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Antidiabetic Action of a Liver X Receptor Agonist Mediated By Inhibition of Hepatic Gluconeogenesis*

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The oxysterol receptors LXR (liver X receptor)-α and LXRβ are nuclear receptors that play a key role in regulation of cholesterol and fatty acid metabolism. We found that LXRs also play a significant role in glucose metabolism. Treatment of diabetic rodents with the LXR agonist, T0901317, resulted in dramatic reduction of plasma glucose. In insulin-resistant Zucker (fa/fa) rats, T0901317 significantly improved insulin sensitivity. Activation of LXR did not induce robust adipogenesis but rather inhibited the expression of several genes involved in hepatic gluconeogenesis, including phosphoenolpyruvate carboxykinase (PEPCK). Hepatic glucose output was dramatically reduced as a result of this regulation. Nuclear run-on studies indicated that transcriptional repression was primarily responsible for the inhibition of PEPCK by the LXR agonist. In addition, we show that the regulation of the liver gluconeogenic pathway by LXR agonists was a direct effect on hepatocytes. These data not only suggest that LXRs are novel targets for diabetes but also reveal an unanticipated role for these receptors, further linking lipid and glucose metabolism.

Type II diabetes mellitus is a prevalent metabolic disease in developed countries, with insufficient therapies for treatment and prevention (1, 2). Studies in recent years have suggested that nuclear receptors are intimately linked to the pathophysiology of diabetes. The antidiabetic thiazolidinediones have been identified as ligands of proxisome proliferator-activated receptor γ (PPARγ)[1] (3, 4). Retinoid X receptor (RXR) ligands have also been shown to lower plasma glucose levels in rodent diabetic models (3–5).

Originally identified as orphan members of the nuclear receptor superfamily, liver X receptors exist as two isoforms, LXRα and LXRβ. The two isoforms display distinct patterns of expression with LXRα being primarily expressed in liver, intestine, and kidney, whereas LXRβ is expressed ubiquitously (6). Oxysterols were identified as the putative physiological ligands for the LXRs (7), and additional studies have demonstrated that these receptors act as sensors for these cholesterol metabolites and are essential components of a physiological feedback loop regulating cholesterol metabolism and transport (8). Consistent with their role in regulation of these metabolic pathways, several LXR-regulated genes involved in lipid metabolism and cholesterol transport have been identified including ABCA1, ABCG1, ABCG5, ABCG8, ApoE, CETP, Cyp7a, LPL, SREBP1c, and FAS (8–14).

As a result of the close relationship between lipid and carbohydrate metabolism, we examined the potential role LXRs may play in glucose homeostasis by using a specific LXR agonist, T0901317, (11) in rodent models of diabetes. Our findings indicated that T0901317 dose-dependently lowered plasma glucose level in both db/db and Zucker diabetic fatty (ZDF) rat models. In the fa/fa insulin-resistant rat model, T0901317 significantly improved insulin sensitivity. Examination of the liver gluconeogenesis pathway revealed dramatic repression of key genes involved in this pathway. As a result, hepatic glucose output was dramatically suppressed. PEPCK mRNA suppression appeared to originate primarily from transcriptional repression as indicated by the nuclear run-on experiments. Further studies in cultured hepatocytes indicated that hepatic activation of LXRs was sufficient to mediate the suppression of the hepatic gluconeogenesis pathway. Moreover, in an *in vitro* adipocyte differentiation assay, we showed that LXR agonists only minimally induced adipocyte differentiation compared with the robust effect by classic PPARγ agonists.

MATERIALS AND METHODS

In Vivo Glucose-lowering Studies—Five-week-old male db/db mice were purchased from Harlan (Madison, WI) and acclimated for 2 weeks prior to the start of the study. Mice were provided Purina 5008 food *ad libitum*, and the compounds were dosed once daily via oral gavage for 7 days. Blood samples were taken 1 h after dosing via the tail vein, and plasma glucose and triglyceride levels were measured on a Hitachi 912 clinical chemistry analyzer. Animals were sacrificed in the morning, 1 h after the eighth dose, and tissues were collected and frozen in liquid nitrogen for processing. A similar protocol was used for ZDF rats that were purchased from Charles River/Genetic Models, Inc. (Zionsville, IN). The rats were 8 weeks old at the start of the study.

Oral Glucose Tolerance Study in fa/fa Rats—Obese insulin-resistant female Zucker (fa/fa) rats (Charles River/Genetic Models, Inc.), 10 weeks of age, were orally gavaged for 9 days with either vehicle or T0901317 (3 mg/kg/d). A pair-fed group was also included, to ascertain the effects of a mild reduction in food consumption noted in the T0901317 group. Eight hours after the last dose, animals were fasted overnight and on the following morning subjected to an oral glucose tolerance test. Briefly, blood was obtained from the animals in the conscious state, via the tail vein, at time 0 and times 15, 30, 60, and 120

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[1] The abbreviations used are: PPAR, proxisome proliferative-activated receptor; PEPCK, phosphoenolpyruvate carboxykinase; G6P; glucose-6-phosphatase; ABC, ATP binding cassette transporter; CETP, cholesterol ester transport protein; ApoE, Apolipoprotein E; SREBP, sterol responsive element-binding protein; LXR, liver X receptor; RXR, retinoid X receptor; LPL, lipoprotein lipase; FAS, fatty acid synthase; Cyp7a, cholesterol 7α-hydroxylase; ZDF, Zucker diabetic fatty rat.

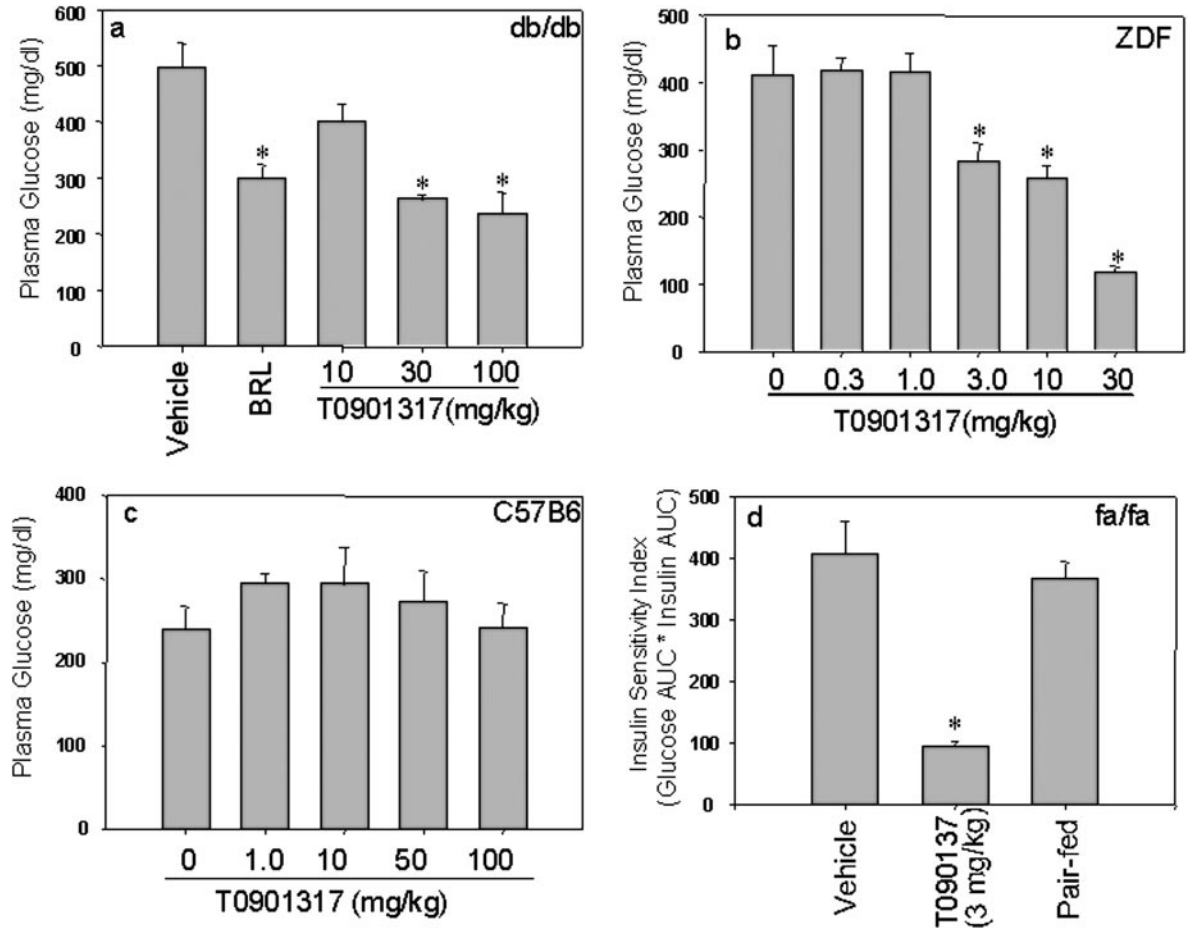

FIG. 1. **LXR agonist T0901317 lowers plasma glucose in male db/db mice and male Zucker diabetic fatty rats and improves insulin sensitivity in female Zucker (fa/fa) rats.** Five animals in each group were orally dosed with T0901317 for 7 days, and plasma glucose levels were measured. Female Zucker (fa/fa) rats were dosed for 9 days and subjected to an oral glucose tolerance test. Insulin sensitivity index is glucose AUC × insulin AUC. *, $p < 0.05$.

min after an oral glucose challenge (2.5 g of glucose/kg body weight). Plasma glucose and insulin levels were analyzed on all samples, and the results are expressed as the product of glucose AUC and insulin AUC.

Glucose Output with ZDF Rat Liver Slices—Precision-cut liver slices were generated from control, T0901317-treated (10 and 30 mg/kg/d for 7 days), and pair-fed to male ZDF rats following 7 days of treatment and an overnight fast. After preincubation and wash phases, the slices were incubated for 2 h at 29 °C in Krebs-Henseleit bicarbonate buffer containing 40 mM mannitol in either the presence or absence of 10 mM lactate. Incubation media glucose levels were assessed at the 2-h time point. Lactate-stimulated glucose output for each condition was derived by subtracting the basal rate of glucose output per gram of liver tissue from the substrate-stimulated rate of glucose output per gram of liver tissue. This rate of glucose output largely reflects the gluconeogenic rate because no group displayed a net increase in glycogen formation during the incubation period (data not shown).

Nuclear Run-on Experiment—A nuclear run-on experiment was performed essentially as described (15). Briefly, db/db mice were treated by T0901317 as described above. Liver samples were collected after animals were sacrificed. Nuclei were isolated, and *in vivo* elongation reaction was performed. The radiolabeled RNA was then subjected to slot blot to probes.

mRNA Measurement—Total RNAs were prepared from frozen tissue samples or cells with TRIzol reagent (Invitrogen) or Qiagen RNA prep kit. Mouse PEPCK and G6P mRNA were measured by RNase protection assay and quantified with a Molecular Dynamics Phosphorimager Model 51. Rat mRNA was subjected to reverse transcription reactions using the Omniscript reverse transcriptase kit (Qiagen) according to the manufacturer's directions. The resulting cDNA was amplified using TaqMan 2× PCR master mix (Applied Biosystems). The PCR products were detected in real time using an ABI-7900HT sequence detection system (Applied Biosystems). The rat PEPCK bDNA was performed as described (16).

In Vitro Adipocyte Differentiation—3T3-L1 preadipocytes were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. Two-day postconfluent 3T3-L1 cells (designated day 0) were induced to differentiate by exposure for 4 days to DMEM with 10% fetal bovine serum containing either DII (1 μM dexamethasone, 1 μM insulin, and 0.5 mM isobutylmethylxanthine) or single compound (10 μM each of T0901317 or 22(R)-hydroxycholesterol or rosiglitazone). From day 4 to day 8, cells were exposed to DMEM with 10% fetal bovine serum. Oil Red O staining was performed as described (17). C3H10T1/2 mouse preadipocyte cells were grown up in DMEM and 10% calf serum. Cells at passage six were plated at 1.0×10^4 cells per well in 96-well clear bottom plates. The next day, compounds were diluted in media containing 0.03 mg/ml insulin. Media was aspirated off cells and replaced with 100 μl of media containing insulin and different concentrations of compound in quadruplicate. The cells were treated for 6 days, followed by washing once with 200 μl/well phosphate-buffered saline, and lysed in 50 μl/well 0.1% IGEPAL in phosphate-buffered saline for 10 min at room temperature. 100 μl/well of Sigma infinity triglyceride reagent (Sigma Diagnostics no. 343) was added, and absorbencies were read at 490 nm using a Molecular Devices THERMOmax microplate reader.

RESULTS

In 7-week-old male diabetic db/db mice, the specific LXR agonist, T0901317, dose-dependently lowered plasma glucose (Fig. 1*a*). The maximum efficacy in plasma glucose-lowering achieved with T0901317 was comparable with rosiglitazone (Invitrogen; Avandia®) treatment. Food consumption and body weight gain were similar to control in all dose groups with the exception of the 100 mg/kg T0901317 treatment, which trended downward (Table I). Subsequently we tested T0901317 in the

TABLE I
Metabolic parameters of db/db mice treated with either BRL (30 mg/kg) or various doses of T0901317

Parameters	Vehicle	BRL 30 mg/kg	T0901317		
			10 mg/kg	30 mg/kg	100 mg/kg
Body weight (g)	36 ± 0.3	35.5 ± 0.6	34.6 ± 1.1	35.7 ± 0.7	35.8 ± 0.9
Food intake (g)	7.1	6.5	7.0	6.9	5.1
Plasma triglyceride (mmol/liter)	4.3 ± 0.6	1.4 ± 0.3	9.5 ± 6.0	23.4 ± 38.5	23.9 ± 20.6
Liver triglyceride (mg/g)	48.2 ± 19.3	92.5 ± 24.0	176.8 ± 40.0	158.0 ± 19.8	165.6 ± 10.0
Plasma insulin (ng/ml)	7.0 ± 3.2	6.2 ± 2.8	11.9 ± 5.3	12.0 ± 02.6	8.9 ± 3.3

TABLE II
Metabolic parameters of ZDF rats treated with either vehicle or various doses of T0901317

Parameters	Vehicle	T0901317				
		0.3 mg/kg	1.0 mg/kg	3.0 mg/kg	10 mg/kg	30 mg/kg
Body weight (g)	302.5 ± 5.4	307.8 ± 6.7	310.0 ± 5.8	302.9 ± 5.1	309.7 ± 4.5	289.9 ± 6.6
Food intake (g)	29.0 ± 0.9	29.4 ± 0.6	30.4 ± 0.9	22.4 ± 4.0	27.7 ± 0.8	20.1 ± 1.4
Plasma triglyceride (mmol/liter)	8.0 ± 2.3	10.6 ± 2.1	11.7 ± 2.3	13.1 ± 5.6	30.2 ± 10.7	14.9 ± 6.8
Liver triglyceride (mg/g)	10.0 ± 4.0	12.9 ± 4.6	9.8 ± 3.0	7.7 ± 2.7	83.3 ± 55.9	143.2 ± 23.8
Plasma insulin (ng/ml)	6.3 ± 4.0	8.9 ± 5.1	11.1 ± 4.8	9.2 ± 2.8	8.1 ± 1.7	3.8 ± 1.7

male ZDF model. Eight-week-old ZDF rats were treated orally with various doses of T0901317 for 7 days. Consistent with the data from the db/db model, plasma glucose levels were significantly reduced at 3 and 10 mg/kg doses. At a greater dose of T0901317 (30 mg/kg) a more striking reduction in plasma glucose was noted but was associated with a significant decrease in food consumption and weight loss, possibly as a result of toxicity of the high dose of compound used (Table II). Both plasma and liver triglycerides in db/db and ZDF rat studies increased dramatically (Tables I and II), which is consistent with earlier reports in C57B6 mice (11). There was no significant change in plasma insulin levels in these studies (Tables I and II). Treatment of normal C57BL6 mice resulted in no significant change in plasma glucose levels (Fig. 1*c*). An oral glucose tolerance test in female obese insulin-resistant Zucker (fa/fa) rats, subsequent to 9 days of treatment with T0901317 (3 mg/kg/d), revealed a significant improvement in glucose tolerance in the treated animals relative to both vehicle control and pair-fed control groups. Although the insulin response to the glucose challenge was not significantly altered, the insulin sensitivity index, calculated as the product of the glucose AUC and the insulin AUC during the oral glucose tolerance test, was significantly improved in the treated group (Fig. 1*d*). Thus T0901317, presumably functioning as an LXR agonist, effectively lowers glucose in diabetic rodents and improves insulin sensitivity in insulin-resistant rodents but does not cause hypoglycemia in normal mice.

In assessing potential mechanisms underlying the antidiabetic actions, we found significant reductions in mRNA levels of two key gluconeogenic enzymes, PEPCK and glucose 6-phosphatase (G6P), in liver samples from T0901317-treated db/db mice. PEPCK mRNA levels in T0901317-treated liver samples were reduced dose-dependently and correlated well with the glucose-lowering effects (Fig. 2*a*). Similar alterations were also observed in liver samples from C57BL6 mice but to a lesser extent (Fig. 2*c*). G6P mRNA levels were reduced more than 50% in a dose-dependent fashion in liver samples from T0901317-treated db/db mice (Fig. 2*b*). We then measured lactate-stimulated glucose output from precision-cut liver slices derived from ZDF rats treated with either vehicle, T0901317 (10 mg/kg), or T0901317 (30 mg/kg). Compared with either control or a pair-fed group (matched to T0901317 30 mg/kg), T0901317 at 10 mg/kg inhibited lactate-stimulated glucose output by ~80%, whereas the 30 mg/kg treatment resulted in virtually complete inhibition of glucose output (Fig. 2*d*). Very similar trends were observed for lactate utilization as the vehicle control; pair-fed groups displayed the greatest rates, followed distantly by T0901317 at 10 mg/kg and T0901317 at 30 mg/kg, which utilized essentially no lactate. These results indicate that the LXR agonist, T0901317, improves glucose homeostasis in diabetic rodents, at least in part, through down-regulation of key enzymes in the hepatic gluconeogenesis pathway.

To investigate the mechanism of PEPCK mRNA reduction upon LXR activation, we performed nuclear run-on experiments with liver samples from db/db mice treated with either T0901317 or vehicle (Fig. 2*e*). The results suggested that reduction of PEPCK mRNA upon T0901317 treatment in db/db mice was largely from transcriptional repression. To determine whether the aforementioned alterations were the result of T0901317 acting directly on hepatocytes, we treated rat hepatoma Fao cells with either 0.2 nM insulin or 100 nM T0901317 or a combination of both for 24 h. The mRNA levels of PEPCK, G6P, pyruvate carboxylase, and fructose 1,6-bisphosphatase decreased dramatically upon either insulin or T0901317 treatment. The combination of both agents did not result in an additive effect (Fig. 3*a*). To confirm our observations, we treated rat hepatoma H4IIE cells with either T0901317 or another structurally distinct synthetic LXR agonist, GW3965, (18) and measured PEPCK mRNA. Both compounds showed dose-dependent reductions of PEPCK mRNA levels. The calculated IC_{50} values for PEPCK inhibition of these two compounds are 26 nM and 108 nM, respectively (Fig. 3*b*), which agrees closely with their respective described LXR potencies (11, 18). These results suggest that the *in vivo* regulation of hepatic gluconeogenic genes was a direct action of the LXR agonist on the liver.

As a result of recent studies indicating that LXRα is a target gene of PPARγ (19, 20), we further explored the mechanisms of LXR action by comparing the effects of LXR agonists and a PPARγ agonist, rosiglitazone, on adipocyte differentiation *in vitro*. Although rosiglitazone induced dramatic adipocyte differentiation, both the natural LXR ligand, 22(R)-hydroxycholesterol, and T0901317 failed to induce robust adipocyte differentiation as assessed by Oil Red O staining (Fig. 4*a*). Similar results were obtained in C3H10T1/2 cells where adipocyte dif-

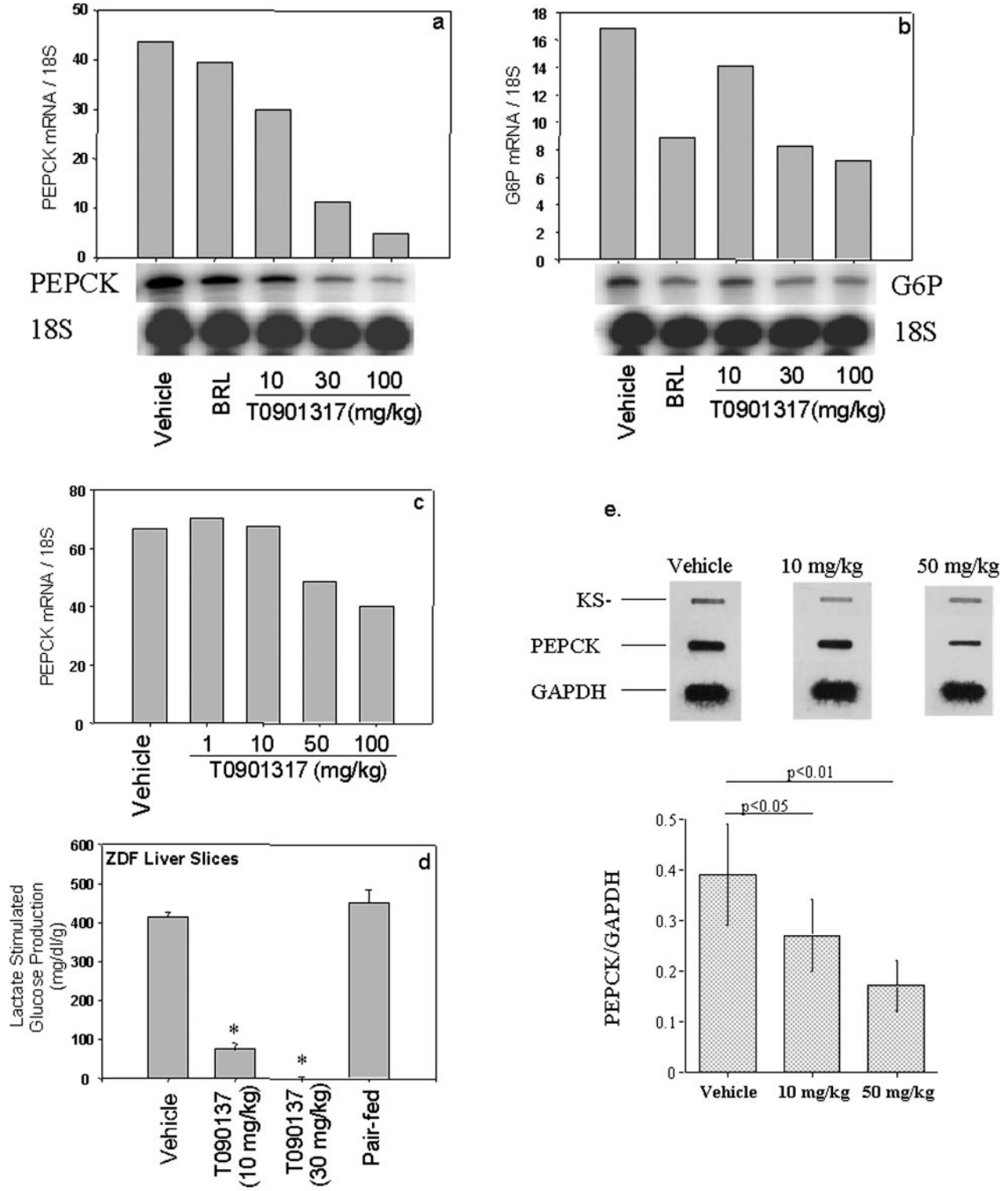

FIG. 2. **LXR agonist T0901317 down-regulates the hepatic gluconeogenesis pathway *in vivo*.** Animals were treated as described under "Materials and Methods." Tissues were collected, and total RNAs were prepared and pooled for analysis. Mouse PEPCK and G6P mRNA were measured by RNase protection assay using the Ambion RPAIII kit. Signal was quantified with a Molecular Dynamics Phosphorimager Model 51. 28 S RNA was used as control (*a–c*). Lactate-stimulated glucose production from precision-cut liver slices derived from vehicle control, pair-fed control, and T0901317-treated ZDF rats. *, $p < 0.05$ (*d*). Nuclear run-on studies were performed on db/db mice as described under "Materials and Methods." Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control for normalization (e).

ferentiation was quantitated (Fig. 4*b*). Examination of aP2 mRNA in 3T3L1 cells did not reveal any regulation by the LXR agonist, T0901317 (data not shown). These results show that although LXRα is a direct target gene of PPARγ, LXR agonists do not merely mimic PPARγ action in adipocytes, suggesting a unique mechanism for LXR-mediated antidiabetic action. Our results however, do not rule out the potential involvement of LXR-mediated contributions in peripheral tissues.

DISCUSSION

Our studies reveal for the first time that an LXR agonist, T0901317, exerts antidiabetic effects through suppression of

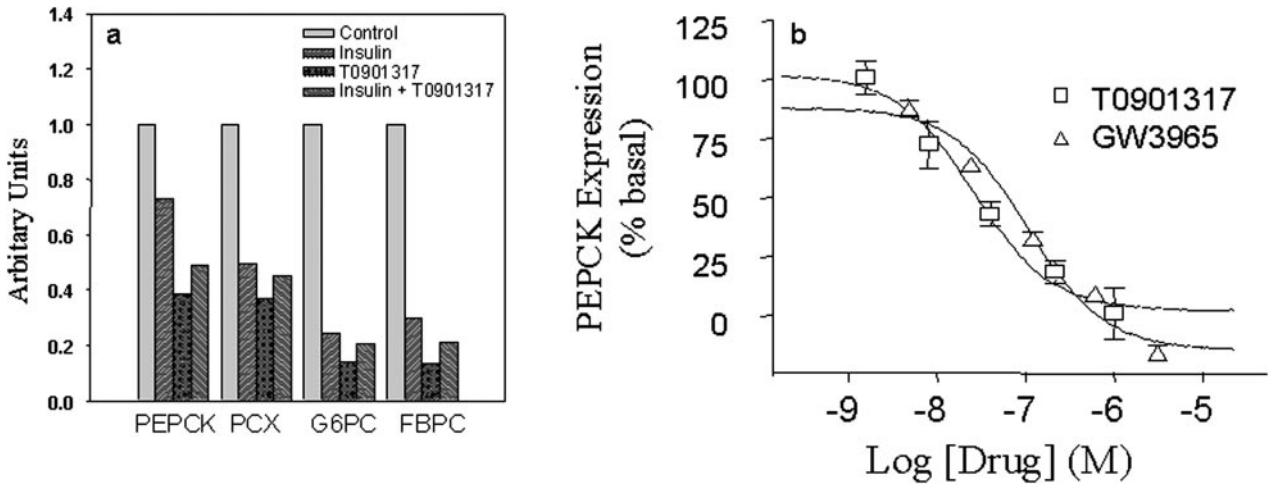

FIG. 3. **Direct regulation of key gluconeogenic genes by LXR agonists in rat hepatoma cells.** Fao cells were treated with either insulin (0.2 nM) or T0901317 (100 nM) or the combination for 24 h, and RNAs were prepared. Real-time PCR experiments were carried out using 18 S RNA as control as recommended by the manufacturer (*a*). H4IIE cells were treated with various concentrations of either T0901317 or GW3965 for 24 h, and PEPCK mRNA was measured by the bDNA method (*b*).

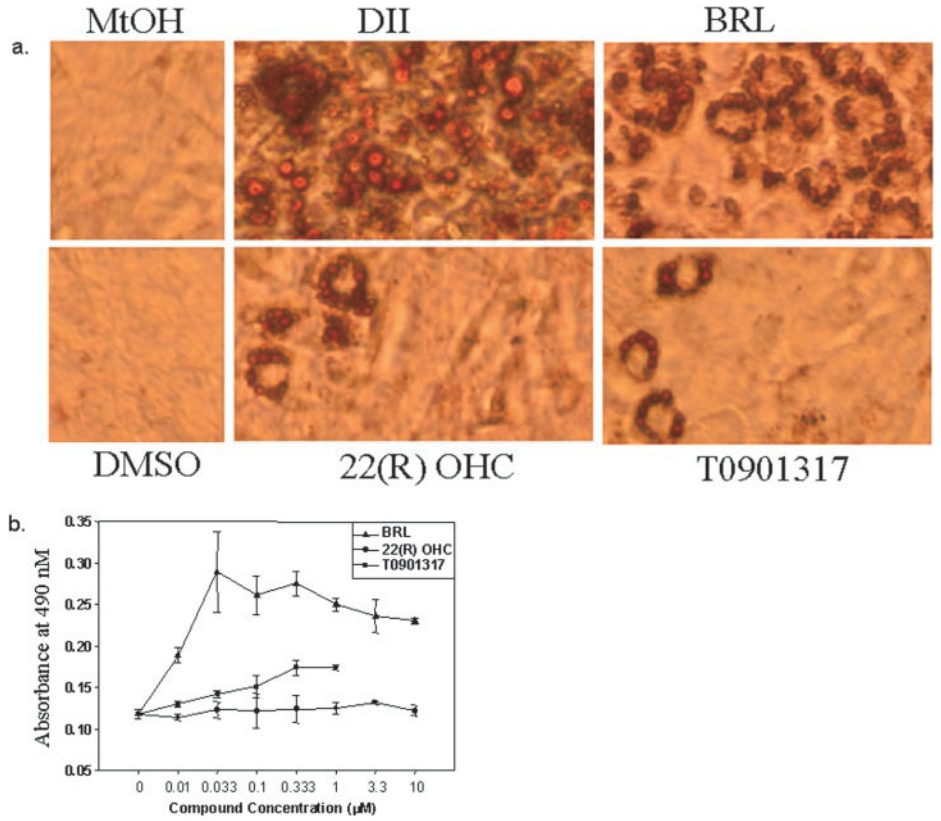

FIG. 4. **LXR agonists fail to induce robust adipocyte differentiation.** 3T3L1 preadipocytes were cultured and differentiation was induced as described under "Materials and Methods." Cells were then fixed and stained with Oil Red O. DII: dexamethasone, insulin, and isobutylmethylxanthine. 22(R)-OHC: 22(R)-hydroxycholesterol (*a*). 10T1/2 cells were treated as described under "Materials and Methods," and adipocyte differentiation was quantified (*b*).

the hepatic gluconeogenic process. Because inhibition of hepatic glucose production has been identified as an effective approach for lowering hyperglycemia (2), LXRs potentially represent novel targets for treating diabetes.

LXR and RXR function as permissive heterodimers (21), and our results suggest that the glucose-lowering effect of rexinoids may be mediated, at least partially, through decreased hepatic gluconeogenesis via activation of the LXR/RXR heterodimer. Recent studies indicated that LXR/RXR heterodimers regulate a spectrum of important gene products involved in lipid metabolism. One of the target genes, *SREBP1c*, has been identified as the master transcription factor controlling the entire fatty acid biosynthetic pathway (22, 23). In our studies, we observed dose-dependent plasma and liver triglyceride increases in both db/db mice and ZDF rats (Tables I and II), which is consistent with previous reports in C57BL6 mice. It is interesting to note that despite the increase in triglyceride levels, hyperglycemia was reduced dose-dependently. This observation is strikingly similar to the previous report on the effects of RXR agonists (24).

Although LXRs have been regarded as potential targets for mediating cardiovascular benefits, the induction of hypertri-

glyceridemia and liver steatosis has severely hampered its development. A selective modulator that does not lead to accumulation of liver triglycerides will be essential if therapeutic potentials of LXRs for both cardiovascular and diabetic diseases can be realized.

In contrast to previous reports that have identified positively regulated target genes for LXR, we have identified several gene products that are down-regulated by LXR activation. Traditional LXR target genes possess an LXR-responsive element (LXRE) in their promoter or intron; however, it is unclear whether LXR represses genes through a negative LXRE or through indirect regulation similar to farnesoid X receptor repression of cholesterol 7α-hydroxylase expression (25, 26).

Our novel findings suggest that LXR activation alters liver metabolism in a manner reminiscent of insulin, increased lipogenesis and decreased gluconeogenesis. Despite the similarities, T0901317 does not appear to work through the classic insulin signaling cascade because we found that LXR activation does not alter AKT phosphorylation or PGC-1 expression (data not shown). In contrast, insulin has been shown to regulate LXRα in hepatocytes, and thus it is plausible that the effects of insulin on lipogenesis and gluconeogenesis may be regulated, at least in part, through changes in LXR expression.

In summary, we have discovered an additional metabolic pathway regulated by LXRs. Activation of this pathway by an LXR agonist leads to a significant reduction in hyperglycemia and an improvement in insulin sensitivity in preclinical models. These studies strongly implicate LXRs as alternative targets for intervention in diabetes mellitus.

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