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Stimulation of Lipogenesis by Pharmacological Activation of the Liver X Receptor Leads to Production of Large, Triglyceride-rich Very Low Density Lipoprotein Particles*

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The oxysterol-activated liver X receptor (LXR) provides a link between sterol and fatty acid metabolism; activation of LXR induces transcription of lipogenic genes. This study shows that induction of the lipogenic genes *Srebp-1c*, *Fas*, and *Acc1* upon administration of the synthetic LXR agonist T0901317 to C57BL/6J mice (10 mg/kg/day, 4 days) is associated with massive hepatic steatosis along the entire liver lobule and a 2.5-fold increase in very low density lipoprotein-triglyceride (VLDL-TG) secretion. The increased VLDL-TG secretion was fully accounted for by formation of larger (129 ± 9 nm *versus* 94 ± 12 nm, a 2.5-fold increase of particle volume) TG-rich particles. Stimulation of VLDL-TG secretion did not lead to elevated plasma TG levels in C57BL/6J mice, indicating efficient particle metabolism and clearance. However, T0901317 treatment did lead to severe hypertriglyceridemia in mouse models of defective TG-rich lipoprotein clearance, *i.e.* APOE*3-Leiden transgenic mice (3.2-fold increase) and apoE−/− LDLr−/− double knockouts (12-fold increase). Incubation of rat hepatoma McA-RH7777 cells with T0901317 also resulted in intracellular TG accumulation and enhanced TG secretion. We conclude that, in addition to raising high density lipoprotein cholesterol concentrations, pharmacological LXR activation in mice leads to development of hepatic steatosis and secretion of atherogenic, large TG-rich VLDL particles.

The nuclear liver X receptor α (LXRα[1]; NR1H3) and LXRβ (NR1H2) are involved in the control of cholesterol and fatty acid metabolism. LXRα is expressed mainly in the liver whereas LXRβ is ubiquitously expressed (1, 2). Activated LXRs heterodimerize with the retinoid X receptor (NR2B1) and bind to a LXR response element, consisting of two hexameric nucleotide direct repeats separated by four nucleotides, to induce gene transcription (1, 2). Oxysterols constitute the physiological ligands for LXR. The most potent LXR-activating oxysterols are 22(*R*)-hydroxycholesterol, 24(*S*)-hydroxycholesterol, and 24(*S*),25-epoxycholesterol (3). Activation of LXR in macrophages results in increased expression of genes encoding ATP-binding cassette (ABC) cholesterol transporters ABCA1 (4–7) and ABCG1 (8, 9) and apolipoprotein E (apoE) (9). ABCA1, ABCG1, and apoE are involved in cholesterol efflux from macrophages toward high density lipoproteins (HDL), which is considered the critical first step of reverse cholesterol transport. In the liver, LXR is involved in transcriptional control of *Cyp7A1*, encoding a critical enzyme in the conversion of cholesterol into bile acids (3, 10), as well as in transcriptional control of ABCG5/ABCG8 (11, 12), ABC transporters implicated in biliary cholesterol excretion. Induction of intestinal *Abca1*, *Abcg5*, and *Abcg8* expression upon LXR activation is thought to reduce the efficiency of cholesterol absorption and hence to accelerate fecal cholesterol disposal (4). The physiological role of LXR in the control of cholesterol metabolism and the possibility of pharmacological interventions aimed at prevention of atherosclerosis via this regulatory system have recently been reviewed extensively (13–17).

In addition to cholesterol transport genes, LXR has been reported to control genes that encode proteins involved in *de novo* lipogenesis. Induced transcription has been reported for the gene encoding the sterol-regulatory element-binding protein-1c (SREBP-1c) (18–20), the transcription factor that regulates expression of various lipogenic genes, including those encoding acetyl-CoA carboxylase (ACC) and fatty acid synthase (21). Apart from indirect actions through SREBP-1c, LXR also directly influences transcription of *Fas* (22) and of genes encoding lipoprotein lipase (23), cholesterol ester transfer protein (24), and stearoyl-CoA desaturase-1 (25).

Recently, the availability of synthetic LXR agonists, *e.g.* T0901317, has provided more insight into metabolic consequences of LXR activation. Oral administration of T0901317 to C57BL/6 mice increased HDL cholesterol concentrations in plasma, probably related to induction of the *Abca1* and *Abcg1* expression (18). In addition to this potentially beneficial effect, treatment with LXR agonists also led to a marked increase in hepatic triglyceride (TG) content (18). In *Lxr*α/*Lxr*β double

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[1] The abbreviations used are: LXR, liver X receptor; ABC, ATP-binding cassette; apo, apolipoprotein; HDL, high density lipoprotein; SREBP-1c, sterol-regulatory element-binding protein-1c; ACC, acetyl-CoA carboxylase; TG, triglyceride; VLDL, very low density lipoprotein; FFA, free fatty acid; PLTP, phospholipid transfer protein; FPLC, fast protein liquid chromatography; IDL, intermediate density lipoprotein; HBSS, Hanks' buffered saline solution; RT, reverse transcription.

knockout mice, hepatic steatosis did not occur upon treatment with high doses of the agonist (18). In wild type mice, but not in *Lxrα* knockout mice, hepatic TG accumulation was also observed upon feeding a high cholesterol diet (10). Hepatic steatosis is thought to be a result of increased fatty acid synthesis caused by induction of lipogenic genes. *De novo* lipogenesis, in turn, is thought to contribute to regulation of hepatic very low density lipoprotein (VLDL) production (26), but data on VLDL production upon LXR activation are not available. In this study, we examined the effects of T0901317 treatment on VLDL-TG production rates in relation to steady state plasma TG levels in mice. Our results demonstrate that administration of the synthetic LXR agonist resulted in an increased hepatic production rate of VLDL-TG, caused by formation of large TG-rich particles. In wild type mice, increased VLDL-TG production was compensated for by efficient metabolism and clearance, and no effects on fasting TG levels were seen. However, in mice with impaired particle clearance (apoE−/− LDLr−/− double knockouts, APOE*3-Leiden transgenics) treatment with the LXR agonist resulted in a marked hypertriglyceridemia.

EXPERIMENTAL PROCEDURES

Animals—Male C57BL/6J mice (Harlan, Horst, The Netherlands), were housed in a light- and temperature-controlled facility. The animals were fed a commercially available lab chow (RMH-B, Hope Farms BV, Woerden, The Netherlands), containing 6.2% fat and ~0.01% cholesterol (w/w), and water *ad libitum*. Male transgenic mice expressing the human APOE*3-Leiden gene (27, 28) and apoE−/− LDLr−/− double knockout mice (29) were bred at the animal facility of the Gaubius Laboratory (Leiden, The Netherlands) and were fed the same standard diet. The mice received humane care, and experimental procedures were in accordance with local guidelines for use of experimental animals.

Plasma and Liver Tissue Sampling—C57BL/6J mice received 10 mg/kg LXR agonist T0901317 (kindly donated by Organon Laboratories, Lanarkshire, United Kingdom) or its solvent by gavage daily for 4 days. T0901317 was dissolved in Me_2SO and Chremophor (both from Sigma) in 5% mannitol/water, to a final concentration of 2.5 mg/ml. On the morning of the fifth day, a large blood sample was collected by cardiac puncture and centrifuged. The obtained plasma was stored at −20 °C until analyzed. The liver was quickly removed, weighed, and frozen in separate portions for RNA isolation and lipid analysis. Parts of the liver were frozen in isopentane and used for microscopic examination.

ApoE−/− LDLr−/− double knockout mice and APOE*3-Leiden transgenic mice were treated for 4 days with T0901317 (10 mg/kg/day) or its solvent as described. Before onset of treatment (day 0) and on day 2 of treatment, a small EDTA blood sample was collected by tail bleeding and plasma was obtained for measurement of triglyceride concentrations. On day 4 of treatment, a large blood sample was obtained by cardiac puncture.

In Vivo VLDL Triglyceride Production Rate—C57BL/6J wild type mice received T0901317 or the solvent only exactly as described. On the fifth day, after a fasting period of 10 h, mice received an injection of 12.5 mg of Triton WR-1339 in 100 μl of phosphate-buffered saline via the penile vein. Tail blood samples were taken under light halothane anesthesia before and 1, 2, and 3 h after the injection of Triton WR-1339. At 4 h after Triton WR-1339, a large blood sample was collected by cardiac puncture. The collected blood samples were used for triglyceride measurements. VLDL triglyceride production rate was calculated from the slope of the triglyceride concentration *versus* time curve (30). The large blood sample was used for isolation of VLDL.

Hepatic RNA Isolation and Measurement of mRNA Levels by Real-time PCR (Taqman)—Total RNA was isolated from ~30 mg of liver tissue with the TRIzol method (Invitrogen, Paisley, United Kingdom). RNA was converted to single stranded cDNA by a reverse transcription procedure with Moloney murine leukemia virus-RT (Roche Diagnostics, Mannheim, Germany) according to the protocol of the manufacturer using random primers. cDNA levels were measured by real-time PCR using the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA).

For the PCR amplification studies, an amount of cDNA corresponding to 20 ng of total RNA was amplified using the qPCR core kit (Eurogentec, Seraing, Belgium) essentially according to the protocol of the manufacturer and optimized for amplification of the particular gene using the appropriate forward and reverse primers (Invitrogen) and a template-specific 3′-TAMRA (6-carboxytetramethylrhodamine)/5′-FAM (6-carboxyfluorescein)-labeled double dye oligonucleotide probe (Eurogentec). In the same experiments, calibration curves were run on serial dilutions of pooled 8× concentrated cDNA solution as used in the assay, resulting in series containing 4, 2, 1, 0.5, 0.25, 0.125, 0.062, and 0.031 × cDNA present in the assay incubation. The data obtained were processed using the software program ABI Sequence Detector (version 1.6.3; System Applied Biosystems, Foster City, CA). All quantified expression levels were within the linear part of the calibration curves and were calculated by using these curves. PCR results were normalized to *β-Actin* mRNA levels. The sequences of the primers and probes used in this study are listed in Table I.

Hepatic Lipid and Protein Analyses—Livers were homogenized and hepatic concentrations of triglycerides, free cholesterol and total cholesterol were measured using commercial kits (Roche Diagnostics and Wako Chemicals, Neuss, Germany) after lipid extraction according to Bligh and Dyer (31) and redissolving the lipids in 2% Triton X-100 in water. Phospholipid content of the liver was determined according to Böttcher *et al.* (32) after lipid extraction. Protein concentrations in livers were determined according to Lowry *et al.* (33) using bovine serum albumin as standard (Pierce). Fatty acid composition was determined by gas chromatography after methylation as described previously (34).

Histology—Liver histology was examined on frozen liver sections after Oil-Red-O staining for neutral lipids by standard procedures.

Plasma Lipid Analyses—Plasma triglycerides, phospholipids, free fatty acids, HDL cholesterol, free cholesterol, and total cholesterol were determined using commercially available kits (Roche Diagnostics and Wako Chemicals). Lipoproteins of C57BL/6J mice, apoE−/− LDLr−/− double knockout mice and APOE*3-Leiden transgenic mice were separated using fast protein liquid chromatography (FPLC) on a Superose 6B 10/30 column (Amersham Biosciences). The contents of apoB100, apoB48, and apoA-I in the fractions were visualized by Western blotting. Proteins in the fractions were separated by SDS-PAGE using 4–15% gradient gels (Ready Gels, Bio-Rad) and transferred to nitrocellulose membranes (Hyperbound, Amersham Biosciences, Roosendaal, The Netherlands). The membranes were incubated with sheep anti-human apoB (Roche Diagnostics) or rabbit anti-human apoA-I (Calbiochem, San Diego, CA), followed by incubation with donkey anti-sheep IgG horseradish peroxidase or donkey anti-rabbit IgG horseradish peroxidase and visualized by ECL detection (all three from Amersham Biosciences, Uppsala, Sweden) according to the instructions from the manufacturer.

VLDL Isolation and Analyses—Plasma VLDL/IDL ($d < 1.019$ g/ml) was isolated by density gradient ultracentrifugation. For this, 300 μl of plasma was adjusted to 800 μl with a NaCl/KBr solution with a density of 1.019 g/ml, containing 1 mM EDTA and NaN_3, and centrifuged at 120,000 rpm in a Optima TM LX tabletop ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA). VLDL was isolated by tube slicing, and the volume was recorded by weight. ApoB100 and apoB48 were determined by Western blotting, using antibodies against human apoB raised in sheep. Triglyceride and cholesterol content were determined as described for plasma. Phospholipids were determined using a commercial kit (Wako Chemicals). Fatty acid composition was determined as described previously (34).

VLDL Size Determination—VLDL size and volume distribution profiles were analyzed by dynamic scattering using a Nicomp model 370 submicron particle analyzer (Nicomp Particle Sizing Systems, Santa Barbara, CA). Particle diameters were calculated from the volume distribution patterns provided by the analyzer.

Cell Culture Experiments—McA-RH7777 (rat hepatoma) cells were plated in 35-mm six-well plastic dishes (Costar Corp., Cambridge, MA) in 2 ml of Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 10% natural horse serum, penicillin/streptomycin, and geneticin. At a confluence of ~30%, the medium was removed and cells were washed with Hanks' balanced salt solution (HBSS) and subsequently incubated with Dulbecco's modified Eagle's medium + 10% fetal calf serum + 10% natural horse serum containing either 0 or 10 μM T0901317 and the above mentioned antibiotics. After 24 h, the medium was removed and half of the wells were incubated with the same medium for another 41 h. The other half of the wells were incubated with the same medium to which was added 25 μM [^{3}H]glycerol (4.4 μCi/well) for 41 h. The medium was then collected, centrifuged to remove debris, and stored at 4 °C until further analysis. Cells were washed with ice-cold HBSS and either used for RNA isolation or

TABLE I
Primers and probes used for real-time PCR analysis of hepatic gene expression

Gene		Sequences (5′ to 3′)	GenBank™ accession no.
β-Actin	Forward	AGCCATGTACGTAGCCATCCA	NM_007393 (mouse)
	Reverse	TCTCCGGAGTCCATCACAATG	NM_031144 (rat)
	Probe	TGTCCCTGTATGCCTCTGGTCGTACCAC	
Srebp-1c	Forward	GGAGCCATGGATTGCACATT	AF286470 (mouse)
	Reverse	CCTGTCTCACCCCCAGCATA	Ref. 50 (rat)
	Probe	CAGCTCATCAACAACCAAGACAGTGACTTCC	
Srebp-1a	Forward	GAGGCGGCTCTGGAACAGA	Ref. 50
	Reverse	TGTCTTCGATGTCGTTCAAAACC	
	Probe	TGTGTCCAGTTCGCACATCTCGGC	
Acc1	Forward	GCCATTGGTATTGGGGCTTAC	AF374170 (mouse)
	Reverse	CCCGACCAAGGACTTTGTTG	NM_022193 (rat)
	Probe	CTCAACCTGGATGGTTCTTTGTCCCAGC	
Fas	Forward	GGCATCATTGGGCACTCCTT	AF127033 (mouse)
	Reverse	GCTGCAAGCACAGCCTCTCT	NM_017332 (rat)
	Probe	CCATCTGCATAGCCACAGGCAACCTC	
Apob	Forward	GCCCATTGTGGACAAGTTGATC	AW012827
	Reverse	CCAGGACTTGGAGGTCTTGGA	
	Probe	AAGCCAGGGCCTATCTCCGCATCC	
Apobec-1	Forward	TCGTCCGAACACCAGATGCT	NM_031159
	Reverse	GGTGTCGGCTCAGAAACTCTGT	
	Probe	CCTGGTTCCTGTCCTGGAGTCCCTG	
Mttp	Forward	CAAGCTCACGTACTCCACTGAAG	NM_008642
	Reverse	TCATCATCACCATCAGGATTCCT	
	Probe	ACCGCAAGACAGCGTGGGCTACA	
Apoe	Forward	CCTGAACCGCTTCTGGGATT	NM_009696
	Reverse	GCTCTTCCTGGACCTGGTCA	
	Probe	AAAGCGTCTGCACCCAGCGCAGG	
Hl	Forward	ACTGCAGGAGTGTGGCTTCAAC	NM_008280
	Reverse	TGGGACTGTCGGGACTTCAG	
	Probe	CAAGCCATCCACCGACCACCCG	
Lpl	Forward	AAGGTCAGAGCCAAGAGAAGCA	NM_008509
	Reverse	CCAGAAAAGTGAATCTTGACTTGGT	
	Probe	CCTGAAGACTCGCTCTCAGATGCCCTACA	
Pltp	Forward	TCAGTCTGCGCTGGAGTCTCT	NM_011125
	Reverse	AAGGCATCACTCCGATTTGC	
	Probe	TCCCACTGCAGGCCCCACTGAA	
Vldlr	Forward	CCACAGCAGTATCAGAAGTCAGTGT	NM_013703
	Reverse	CACCTACTGCTGCCATCACTAAGA	
	Probe	CAGCTGCCTGGGCCATCCTTCC	
Ldlr	Forward	GCATCAGCTTGGACAAGGTGT	X64414
	Reverse	GGGAACAGCCACCATTGTTG	
	Probe	CACTCCTTGATGGGCTCATCCGACC	
Lxra	Forward	GCTCTGCTCATTGCCATCAG	AF085745
	Reverse	TGTTGCAGCCTCTCTACTTGGA	
	Probe	TCTGCAGACCGGCCCAACGTG	

scraped into 2 ml of HBSS for lipid extraction ([^{3}H]glycerol-labeled cells).

Lipid Analyses of Cells and Medium—Lipids secreted by McA-RH7777 cells into medium and cellular lipids were extracted as described previously (35). [^{3}H]TG was separated from the other lipids by thin layer chromatography with hexane/diethyl ether/acetic acid (80/20/1 v/v/v) as developing solvent. After iodine staining, the spots containing [^{3}H]TG (as a measure for VLDL-TG secretion) were scraped into vials and assayed for radioactivity by scintillation counting. Part of the extracted lipids was dissolved in chloroform containing 2% Triton X-100. The chloroform was replaced by the same volume of water after evaporation of the chloroform, the lipids were resuspended, and total cellular TG concentration was determined using the commercially available triglyceride assay kit (Roche Diagnostics).

Western Blotting of ApoB in Medium—Secreted apoB in medium of McA-RH7777 cells was concentrated with fumed silica according to the methods described by Vance *et al.* (36). ApoB was separated by SDS-PAGE using 4–15% gradient gels as described.

Protein Measurements of Cells—Protein concentrations of cell suspensions were determined according to Lowry *et al.* (33) using bovine serum albumin as standard (Pierce).

mRNA Expression Levels in Cells—Total RNA from McA-RH7777 cells was isolated by using the SV Total RNA Isolation System (Promega RNA, Madison, WI) according to the instructions from the manufacturer. RNA was processed for real-time PCR as described under "Hepatic RNA Isolation and Measurement of mRNA Levels by Real-time PCR (Taqman)."

Statistics—All values represent mean ± standard deviation for the number of animals, wells, or experiments indicated. Statistical analysis of two groups was assessed by Mann-Whitney *U* test. Level of significance was set at $p < 0.05$. Analyses were performed using SPSS for Windows software (SPSS, Chicago, IL).

RESULTS

Effects of T0901317 on Hepatic and Plasma Lipids in C57BL/6J Mice—Livers from mice that had received T0901317 were clearly heavier than those in the control group (Table II). The hepatic TG content in treated mice was ~10 times higher than in controls. There were no differences in protein, phospholipid, and free cholesterol contents, whereas the cholesteryl ester content was slightly decreased after treatment (Table II). Oil-Red-O staining for neutral lipids on frozen liver sections showed massive lipid accumulation in periportal (zone 1) as well as in perivenous (zone 3) hepatocytes in mice receiving T0901317 (Fig. 1). The hepatic fatty acid composition was changed upon treatment: the relative amount of oleate (C18:1) was sharply increased (Fig. 2), whereas the relative

TABLE II
Comparison of hepatic lipid parameters of mice treated with T0901317 or its solvent

Male C57BL/6J mice were treated with 10 mg/kg synthetic LXR agonist T0901317 or its solvent (control) during 4 days. Livers were weighed, and concentrations of lipids were measured as described under "Experimental Procedures." Each value represents the mean ± S.D.; $n = 10$. *, $p < 0.05$.

	Control	T0901317
Liver weight (% of body weight)	4.6 ± 0.6	6.6 ± 0.3*
Protein (mg/g liver)	225 ± 17	225 ± 17
Triglycerides (nmol/mg liver)	6.92 ± 2.65	57.74 ± 16.61*
Free cholesterol (nmol/mg liver)	4.21 ± 0.50	3.83 ± 0.71*
Cholesterylester (nmol/mg liver)	1.44 ± 0.52	0.98 ± 0.40*
Phospholipids (nmol/mg liver)	35.74 ± 3.48	36.43 ± 3.63

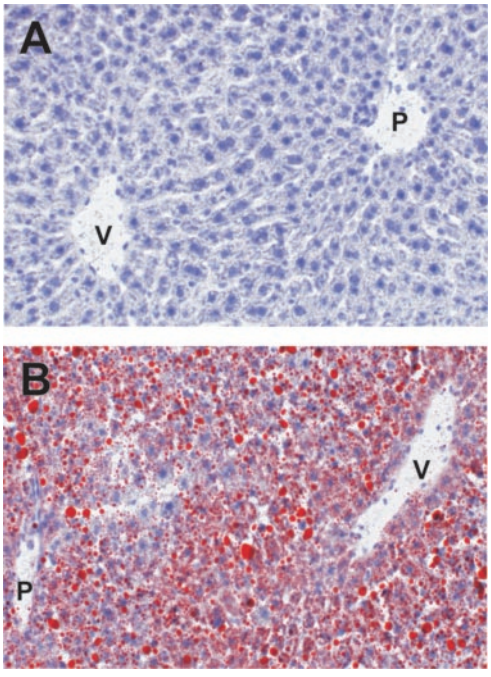

FIG. 1. **Uniform Oil-Red-O staining for neutral fat in livers of T0901317-treated mice.** No staining was observed in liver sections from male C57BL/6J mice receiving the solvent (*A*). Massive staining was found in liver sections from mice treated 4 days with 10 mg/kg LXR agonist T0901317 (*B*). *V*, central vein; *P*, portal vein. Original magnification, ×50.

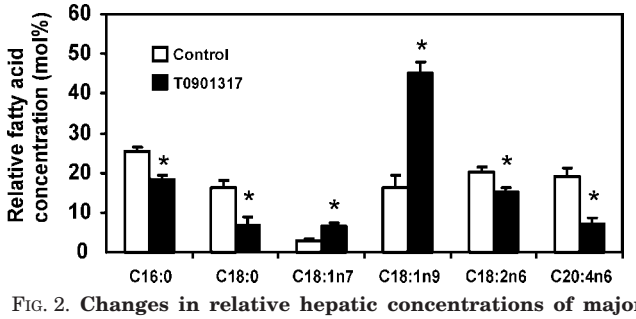

FIG. 2. **Changes in relative hepatic concentrations of major fatty acid species upon LXR activation.** Fatty acid composition was determined in ~15 mg of liver tissue by gas chromatography after methylation, with heptadecanoic acid (C17:0) as internal standard. *Open bars*, livers from male C57BL/6J mice receiving the solvent (control). *Closed bars*, livers from mice treated 4 days with 10 mg/kg LXR agonist T0901317. Values represent the mean percentage ± S.D.; $n = 10$; *, $p < 0.05$.

amounts of palmitate (C16:0), linoleate (C18:2), and arachidonate (C20:4) were markedly decreased upon LXR activation.

Hepatic expression of LXRα was slightly reduced upon treatment with the agonist (Fig. 3*A*), but expression of genes involved

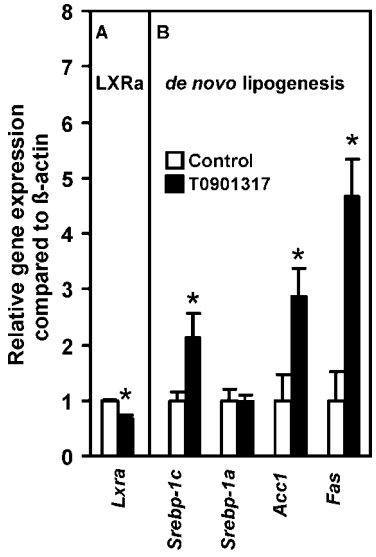

FIG. 3. **Changes in relative hepatic gene expression upon LXR activation, determined by real-time PCR.** RNA was isolated from ~30 mg of liver tissue from male C57BL/6J mice and converted to cDNA. Levels of cDNA were measured by real-time PCR as described under "Experimental Procedures." Results were normalized to *β-Actin* mRNA levels. *A*, expression of the gene encoding LXRα. *B*, expressions of genes encoding proteins involved in *de novo* lipogenesis. *Open bars*, mice receiving the solvent (control), arbitrarily defined as 1. *Closed bars*, mice treated 4 days with 10 mg/kg LXR agonist T0901317. *Lxra*, liver X receptor α; *Srebp*, sterol-regulatory element-binding protein; *Acc1*, acetyl-CoA carboxylase-1; *Fas*, fatty acid synthase. Values represent the mean ± S.D.; $n = 4$; *, $p < 0.05$.

in *de novo* lipogenesis was clearly increased (Fig. 3*B*). Expression of *Srebp-1c* was more than doubled after LXR activation, whereas mRNA levels of *Acc1* and *Fas* were 3.5–4.5 times higher than in controls. *Acc1* encodes the cytosolic isoform of ACC. No induction of mRNA levels of *Srebp-1a* was observed.

The plasma concentrations of free cholesterol, cholesteryl esters, phospholipids, and free fatty acids (FFA) were significantly elevated in the treated group as compared with controls (Table III). As expected, HDL cholesterol was increased by ~60%, but the TG concentration was not affected by LXR activation in these experiments. Upon FPLC separation of plasma lipoproteins, distribution of TG appeared to be unaffected after LXR activation (Fig. 4*A*). Cholesterol contents of fractions 25–29 were higher in the group treated with T0901317 (Fig. 4*B*). Fig. 4*C* shows that apoA-I was present in these earlier fractions after LXR activation, indicating that the "shoulder" in the cholesterol profile could be attributed to the presence of large HDL particles.

LXR Activation Leads to Stimulation of VLDL-TG Secretion through Formation of Larger Particles—Fig. 5 shows the plasma TG concentration *versus* time curve after injection of Triton WR-1339 in control and T0901317-treated mice. The VLDL-TG production rates were calculated from these curves. Upon LXR activation, the production rate was 2.56 times higher than control values: 201.9 ± 36.4 *versus* 78.8 ± 18.7 μmol/kg/h (Fig. 5, *inset*). The concentrations of free cholesterol, cholesteryl esters, phospholipids, and TG in nascent VLDL particles isolated from plasma obtained at 4 h after Triton WR-1339 injection are summarized in Table IV. The mol% of surface lipids (free cholesterol and phospholipids) was reduced relative to that of core lipids (TG and cholesteryl esters), leading to an increase of the core:surface ratio by 14.4%. Accordingly, direct measurement of particle sizes revealed that after LXR activation the mean diameter of the VLDL particles was increased by 48% (129 ± 9 *versus* 94 ± 12 nm, $p < 0.001$). The diameter was used to calculate the particle volume. The in-

TABLE III
Comparison of plasma lipid parameters of mice treated with T0901317 or its solvent

Male C57BL/6J mice were treated with 10 mg/kg synthetic LXR agonist T0901317 or its solvent (control) during 4 days. Blood was collected by cardiac puncture, and plasma obtained by centrifugation. Concentrations of lipids were measured as described under "Experimental Procedures." Each value represents the mean ± S.D.; $n = 10$. *, $p < 0.05$.

	Control	T0901317
Free cholesterol (mM)	0.60 ± 0.07	0.87 ± 0.05*
Cholesterylester (mM)	1.36 ± 0.31	2.27 ± 0.21*
HDL cholesterol (mM)	1.35 ± 0.27	2.15 ± 0.14*
Phospholipids (mM)	2.55 ± 0.41	3.94 ± 0.21*
Triglycerides (mM)	0.46 ± 0.16	0.49 ± 0.21
Free fatty acids (mM)	0.34 ± 0.07	0.47 ± 0.08*

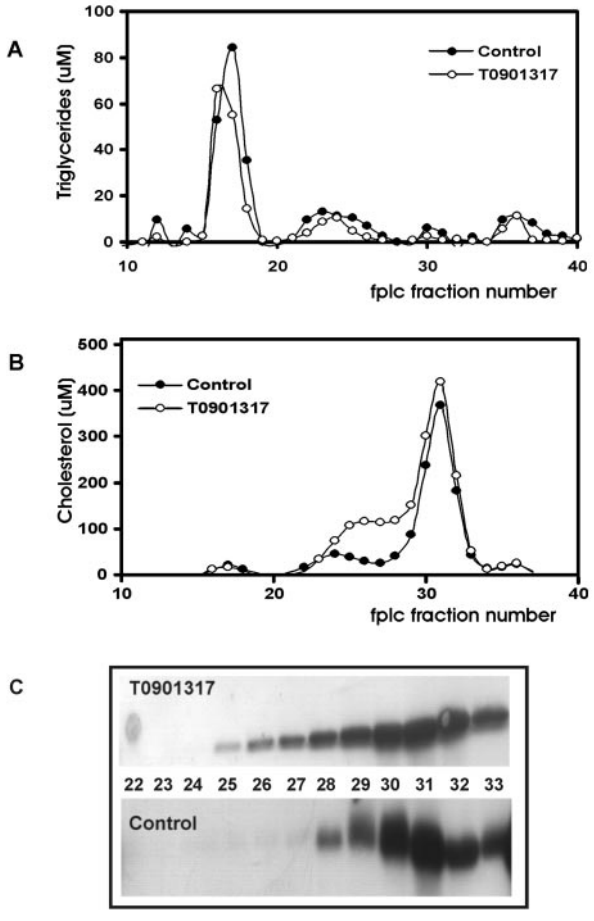

FIG. 4. **Effect of LXR activation on distribution of triglycerides and cholesterol in plasma lipoprotein fractions.** Lipoproteins were separated using FPLC on a Superose 6B 10/30 column (Amersham Biosciences). Plasma of 10 male C57BL/6J mice per group was pooled, and 0.2 ml was applied to the column and eluted with phosphate-buffered saline at a flow of 0.5 ml/min. Triglycerides and cholesterol were measured as described under "Experimental Procedures." *A*, profiles of triglycerides. *B*, profiles of cholesterol. ○, mice receiving the solvent (control). ●, mice treated 4 days with 10 mg/kg LXR agonist T0901317. *C*, SDS-polyacrylamide gel electrophoresis followed by Western blotting of apoA-I in the indicated FPLC fractions. *Top panel*, mice treated 4 days with 10 mg/kg LXR agonist T0901317; *bottom panel*, mice receiving the solvent (control).

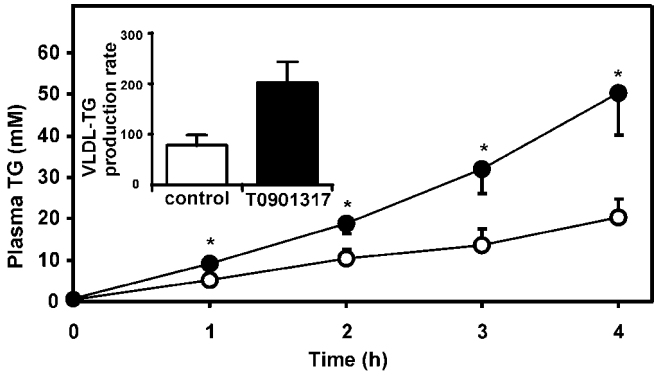

FIG. 5. **Plasma triglyceride concentrations in T0901317-treated and control mice after injection of Triton WR-1339.** Male C57BL/6J mice received at $t = 0$ an injection of 12.5 mg of Triton WR-1339 in 100 μl of phosphate-buffered saline via the penile vein. Triglyceride concentration was measured in the plasma samples collected at indicated time points as described under "Experimental Procedures." ○, plasma from mice receiving the solvent (control). ●, plasma from mice treated 4 days with 10 mg/kg LXR agonist T0901317. The *inset* shows the VLDL-TG production rate in μmol/kg/h, calculated from the plasma TG *versus* time curve. *Open bar*, mice receiving the solvent (control). *Closed bar*, mice treated 4 days with 10 mg/kg LXR agonist T0901317. Values represent the mean ± S.D.; $n = 6$ (T0901317), $n = 7$ (control); *, $p < 0.05$.

TABLE IV
Comparison of composition and size of nascent VLDL particles of mice treated with T0901317 or its solvent

Male C57BL/6J mice were treated with 10 mg/kg synthetic LXR agonist T0901317 or its solvent (control) during 4 days. Mice were injected with Triton WR-1339 on the fifth day. At 4 h after injection, blood was collected by cardiac puncture and VLDL was isolated from plasma by density gradient ultracentrifugation. Concentrations of lipids were measured as described under "Experimental Procedures" and particle diameter using a particle sizer. Particle volume was calculated using the equation volume = $4/3 \times \pi \times (0.5 \times \text{diameter})^3$. Each value represents the mean ± S.D.; $n = 7$ (control); $n = 6$ (T0901317); *, $p < 0.05$.

	Control	T0901317
Free cholesterol (mM)	1.50 ± 0.31	2.01 ± 0.31*
Cholesterylester (mM)	0.63 ± 0.10	1.34 ± 0.19*
Phospholipids (mM)	2.95 ± 0.62	4.47 ± 0.45*
Triglycerides (mM)	28.53 ± 5.86	47.10 ± 8.66*
Core:surface lipids	6.55 ± 0.35	7.49 ± 1.10
Diameter (nm)	94 ± 12	129 ± 9*
Volume (10^5 nm^3)	4.5 ± 1.7	11.3 ± 2.3*

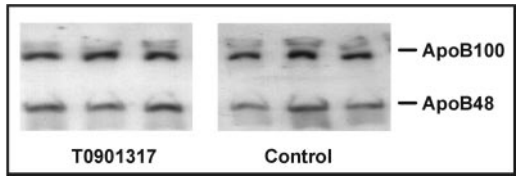

FIG. 6. **Apolipoprotein B contents in nascent VLDL particles of control and T0901317-treated mice.** Plasma was obtained by cardiac puncture at 4 h after injection of Triton WR-1339. 300 μl of plasma was adjusted to 800 μl with a NaCl/KBr solution ($d = 1.019$ g/ml), containing 1 mM EDTA and NaN_3, and centrifuged at 120,000 rpm. VLDL was isolated by tube slicing, and this fraction was applied to SDS-polyacrylamide gel electrophoresis followed by Western blotting. Each *lane* represents VLDL from an individual mouse. *Right panel*, mice receiving the solvent (control). *Left panel*, mice treated 4 days with 10 mg/kg LXR agonist T0901317.

crease in mean particle volume (factor 2.54) was virtually identical to the increase in VLDL-TG production (factor 2.56), indicating that the latter increase was caused by the production of *larger* particles rather than by the production of *more* particles. In accordance with this notion, no differences in apoB contents were found in isolated VLDL fractions by Western blotting (Fig. 6).

The fatty acid composition of the VLDL fraction was changed upon treatment, resulting in changes similar as seen for the hepatic fatty acids (Fig. 7). The relative amount of VLDL-associated oleate (C18:1) was increased, and relative amounts

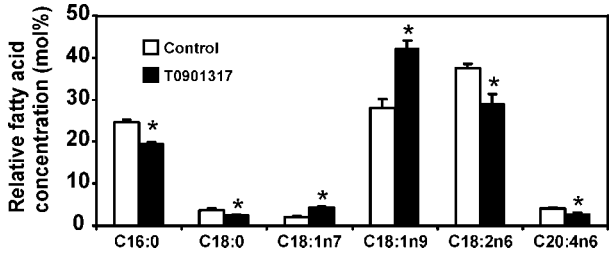

FIG. 7. **Changes in relative concentrations of major fatty acid species in VLDL fractions upon LXR activation.** VLDL fractions were isolated from plasma obtained by cardiac puncture at 4 h after injection of Triton WR1339. Fatty acids were determined by gas chromatography after methylation, with heptadecanoic acid (C17:0) as internal standard. *Open bars*, VLDL from mice receiving the solvent (control). *Closed bars*, VLDL from mice treated 4 days with 10 mg/kg LXR agonist T0901317. Values represent the mean percentage ± S.D.; $n = 10$; *, $p < 0.05$.

of palmitate (C16:0), linoleate (C18:2), and arachidonate (C20:4) were decreased upon LXR activation.

To gain further insight into the molecular background of LXR-induced stimulation of VLDL-TG secretion, the expression of genes involved in the control of this process was investigated. Fig. 8*A* shows that expression of the *Apob* gene was slightly but significantly reduced upon LXR activation. No changes in expression of *Mttp* and *Apoe* were noted. Likewise, expression of *Apobec-1*, encoding the apoB mRNA-editing protein, was not affected. In contrast, a marked induction of *Lpl* and *Pltp* mRNA levels was found (Fig. 8*B*), the latter encoding the phospholipid transfer protein. Expression of *Ldlr*, encoding the low density lipoprotein receptor involved in the clearance of TG-rich lipoproteins, was slightly induced.

*LXR Activation Leads to Hypertriglyceridemia in ApoE−/− LDLr−/− Double Knockout and APOE*3-Leiden Transgenic Mice*—Increased VLDL-TG production in C57BL/6J mice was not accompanied by elevated fasting TG levels (Table III), possibly as a result of efficient particle metabolism and clearance. To test this option, effects of LXR activation on plasma TG levels were also studied in mouse models with defective particle clearance, *i.e.* apoE−/− LDLr−/− double knockout and APOE*3-Leiden transgenic mice. Fig. 9 shows that, in both apoE−/− LDLr−/− mice and in APOE*3-Leiden transgenic mice, treatment with T0901317 resulted in a very strong, progressive increase in plasma TG concentrations. As expected, TG was almost exclusively present in VLDL-sized lipoprotein fractions in T0901317-treated apoE−/− LDLr−/− double knockouts and in APOE*3-Leiden transgenics upon FPLC separation of plasma lipoproteins (data not shown).

Effects of LXR Activation on VLDL-TG Production in Cultured Liver Cells—To determine whether LXR effects on VLDL production can be achieved via direct actions on liver cells, the rat hepatoma cell line McA-RH7777 was exposed to the LXR agonist T0901317 for 65 h. Fig. 10 shows the effects of LXR activation on mRNA expression of genes involved in VLDL assembly and fatty acid synthesis. After incubation with T0901317, a 7-fold increase in *Srebp-1c* mRNA levels was observed as well as a 2-fold increase in both *Acc1* and *Fas* mRNA levels. Similar to the *in vivo* situation, a 20% decrease in *Apob* and a slight decrease in *Lxrα* mRNA levels were observed after exposure of the cells to the LXR agonist.

After 24 h of incubation with T0901317 alone, the medium was replaced by medium also containing [^{3}H]glycerol. After another 41-h incubation, lipids were isolated from cells and media to determine the amount and secretion of newly synthesized VLDL-TG. Incubation with T0901317 resulted in a 2.6-fold induction of VLDL-TG secretion and a 3.4-fold induction of TG synthesis (Fig. 11*A*). Intracellular total TG mass was also

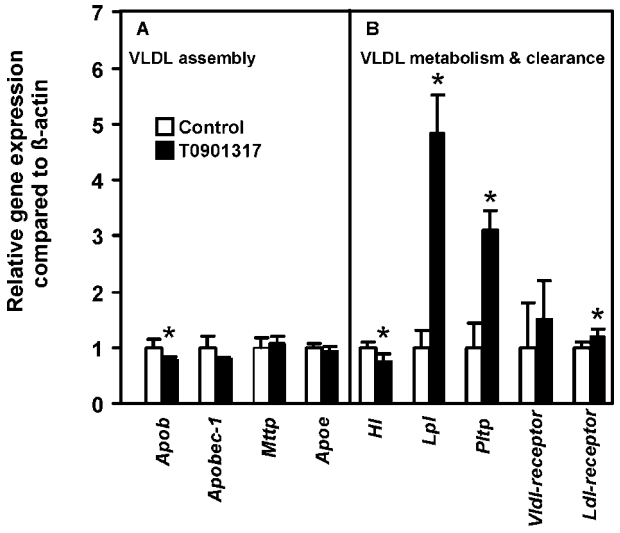

FIG. 8. **Changes in relative hepatic gene expression upon LXR activation, determined by real-time PCR.** RNA was isolated from ~30 mg of liver tissue from male C57BL/6J mice and converted to cDNA. Levels of cDNA were measured by real-time PCR as described under "Experimental Procedures." Results were normalized to *β-Actin* mRNA levels. *A*, expressions of the genes encoding proteins involved in VLDL assembly and secretion. *B*, expressions of genes encoding proteins involved in VLDL metabolism and clearance. *Open bars*, mice receiving the solvent (control), arbitrarily defined as 1. *Closed bars*, mice treated 4 days with 10 mg/kg LXR agonist T0901317. *Apob*, apolipoprotein B; *Apobec-1*, apolipoprotein B editing complex-1; *Mttp*, microsomal triglyceride transfer protein; *Apoe*, apolipoprotein E; *Hl*, hepatic lipase; *Lpl*, lipoprotein lipase; *Pltp*, phospholipid transfer protein. Values represent the mean ± S.D.; $n = 4$; *, $p < 0.05$.

increased upon incubation with T0901317 (1.4-fold compared with control situation). The amount of apoB isolated from medium after incubation with T0901317 was compared with that in the control medium. No significant differences in apoB100 or apoB48 were detected (Fig. 11*B*).

DISCUSSION

Pharmacological activation of LXR leading to accelerated reverse cholesterol transport has been advocated as a potential novel treatment or prevention of atherosclerosis (17). This study shows that the beneficial raise of HDL levels is accompanied by potentially adverse effects on triglyceride metabolism. In mice, an increased VLDL-TG production was seen upon LXR activation by the LXR agonist T0901317, caused by the formation of large, TG-rich particles. Large VLDL particles, also known as VLDL1 particles, are considered pro-atherogenic and are metabolized to small dense low density lipoprotein. Increased production of VLDL1 is a hallmark of diabetes type 2-related hyperlipidemia (37).

The development of a severe hepatic steatosis, also reported previously (18), is likely related to increased *de novo* lipogenesis in combination with an increased FFA flux toward the liver. Slight quantitative differences in T0901317-induced gene expression patterns between our and other studies (18, 22, 25) are probably the result of differences in treatment protocols. For instance, Liang *et al.* (25) supplied T0901317 via the food during 12 h, which may have resulted in a more constant exposure of the liver to the agonist. Livers were removed immediately after this 12-h period. In our study mice received T0901317 daily for 4 days, and livers were taken out ~20 h after the last administration. Second, effects of the agonist may be transient because Joseph *et al.* (22) showed that hepatic expression of *Fas* and *Scd-1* was higher after 3 days of LXR activation than after 7 days.

The accumulated neutral lipids were distributed uniformly

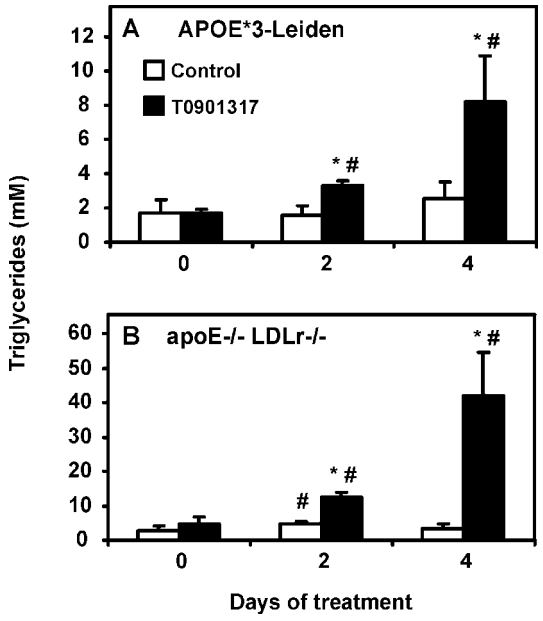

FIG. 9. **Plasma triglyceride concentrations in apoE−/− LDLr−/− mice and APOE*3-Leiden transgenic mice upon LXR activation.** Male apoE−/− LDLr−/− mice and APOE*3-Leiden transgenic mice were treated for 4 days with T0901317 or its solvent. Before onset of treatment (day 0) and on the second day of treatment, a small EDTA blood sample was collected by tail bleeding. On the fourth day, blood was obtained by cardiac puncture. Triglyceride concentration was measured in plasma as described under "Experimental Procedures." *A*, plasma triglyceride concentrations in APOE*3-Leiden transgenic mice. *B*, plasma triglyceride concentrations in apoE−/− LDLr−/− double knockout mice. *Open bars*, mice receiving the solvent (control). *Closed bars*, mice treated with 10 mg/kg LXR agonist T0901317. Values represent the mean ± S.D.; $n = 4$ (APOE*3-Leiden); $n = 3$ (apoE−/− LDLr−/− double knockouts); *, $p < 0.05$ for treated *versus* control; #, $p < 0.05$ compared with concentration at $t = 0$.

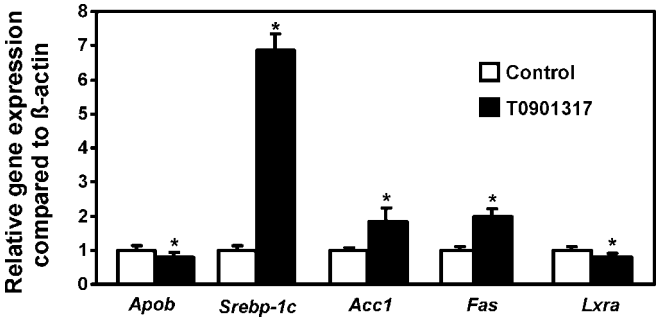

FIG. 10. **Changes in relative hepatic gene expression, determined by real-time PCR, upon LXR activation in McA-RH7777 cells.** RNA was isolated from McA-RH7777 cells exposed to T0901317-containing medium or control medium and converted to cDNA. Levels of cDNA were measured by real-time PCR as described under "Experimental Procedures." Results were normalized to *β-Actin* mRNA levels. *Open bars*, cells without T0901317 (control), arbitrarily defined as 1. *Closed bars*, cells incubated with 10 μM LXR agonist T0901317 during 65 h. *Apob*, apolipoprotein B; *Srebp-1c*, sterol-regulatory element-binding protein-1c; *Acc1*, acetyl-CoA carboxylase-1; *Fas*, fatty acid synthase; *Lxra*, liver X receptor α. Values represent the mean ± S.D.; $n = 6$; *, $p < 0.05$.

across the liver lobule, in contrast to most models of steatosis, *e.g.* the ob/ob mice (38), in which fat accumulates mainly in perivenous (zone 3) hepatocytes. After LXR activation, TG accumulated both in perivenous (zone 3) hepatocytes, which may reflect increased *de novo* lipogenesis (39), and in periportal (zone 1) hepatocytes. The fatty acid profile showed a specific increase in 18:1 fatty acids, in accordance with increased expression of *Scd-1* after LXR activation (25). The fatty acid composition of VLDL was comparable with that of liver homogenates, indicating that TG containing specific fatty acids are not preferentially recruited for VLDL assembly.

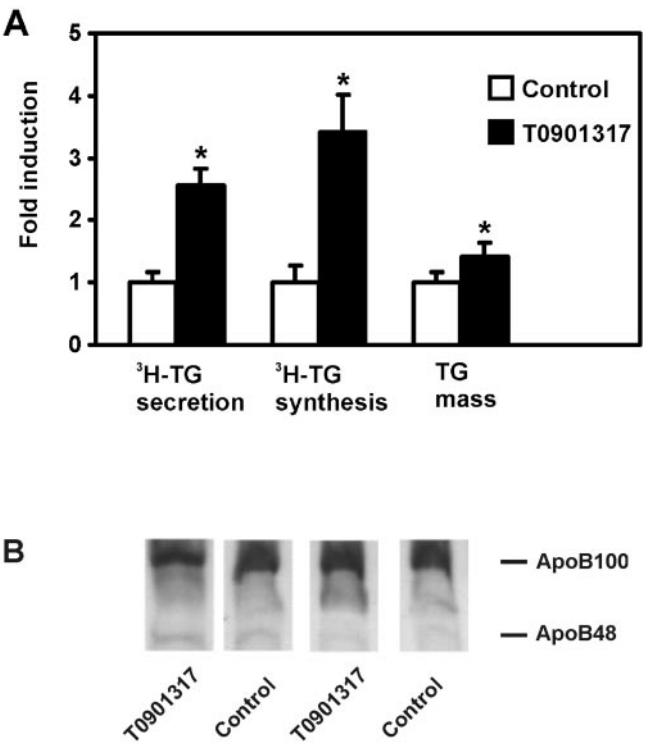

FIG. 11. **Triglyceride synthesis and VLDL-triglyceride secretion in the rat hepatoma cell line McA-RH7777.** McA-RH7777 cells were incubated with or without 10 μM T0901317 for 24 h. After 24 h the medium was removed and replaced by medium containing [^{3}H]glycerol with or without 10 μM T0901317. After 41 h, medium was collected and cells were harvested. Lipids were extracted from medium and cell suspensions, and [^{3}H]TG was separated from other lipids by thin layer chromatography. [^{3}H]TG spots were assayed for radioactivity by scintillation counting. The intracellular TG mass was enzymatically determined as described under "Experimental Procedures." Secreted apoB in the medium was concentrated with fumed silica and separated from other proteins on a 4–15% gradient gel by SDS-PAGE followed by Western blotting. *A*, -fold induction of [^{3}H]TG secretion, [^{3}H]TG synthesis, and intracellular TG mass after a 65-h incubation with 10 μM T0901317 (*closed bars*) compared with the control situation (*open bars*). Values represent mean -fold induction from three independent experiments ± S.D.; *, significantly different from control, $p < 0.05$. *B*, secreted apoB100 and apoB48 after a 65-h incubation with or without 10 μM T0901317. The apoB blot is representative for the results from three independent experiments.

The increase of HDL cholesterol was mainly the result of the presence of larger particles in the circulation. Elevated HDL levels are probably caused by increased cholesterol efflux from peripheral and/or liver cells caused by induction of ABCA1 and ABCG1. The HDL elevating action of the LXR agonist may be, in part, counteracted by the LXR-induced expression of *Pltp*, because overexpression of *Pltp* is shown to reduce HDL in mice (40). However, induction of *Pltp* expression might also contribute to the presence of large HDL particles, as PLTP is a main factor in the regulation of the size and composition of HDL. PLTP has been proposed to catalyze conversion of two HDL particles to yield one *larger* HDL particle and a small lipid-poor apoA-I particle, pre-β-HDL (41, 42). These latter particles are efficient acceptors of cholesterol from peripheral cells.

The induced VLDL-TG production rate upon LXR activation was found to be the result exclusively of formation of larger particles. The increase in VLDL-TG production exactly matched the increased mean VLDL particle volume. The apoB content of isolated VLDL fractions was not affected upon treatment, strengthening our conclusion that LXR activation leads

to formation of *larger* instead of *more* VLDL particles. The increased TG production by the liver did not lead to a raise in plasma TG levels in CL57BL/6J mice, but defective clearance in apoE−/− LDLr−/− double knockout and APOE*3-Leiden transgenic mice clearly unmasked the hypertriglyceridemic actions of LXR activation. Plasma TG levels markedly and progressively increased upon treatment with the agonist in these mice. Interestingly, Schultz *et al.* (18) showed that treatment of wild type C57BL/6 mice with T0901317 during 7 days did lead to elevated plasma TG levels. The discrepancy with our results may be caused by a difference in duration of treatment. Possibly, net effects of T0901317-treatment on plasma lipid levels are determined by time-dependent effects on gene expression levels and their metabolic consequences.

It is tempting to speculate that LXR-stimulated *de novo* lipogenesis is the primary cause of the raised VLDL-TG secretion. The increased VLDL-TG production together with the induced transcription of hepatic *Lpl* might account for the elevated plasma FFA levels observed upon treatment (Table II). Lipoprotein lipase hydrolyzes TG and thereby catalyzes the conversion of VLDL into IDL, and subsequently of IDL into low density lipoprotein. Both conversions result in the release of FFAs into the bloodstream (43) and these FFAs will to large extent be taken up again by the liver. Increased supply of FFAs to liver cells stimulates VLDL-TG production (44); part of the observed stimulation of VLDL-TG production may therefore occur via this indirect mechanism. To investigate whether LXR activation stimulates the VLDL-TG secretion by liver cells directly, we performed a limited series of experiments with McA-RH7777 cells kept under fixed FA concentrations. In these *in vitro* studies, we also found a gene expression profile consistent with increased *de novo* lipogenesis and stimulated cellular TG secretion, again without changes in apoB production. Administration of T0901317 to C57BL/6 mice also results in induction of *Srebp-1* and *Scd-1* mRNA levels in intestine and kidney (18). A recent study from Mak *et al.* (45) showed that incubation of murine macrophages with T0901317 resulted in increased *Srebp-1* and *Fas* mRNA levels. Nevertheless, actions of the LXR agonist in hepatocytes may be responsible for the observed increased VLDL-TG production rate *in vivo* because these cells are primarily involved in this process. It should be stressed that increased hepatic *de novo* lipogenesis is not always associated with increased VLDL-TG secretion. For instance, the strong induction of *de novo* lipogenesis upon inhibition of glucose-6-phosphatase activity in rats is not associated with increased VLDL production (46). Apparently, different regulatory factors dominate under different metabolic conditions.

Genes encoding proteins involved in assembly and secretion of VLDL, *i.e. Apoe* and *Mttp*, were not affected upon treatment. The production of larger VLDL particles might be accounted for by an intracellular action of PLTP. Jiang *et al.* (47) reported that PLTP-deficient mice show a decreased secretion of apoB-containing particles. Because this decreased apoB production rate was observed both *in vivo* and in hepatocytes *in vitro*, it was concluded that PLTP must also be active within the cells. This suggestion is supported by the fact that mice overexpressing the human PLTP show increased VLDL-TG production (48). However, neither of these studies provided information about the size of the particles produced in the presence or absence of PLTP. Alternatively, induction of *Cyp7A1* expression and activity may contribute to increased VLDL-TG production, as mice overexpressing the human *Cyp7A1* gene also show this feature (49). Thus, various mechanisms may be responsible for the production of larger VLDL particles after LXR activation, but the exact mode of action is currently not known.

In conclusion, pharmacological activation of LXR is associated with development of hepatic steatosis and strongly affects the VLDL production process in mice, leading to formation of large, TG-rich particles. This combination of hepatic fat accumulation and overproduction of large VLDL strikingly resembles that observed in human diabetic states.

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Stimulation of Lipogenesis by Pharmacological Activation of the Liver X Receptor Leads to Production of Large, Triglyceride-rich Very Low Density Lipoprotein Particles

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