect on gene expression in skeletal muscle (Burant et al., 1997). Assessment of the effects of rosiglitazone on transcript levels in muscle from ZDF rats is complicated by considerable interanimal variability in both control and treated animals. For this reason, Fig. 5 presents both the individual animal data (○) as well as the aggregate data (mean ± S.D., ●). The levels of TNF-α transcripts in skeletal muscle of either control, TZD, or rexinoid-treated animals were below the detection limits of our very sensitive quantitative PCR assay. The basal level of GLUT4 and MCPT transcripts, however, could be measured, and treatment with either rosiglitazone or LG100268 had no effect on transcript levels in ZDF rats (Fig. 5, A and B). The basal level of SCD1 and CD36 were also measurable. Rosiglitazone produced significant increases in CD36 and SCD1 transcripts in skeletal muscle (Fig. 5, C and D). LG100268 also increased the levels of both SCD1 and CD36 transcripts. Thus, although rosiglitazone and LG100268 have very different effects on gene expression in adipose tissue, they have similar effects on these two genes in skeletal muscle.

Comparison of Rosiglitazone and LG100268 Effects on Hepatic Gene Expression. In contrast to adipose tissue, there is very little PPAR-γ in the liver of ZDF rats. Animals treated with rosiglitazone for 14 days showed no significant changes in MCPT and SCD1 transcripts (Fig. 6, A and B). However, the low levels of PPAR-γ were sufficient to produce modest increases in CD36 transcripts by rosiglitazone (Fig. 6C). The liver has the highest RXR-α mRNA levels and treatment with LG100268 resulted in a marked increase in hepatic gene expression. LG100268 significantly increased

TABLE 3
Standard curve parameters

Assay	Amplicon (bp)	Ct[a,b]	ΔRn_{max}[b]	Slope (Ct/log transcript)	Correlation Coefficient	Lower Limit of Detection (Molecules)
36b4	81	14.3	12	−3.4	0.997	11,000
PPAR-γ	136	13.3	18	−3.4	1.000	1,900
RXR-α	96	13.6	26	−3.4	0.996	12,000
TNF-α	126	13.6	8	−3.4	0.994	170
GLUT4	90	14.1	3	−3.2	0.990	410
mCPT	83	14.7	9	−3.4	0.994	3,000
SCD1	116	14.4	8	−3.4	0.994	7,100
CD36	96	14	12	−3.2	0.998	18,000

[a] Number of PCR cycles required to reach threshold (10 times the S.D. of the baseline).
[b] Values for 2 pg standard of individual sRNAs.

the level of transcripts for MCPT, SCD1, and CD36 in ZDF livers.

Discussion

The goal of our studies has been to investigate the mechanisms involved in the glucose lowering activity of rexinoids. Previous studies from our laboratories have shown that rexinoids resemble TZDs in their ability to suppress the fasting hyperglycemia of genetically obese mice (Mukherjee et al., 1997). In addition, cotransfection experiments have demonstrated that rexinoids and TZDs share the ability to activate RXR/PPAR-γ heterodimers. Thus, a very reasonable model to explain the glucose-lowering activity of rexinoids in vivo is that they mimic the activity of TZDs by activating the same RXR/PPAR-γ heterodimers, inducing transcription of the same set of genes and thereby producing equivalent effects on glucose metabolism and insulin sensitivity. To test this model, we first demonstrated the insulin-sensitizing effect of rexinoids. We show here that LG100268 improves whole-body insulin sensitivity in Zucker fatty rats. Furthermore, LG100268 treatment increases glucose disposal in peripheral tissues and decreases hepatic glucose production during euglycemic-hyperinsulinemic clamp. These results demonstrate that rexinoids produce insulin sensitization in both hepatic and peripheral tissues. We then determined whether rexinoids replicate the effects of TZDs on gene expression. We selected a set of genes that meet at least two of the following criteria: 1) have been shown to be regulated by TZDs (TNF-α, GLUT4); 2) have an identified PPRE for the RXR/PPAR heterodimer (MCPT, SCD1, CD36); 3) the regulation of their expression has been associated with diabetes. We studied the expression of these genes in three tissues that play a critical role in the regulation of glucose and lipid metabolism.

Adipose Tissue. Of the tissues we examined, endogenous levels of TNF-α are only detectable in adipose tissue (Hotamisligil et al., 1993). Increased levels of TNF-α mRNA have been observed in adipose tissue of several rodent models of diabetes. These increased levels correlate with deficient levels of the insulin-sensitive glucose transporter (GLUT4) in animals and treatment with TNF-α decreases GLUT4 mRNA levels in cultured adipocytes. Thiazolidinediones are known to have dramatic effects on gene expression in adipose tissue in vivo. Treatment of diabetic rodents with TZDs has been shown to lower TNF-α mRNA and increase the expression of GLUT4, acyl-CoA synthetase, and lipoprotein lipase in adipose tissue (Young et al., 1995; Schoonjans et al., 1996; Martin et al., 1997; Okuno et al., 1998). To test the consistency of these results by real-time quantitative PCR in ZDF rats, we first measured TNF-α and GLUT4 transcripts. We also found that treatment with rosiglitazone suppressed TNF-α expression and induced GLUT4. LG100268, on the other hand, replicated neither of these effects. In the LG100268-treated animals, the levels of TNF-α mRNA were actually slightly higher than those of control animals and significantly higher than the rosiglitazone-treated animals. Similarly, treatment with LG100268 did not induce GLUT4 levels in adipose tissue; actually, it slightly suppressed them. A limitation in the interpretation of the disparate effects of LG100268 and rosiglitazone on TNF-α and GLUT4 expression is that it is unclear precisely how PPAR-γ ligands regulate the expression of either of these transcripts. We therefore extended our studies to compare the effects of TZDs and

TABLE 4
Levels of PPAR-γ, RXR-α, and RXR-γ transcripts in Zucker diabetic tissues

	PPARγ Levels	RXRα Levels	RXRγ Levels
		% 36b4	
Adipose	3.56 ± 0.36	0.99 ± 0.3	0.45 ± 0.22
Muscle	0.18 ± 0.09	1.46 ± 0.53	5.19 ± 2.2
Liver	0.07 ± 0.01	4.61 ± 1.9	2.72 ± 0.55

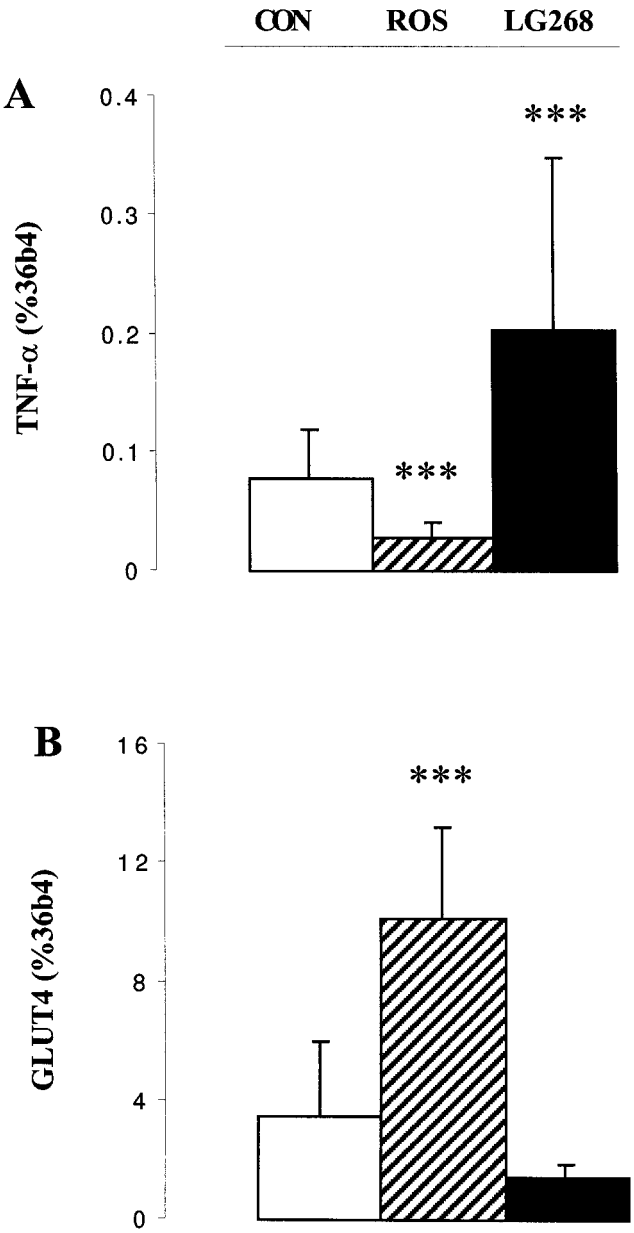

Fig. 3. TNF-α and GLUT-4 transcript levels in adipose tissue of control, rosiglitazone, and LG100268-treated ZDF rats. Total RNA was isolated from control ($n = 10$), rosiglitazone (10 mg/kg, $n = 6$), and LG100268 (30 mg/kg, $n = 5$) treated ZDF rats. One hundred nanograms of RNA from each tissue sample were assayed in triplicate for TNF-α and GLUT4 transcripts by quantitative real time RT-PCR. Absolute values were normalized to and expressed as a percentage of 36B4. Significant within-group difference comparing treatment and control values (ANOVA, $^{***}P < 0.001$).

rexinoids on the expression of three genes, *MCPT*, *SCD1*, and *CD36*, that are known to include a PPRE within their promoters (Miller and Ntambi, 1996; Mascaro et al., 1998; Tontonoz et al., 1998). All three transcripts play important roles in lipid metabolism and are expressed at significant levels in adipose tissue. In each case, treatment of the diabetic rats with rosiglitazone resulted in induction of the genes, and in each case, treatment with LG100268 had the opposite effect.

Comparison of the effects of LG100268 and rosiglitazone on gene expression in adipose tissue clearly establishes that rexinoids do not function as simple TZD-mimetics in vivo. Studies carried out with purified receptors and transfected cells have suggested that the formation of certain heterodimeric complexes, most notably RXR/RAR and RXR/TR, results in a silencing of the ligand-dependent *trans*-activation activity of the RXR component of the heterodimer (Kurokawa et al., 1994; Forman et al., 1995). In these "nonpermissive complexes", the partner receptor is competent to undergo ligand-dependent *trans*-activation, but the activity of the RXR component is effectively suppressed. In "permissive" complexes, on the other hand, both RXR and its partner receptor can bind ligands and contribute equivalently to ligand-dependent *trans*-activation. Although RXR/PPAR has been characterized as a permissive complex, the evidence suggesting its permissiveness has largely been gathered in cotransfection experiments with synthetic reporter genes in the context of fibroblastic cells. Because TZDs regulate gene expression via the activation of RXR/PPAR-γ heterodimers, the results we have obtained suggest that in the context of mature adipose tissue, rexinoids are unable to produce an equivalent activation of this receptor complex. It seems, therefore, that the RXR/PPAR-γ heterodimer is "nonpermissive" in adipose tissue. The levels of receptors, the roster of coactivators associated with the receptors and the physical state of the promoters for the target genes may be very different in tissues in vivo from those present in cultured cells in vitro. These differences apparently play a critical role in determining whether particular complexes are "permissive" or "nonpermissive" in vivo.

The fact that the RXR/PPAR heterodimer is nonpermissive

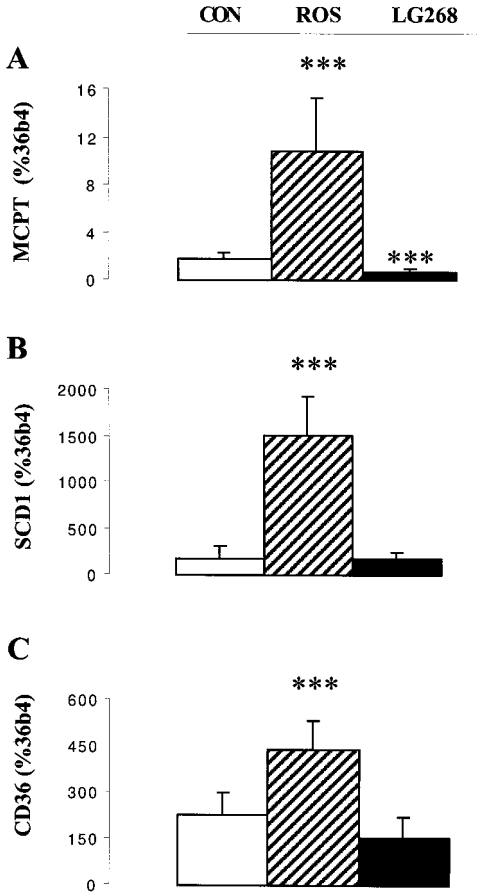

Fig. 4. MCPT, SCD1, and CD36 transcript levels in adipose tissue of control, rosiglitazone-, and LG100268-treated ZDF rats. Total RNA was isolated from control (n = 10) ZDF rat adipose tissue and ZDF rat adipose tissue treated with rosiglitazone (10 mg/kg, n = 6) and LG100268 (30 mg/kg, n = 5). One hundred nanograms of RNA from each tissue sample were assayed in triplicate for MCPT (A), SCD1 (B), and CD36 (C) transcripts by quantitative real time RT-PCR. Absolute values were normalized to and expressed as the percentage of 36B4. Significant within-group difference comparing treatment and control values (ANOVA, ***P < 0.001).

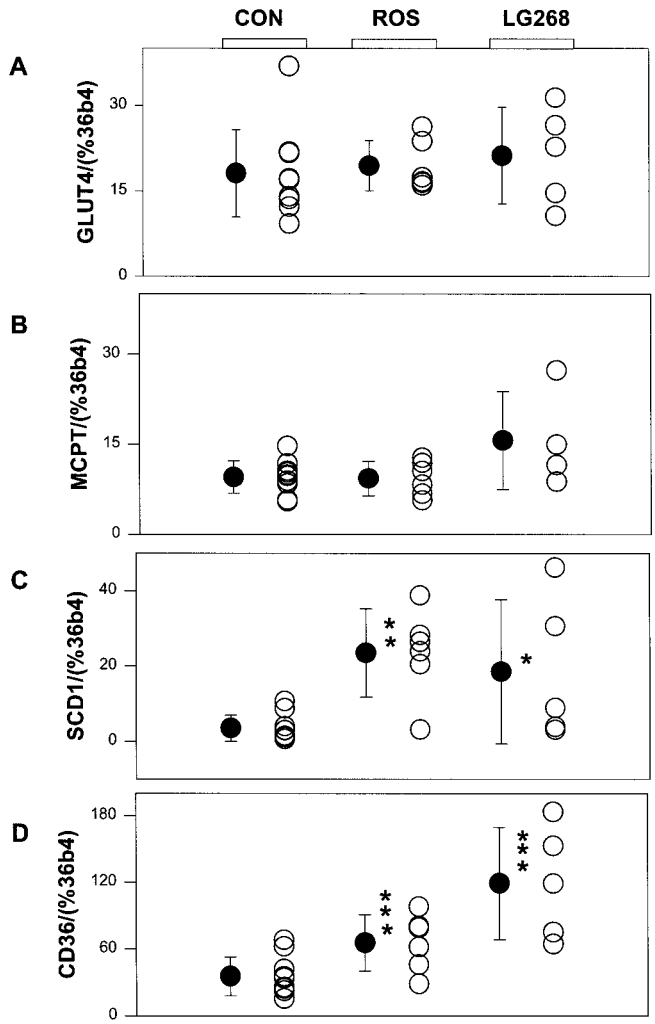

Fig. 5. Transcript levels in skeletal muscle of control, rosiglitazone-, and LG100268-treated ZDF rats. Total RNA was isolated from control (n = 10) ZDF rat muscle tissue and ZDF rat muscle tissue treated with rosiglitazone (10 mg/kg, n = 6) and LG100268 (30 mg/kg, n = 5). One hundred nanograms of RNA from each tissue sample was assayed in triplicate for GLUT4 (A), MCPT (B), SCD1 (C), and CD36 (D) transcripts by real time RT-PCR. Absolute values were normalized to and expressed as the percentage of 36B4. Significant within-group difference comparing treatment and control values (ANOVA, ***P < 0.001 for CD36; **P = 0.002 for SCD1).

in adipose tissue may account for the differential effects of TZDs and rexinoids on adipogenesis. Treatment of diabetic animals with TZDs is frequently associated with an increase in adipogenesis and adipose tissue mass (Hallakou et al., 1997). However, under conditions of comparable lowering of plasma glucose, rexinoids do not produce an observable increase in adipose tissue mass. The failure of rexinoids to activate gene expression in adipose tissue, and specifically the failure to induce CD36 (a fatty acid transporter) or SCD1, a gene associated with increased adiposity, may explain this selective pharmacologic effect.

Liver. Rexinoids are known to produce hepatomegaly accompanied by the induction of several genes associated with fatty acid metabolism, including acyl-CoA oxidase and L-FABP RNA (Poirier et al., 1997; Mukherjee et al., 1998). Our studies extend the list of fatty acid regulatory enzymes whose expression is controlled by rexinoids to include the transmembrane fatty acid transporter (CD36), the mitochondrial fatty acid transporter (MCPT) and SCD1, the rate-limiting enzyme in desaturation of fatty acids, all of which are expressed at very low levels in normal livers (Thiede and Strittmatter, 1985; Abumrad et al., 1993; McGarry and Brown, 1997). Interestingly, although muscle type CPT is expressed abundantly in the heart, skeletal muscle, and adipose tissue (McGarry and Brown, 1997), we also detected low levels in the liver. Both MCPT and LCPT are expressed in several tissue types (McGarry and Brown, 1997), suggesting that although they perform similar functions, they must be under differential regulation. Our results support this in that the effect of LG100268 was specific to MCPT. The rexinoid did not alter transcript levels of the hepatic-enriched liver type isoform of CPT (data not shown).

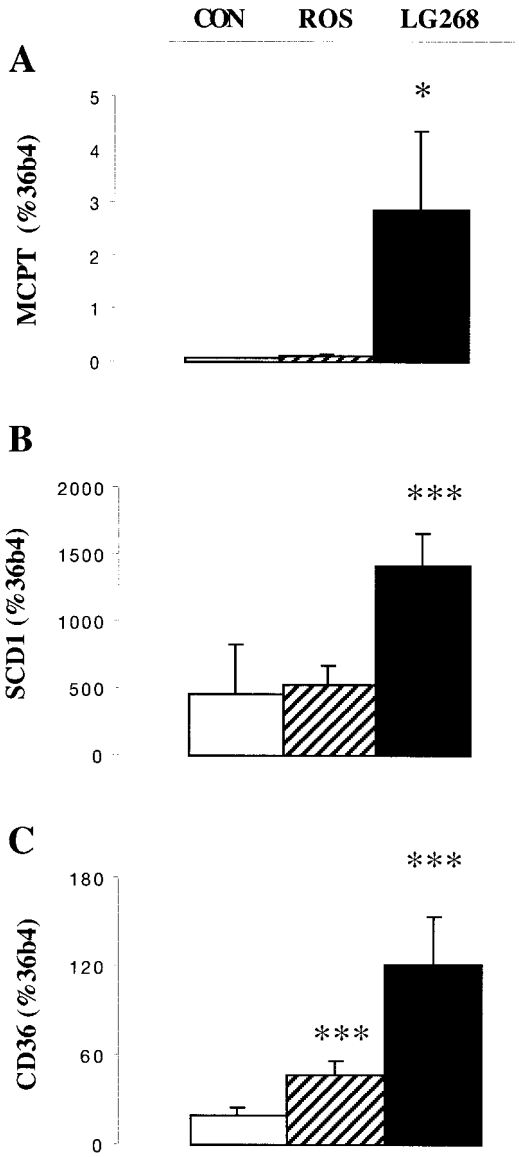

Fig. 6. Transcript Levels in Liver of control, rosiglitazone-, and LG100268-treated ZDF rats. Total RNA was isolated from control (n = 10) ZDF rat livers and ZDF rat livers treated with rosiglitazone (10 mg/kg, n = 6) and LG100268 (30 mg/kg, n = 5). One hundred nanograms of RNA from each tissue sample was assayed in triplicate for MCPT (A), SCD1 (B), and CD36 (C) transcripts by real time RT-PCR. Absolute values were normalized to and expressed as the percentage of 36B4. Significant within-group difference comparing treatment and control values (ANOVA, $^*P = 0.02$ for MCPT; $^{***}P < 0.001$ for SCD1 and CD36).

It seems likely that in the liver, the RXR/PPAR-α heterodimer is capable of being activated by either PPAR-α or RXR ligands, because PPAR-α ligands such as Wy 14,643 and clofibrate induce many of the same effects as rexinoids on hepatic gene expression (Miller and Ntambi, 1996; Motojima et al., 1998). Rosiglitazone has little or no effect on hepatic gene expression, probably because of the low level of expression of PPAR-γ in the liver. Neither TNF-α nor GLUT4 levels are detectable in liver.

Skeletal Muscle. TZDs are very effective in ameliorating hyperglycemia and hyperinsulinemia in mice whose adipose tissue has been genetically ablated, demonstrating their capacity to have direct effects of muscle metabolism (Burant et al., 1997). Much less is known about the effects of rexinoids on muscle gene expression and insulin sensitization. Although both GLUT4 and MCPT are abundantly expressed in muscle, neither transcript is altered by rosiglitazone or LG100268. Unlike either adipose tissue or liver, in which we found major differences between the effect of TZDs and rexinoids on gene expression, in skeletal muscle, both classes of compounds produced similar effects on the expression of two PPAR-γ inducible genes, CD36 (abundantly expressed in muscle, Abumrad et al., 1993) and SCD1. The effects of LG100268 and rosiglitazone on CD36 expression are interesting because of recent studies linking defects in CD36 expression with insulin resistance in some strains of spontaneously hypertensive (SHR) rats (Aitman et al., 1999). These results support the idea that defects in lipid metabolism can ultimately lead to insulin resistance. The similar regulation of a rate-limiting enzyme (SCD1) in the synthesis of unsaturated long-chain fatty acids by both rexinoids and TZDs is consistent with this hypothesis (Enoch et al., 1976). Fatty acids are key constituents of membrane phospholipids and altered levels may be important in diseases including diabetes (Spector and Yorek, 1985).

The promoters of both SCD1 and CD36 contain a well-characterized PPRE (Miller and Ntambi, 1996; Tontonoz et al., 1998). The induction of both genes by TZDs and rexinoids in muscle is compatible with activation of muscle RXR/PPAR-γ heterodimers by both RXR and PPAR ligands. It is possible that even though the RXR/PPAR-γ heterodimer is "nonpermissive" in the context of adipose tissue, the same heterodimer may be "permissive" in skeletal muscle. The tissue-specific activation of heterodimers may well depend

upon the complement of accessory factors available to the ligand-activated receptors in different tissues. For instance, rosiglitazone recruits cAMP response element-binding protein to the RXR/PPAR-γ heterodimer, whereas LG100268 recruits steroid receptor coactivator-1 (Schulman et al., 1998). Further characterization of the contribution of cofactors to the tissue-selective activity of RXR heterodimeric receptors will be necessary to clarify these issues. On the other hand, it is possible and more likely, given the effects in adipose and liver, that the parallel effects of rexinoids and TZDs on SCD1 and CD36 in skeletal muscle reflect the activation of distinct but convergent hormone signaling pathways. In fact, the increased levels of RXR-α and RXR-γ transcript levels in muscle compared with adipose tissue would support the idea of there being ample RXR to partner with nuclear receptors other than PPAR-γ and therefore regulate gene expression. These results are consistent with previous studies demonstrating abundant levels of all three RXR mRNAs (α, β, and γ) in muscle (Mangelsdorf et al., 1992). However, the differences in the abundance of receptor transcript levels may not be directly proportional to differences in receptor levels.

In summary, we show that rexinoids act as insulin sensitizers in diabetic rodents. We have studied the expression of transcripts that are involved in glucose or lipid metabolism and that are known molecular targets of TZDs. Our results show that rexinoids have a profile of pharmacologic activity in vivo that is distinct from TZDs, but do identify two convergent molecular targets (CD36 and SCD1) of both compounds in ZDFs. Future studies will elucidate the roles of these genes in this and other models of insulin resistance.

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Send reprint requests to: Dr. Peter J. A. Davies, Department of Integrative Biology and Pharmacology, University of Texas at Houston School of Medicine, Houston, TX 77030. E-mail: peter.j.davies@.uth.tmc.edu