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Published in:
Journal of Lipid Research

DOI:
[10.1194/jlr.M600170-JLR200](https://doi.org/10.1194/jlr.M600170-JLR200)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2007

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Citation for published version (APA):

van der Veen, J. N., Havinga, R., Bloks, V. W., Groen, A. K., & Kuipers, F. (2007). Cholesterol feeding strongly reduces hepatic VLDL-triglyceride production in mice lacking the liver X receptor alpha. *Journal of Lipid Research*, 48(2), 337-347. DOI: 10.1194/jlr.M600170-JLR200

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Cholesterol feeding strongly reduces hepatic VLDL-triglyceride production in mice lacking the liver X receptor α

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Abstract The oxysterol-activated nuclear receptor liver X receptor α (LXR α) has been implicated in the control of both cholesterol and fatty acid metabolism. In this study, we have evaluated the effects of excess dietary cholesterol on hepatic cholesterol metabolism, lipogenesis, and VLDL production in homozygous (*Lxra*^{-/-}), heterozygous (*Lxra*^{+/-}), and wild-type mice. Mice were fed either chow or a cholesterol-enriched diet (1%, w/w) for 2 weeks. On the high-cholesterol diet, fractional cholesterol absorption was higher in *Lxra*^{-/-} mice than in controls, leading to delivery of more dietary cholesterol to the liver. *Lxra*^{-/-} mice were not able to induce expression of hepatic *Abcg5/Abcg8*, and massive accumulation of free cholesterol and cholesteryl esters (CEs) occurred. Interestingly, despite the inability to upregulate *Abcg5/Abcg8*, the highly increased hepatic free cholesterol content did stimulate biliary cholesterol output in *Lxra*^{-/-} mice. Hepatic cholesterol accumulation was accompanied by decreased hepatic expression of lipogenic genes, probably caused by impaired sterol-regulatory element binding protein 1c processing, lower hepatic triglyceride (TG) contents, strongly reduced plasma TG concentrations (-90%), and reduced VLDL-TG production rates (-60%) in *Lxra*^{-/-} mice. VLDL particles were smaller and CE-enriched under these conditions. *Lxra* deficiency did not affect VLDL formation under chow-fed conditions. Hepatic steryl coenzyme A desaturase 1 expression was decreased dramatically in *Lxra*^{-/-} mice and did not respond to cholesterol feeding, but fatty acid profiles of liver and VLDL were only slightly different between *Lxra*^{-/-} and wild-type mice. Our data indicate that displacement of TGs by CEs during the VLDL assembly process underlies hypotriglyceridemia in cholesterol-fed *Lxra*^{-/-} mice.—van der Veen, J. N., R. Havinga, V. W. Bloks, A. K. Groen, and F. Kuipers. Cholesterol feeding strongly reduces hepatic VLDL-triglyceride production in mice lacking the liver X receptor α . *J. Lipid Res.* 2007. 48: 337–347.

Supplementary key words nuclear receptors • cholesterol absorption • ATP binding cassette transporter g5 • ATP binding cassette transporter

Manuscript received 12 April 2006 and in revised form 1 September 2006 and in re-revised form 1 November 2006.

Published, *JLR Papers in Press*, November 4, 2006.
DOI 10.1194/jlr.M600170-JLR200

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g8 • biliary cholesterol secretion • lipogenesis • fatty acids • very low density lipoprotein

Liver X receptors (LXRs) are nuclear receptors that modulate the expression of target genes upon their activation by certain oxysterol derivatives of cholesterol. Two LXR isoforms (α and β) have been identified in mammals: LXR α (NR1H3), which is expressed predominantly in the liver and intestine, and LXR β (NR1H2), with a more ubiquitous expression pattern. LXRs play important roles in the control of hepatic bile acid and fatty acid synthesis, hepatic glucose metabolism, cellular sterol efflux, and the inflammatory response (1–6). LXR α has particularly been implicated in the coordination of cholesterol and fatty acid metabolism in the liver, as it provides a molecular means to stimulate the synthesis of fatty acids (de novo lipogenesis) when required for storage of cholesterol in the form of cholesteryl esters (CEs) during dietary sterol overload (7).

Feeding a cholesterol-enriched diet to mice activates LXR α , which, in turn, enhances the hepatic turnover of cholesterol by stimulating bile salt synthesis via induction of cholesterol 7 α -hydroxylase (CYP7A1) and biliary cholesterol excretion through upregulation of ABCG5/ABCG8, a heterodimer facilitating cholesterol secretion from liver cells into bile. Peet and colleagues (7) have shown that mice lacking LXR α lose their ability to adequately react to dietary cholesterol, resulting in massive hepatic accumulation of free cholesterol and CEs, eventually leading to liver failure

Abbreviations: Acc-1, acetyl-coenzyme A carboxylase 1; CE, cholesteryl ester; Cyp7A1, cholesterol 7 α -hydroxylase; ER, endoplasmic reticulum; Gpat, glycerol-3-phosphate acyltransferase; Hmgr, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; LXR, liver X receptor; PL, phospholipid; SCAP, sterol-regulatory element binding protein cleavage-activating protein; Scd1, steryl coenzyme A desaturase 1; Srebp-1c, sterol-regulatory element binding protein 1c; TG, triglyceride.

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(7). This finding underscores the essential function of LXR α in the liver as a sensor of cholesterol.

Pharmacological activation of LXR by the synthetic agonist T0901317 in wild-type mice also results in hepatic steatosis; however, under these conditions, the fatty liver is caused by the accumulation of triglycerides (TGs) (1, 8) rather than CEs. LXR α regulates the expression of genes involved in de novo lipogenesis through both direct and indirect mechanisms (1, 2, 8). Thus, activated LXR α directly induces the expression of certain lipogenic genes (e.g., FAS) and indirectly induces it by controlling the levels of sterol-regulatory element binding protein 1c (SREBP-1c). The active form of SREBP-1c controls the transcription of the majority of genes involved in fatty acid synthesis, including acetyl coenzyme A carboxylase, FAS, steryl coenzyme A desaturase 1 (SCD1), glycerol-3-phosphate acyltransferase (GPAT), and others (9). Induction of lipogenic genes by pharmacological LXR activation stimulates fatty acid synthesis in vivo, resulting in hepatic steatosis as well as the stimulation of VLDL-TG secretion by inducing the production of large, TG-rich VLDL particles (1). There is evidence to indicate that different modes of hepatic LXR stimulation (i.e., pharmacological activation, adenoviral overexpression, and cholesterol feeding) have differential physiological effects. For instance, pharmacological activation of LXR by T0901317 induces SREBP-1c gene expression as well as maturation into its nuclear form (10), whereas adenoviral hepatic overexpression of LXR α induced gene expression but not the maturation of SREBP-1c protein in mice fed a Western diet (11).

These studies give rise to the question of whether LXR α has a physiological role in the control of hepatic VLDL production. Therefore, we determined the effects of cholesterol feeding on hepatic cholesterol metabolism, lipogenesis, and VLDL production in wild-type, heterozygous (*Lxr α ^{+/-}*), and homozygous (*Lxr α ^{-/-}*) mice. Our results demonstrate that LXR α deficiency does not affect VLDL formation in mice under chow-fed conditions. Our data confirm that mice deficient in LXR α are not capable of responding adequately to excess dietary cholesterol and accumulate free cholesterol and CE in their livers. The hepatic CE accumulation in *Lxr α ^{-/-}* mice was found to be accompanied by a decrease in lipogenic gene expression, a strongly reduced plasma TG concentration, and a markedly reduced VLDL-TG production attributable to the displacement of TG by CE in newly formed VLDL particles. Furthermore, in contrast to pharmacological activation of LXR α by T0901317, activation of LXR α by dietary sterols did not induce the full lipogenic gene repertoire or VLDL-TG production in wild-type mice, indicating differential effects of physiological and pharmacological LXR activation.

MATERIALS AND METHODS

Animals and diets

Lxr α ^{-/-} mice on a mixed background of Sv129/OlaHsd and C57Bl/6J (12) were generated by Deltagen, Inc. (Redwood City, CA) using standard gene-targeting methods. A 77 bp fragment

corresponding to a segment of exon 2 was replaced by a LacZ-Neo cassette. The construct was linearized and electroporated into embryonic stem cells derived from the Sv129/OlaHsd strain. Cells that harbored the desired mutation were identified by positive selection and injected into recipient C57Bl/6J blastocysts to produce chimeras, which were used for the generation of F1 heterozygotes. F2 wild-type, *Lxr α ^{+/-}*, and *Lxr α ^{-/-}* mice were produced from F1 intercrosses in the expected Mendelian ratios. Mice were genotyped via PCR using allele-specific primers (wild-type: sense, 5'-CAC CCA TTC TCC CGT GCT TCT CTT G-3'; knockout: sense, 5'-GGG CCA GCT CAT TCC TCC CAC TCA T-3'; antisense for both, 5'-GTT TCT CTC CCC TAT CTA GGG AGA C-3'). The absence of LXR α mRNA in the knockout mice was also confirmed by quantitative real-time PCR (see supplemental Fig. 1). *Lxr α ^{-/-}* and *Lxr α ^{+/-}* mice and their wild-type littermates received either standard mouse chow or chow diet containing 1% (w/w) cholesterol (Abdiets, Woerden, The Netherlands) for 2 weeks. Male mice of 3 months of age were used. All experimental procedures were approved by the local Ethical Committee for Animal Experiments.

Experimental procedures

After 2 weeks of either chow diet or cholesterol-enriched diet, nonfasted *Lxr α ^{-/-}*, *Lxr α ^{+/-}*, and wild-type mice ($n = 6$ per group) were anesthetized by intraperitoneal injection with Hypnorm (fentanyl/fluanisone; 1 ml/kg) and diazepam (10 mg/kg). Bile was collected by cannulation of the gallbladder for 30 min. Body temperature was stabilized using a humidified incubator. Subsequently, animals were euthanized by cardiac puncture, livers and small intestines were excised, and livers were weighed. Parts of both liver and intestine were snap-frozen in liquid nitrogen and stored at -80°C for RNA isolation and biochemical analysis. Samples for microscopic evaluation were frozen in isopentane and stored at -80°C or fixed in paraformaldehyde. Liver histology was examined on frozen liver sections after Oil Red O staining for neutral lipids by standard procedures. Feces were collected from individual mice.

Fractional cholesterol absorption measurement

Fractional cholesterol absorption was measured in a separate experiment using the fecal dual-isotope method ($n = 6$ per group). Animals were housed individually 1 week before the experiment. Wild-type and *Lxr α ^{-/-}* mice were fed normal chow or chow diet containing 1% cholesterol for 2 weeks. After 11 days, mice received by gavage 150 μl of medium-chain TG oil containing 1 μCi of [^{14}C]cholesterol (Amersham Bioscience, Buckinghamshire, UK) and 2 μCi of [^3H]sitostanol (American Radiolabeled Chemicals, St. Louis, MO). Feces were collected for the next 4 days. After 4 days, animals were euthanized, the small intestine and liver were removed, and radioactivity was measured upon dissolving the tissues in Soluene. From the collected feces, neutral sterols were extracted. The ^{14}C and ^3H contents of the feces and dosing mixture were counted, and the ratio was used to calculate the percentage cholesterol absorption:

$$\% \text{ cholesterol absorption} = \left(\frac{{}^{14}\text{C}/{}^3\text{H} \text{ dosing mixture}}{{}^{14}\text{C}/{}^3\text{H} \text{ dosing mixture}} - \frac{{}^{14}\text{C}/{}^3\text{H} \text{ feces}}{{}^{14}\text{C}/{}^3\text{H} \text{ dosing mixture}} \right) \times 100$$

In vivo VLDL-TG production rate

Lxr α ^{-/-} and wild-type mice received standard mouse chow, chow diet containing 1% cholesterol for 2 weeks, or chow sup-

plemented with the synthetic LXR agonist T0901317 (0.015%, w/w; Cayman Chemicals, Ann Arbor, MI) ($n = 6$ per group). After a fasting period of 10 h, mice received a retro-orbital injection of 12.5 mg of Triton WR-1339 in 100 μ l of phosphate-buffered saline. Blood samples were taken by retro-orbital puncture before and 1, 2, 3, and 4 h after the injection of Triton WR-1339. The collected blood samples were used for TG and cholesterol measurements. VLDL-TG production rate was calculated from the slope of the TG concentration versus time curve. At the end of the experiment, a blood sample was collected by cardiac puncture, which was used for isolation of the VLDL fraction.

VLDL isolation and analysis

Plasma VLDL ($d < 1.006$ g/ml) was isolated by density gradient ultracentrifugation. Plasma (300 μ l) was adjusted to 1 ml with a NaCl/KBr solution ($d = 1.006$ g/ml) containing 1 mM EDTA and centrifuged at 118,000 rpm for 120 min at 4°C in an Optima TM LX tabletop ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA). VLDL volume was recorded by weight. TG and cholesterol contents were determined as described for plasma. Phospholipids (PLs) were determined using a commercially available kit (Wako Chemicals), and fatty acid composition was determined as described previously (13).

VLDL size and volume distribution profiles were analyzed by dynamic light 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. Particle volume was calculated using the following equation: volume = $4/3 \times \pi \times (0.5 \times \text{diameter})^3$. Apolipoprotein B-100 and apolipoprotein B-48 were determined by Western blotting, as described previously (1).

Immunoblot analysis of SREBP-1

To prepare nuclear extracts for immunoblot analyses, ~100 mg of frozen liver was homogenized in 1.5 ml of homogenization buffer (20 mM Tris-HCl at pH 7.4, 2 mM MgCl₂, 0.25 M sucrose, 10 mM sodium EDTA, and 10 mM sodium EGTA) supplemented with protease inhibitor cocktail. After centrifugation at 1,500 g for 5 min at 4°C in a microcentrifuge, the pellet was resuspended in 1 ml of homogenization buffer and centrifuged at 1,000 g for 5 min at 4°C. The pellet was resuspended in 300 μ l of buffer [20 mM HEPES/KOH at pH 7.6, 2.5% (v/v) glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 1 mM sodium EDTA, and 1 mM sodium EGTA] supplemented with protease inhibitor cocktail. Samples were rotated for 1 h at 4°C followed by centrifugation at 10⁵ g for 30 min at 4°C in a Beckman TLA 100.2 rotor. After removal of an aliquot of the nuclear fraction for measurement of protein concentration (BCA kit; Pierce Biotechnology, Inc., Rockford, IL), 225 μ l of the remaining fraction was mixed with 75 μ l of 4 \times SDS loading buffer. Equal amounts of protein from six mice per group were pooled, and aliquots (20 μ g) of the pooled nuclear fraction were subjected to SDS-PAGE on 10% gels. Western blot analyses were performed by overnight incubation with SREBP-1 antibody (K-10; Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:1,000 dilution.

Analytical procedures

Livers were homogenized, and hepatic and biliary lipids were extracted according to Bligh and Dyer (14). Hepatic, biliary, and plasma concentrations of total cholesterol, TGs, and PLs were determined as described previously (5). Free cholesterol concentrations were determined using a commercially available kit (Wako, Neuss, Germany). Where applicable, CE concentrations were calculated as the difference between total and free cholesterol. Pooled plasma samples were used for lipoprotein separation by fast-protein

liquid chromatography. Fecal neutral sterols were analyzed by gas chromatography. Bile salts in feces and in bile were measured enzymatically. Biliary bile salt composition was determined by capillary gas chromatography as described (15) after extraction of the bile salts from bile by use of Sep-Pak C₁₈ cartridges.

Hepatic lipid extracts were fractionated into PLs, CEs, TGs, and free fatty acids using TLC (20 \times 20 cm, Silica gel 60 F254; Merck), and relative fatty acid composition in these fractions was determined by gas chromatography after methylation (16).

RNA isolation and measurement of mRNA levels by quantitative real-time PCR

RNA isolation, cDNA synthesis, and real-time quantitative PCR were performed as described by Plösch et al. (5). PCR results from liver and intestine were normalized to β -actin mRNA levels. Primer and probe sequences for *Abcg5*, *Abcg8*, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*Hmgr*), *Cyp7a1* (5), *Srebp-1a*, *Srebp-1c*, acetyl-coenzyme A carboxylase 1 (*Acc-1*), *Fas*, β -actin (1), and *Npc1l1* (17) have been published. Furthermore, the following primers/probes were used. For *Scd1*, sense, 5'-ATG CTC CAA GAG ATC TCC AGT TCT-3'; antisense, 5'-CTT CAT CTT CTC TCG TTC ATT TCC-3'; and probe, 5'-CCA CCA CCA CCA TCA CTG CAC CTC-3' (accession number NM_009127). For *Gpat*, sense, 5'-GCT ATC ATG TCC ACC CAC ATT G-3'; antisense, 5'-ACT TCC TCC TTC ATC ACA AAG AAG TC-3'; and probe, 5'-CTC CTC TAC AGA CAC AGG CAG GGA ATC C-3' (accession number NM_008149). For phosphatidylethanolamine N-methyltransferase (*Pemt*), sense, 5'-GGG ACC TTT CTA GGT GAC TAC TTT G-3'; antisense, 5'-CCA GCC TAG GTA GTT GGC TGT AC-3'; and probe, 5'-TGA CCA CAT TTC CCT TCA GCG TGC T-3' (accession number NM_008819). For phosphatase cytidyltransferase 1, choline, alpha isoform (*Ctptc1*), sense, 5'-GGA GTT GAG TTA AAA GAA GAT GGA TG-3'; antisense, 5'-GCT GCA CTT TGG AAG GAA TTC-3'; and probe, 5'-CCT CTG TTG CTC CAT TAG GGC CAG G-3' (accession number NM_009981).

Statistics

All values represent means \pm SD for the number of animals indicated. Statistical analysis was assessed by one-way ANOVA followed by post hoc Bonferroni correction. The level of significance was set at $P < 0.05$. Analyses were performed using SPSS version 12 for Windows (SPSS, Chicago, IL).

RESULTS

Hepatic CE accumulation in *Lxra*^{-/-} mice fed a cholesterol-enriched diet

Table 1 shows that body weights of wild-type, *Lxra*^{+/-}, and *Lxra*^{-/-} mice were similar and not influenced by cholesterol feeding. Relative liver weights were increased in cholesterol-fed *Lxra*^{-/-} mice only. Plasma cholesterol levels were increased slightly upon feeding the 1% cholesterol diet in both wild-type and *Lxra*^{-/-} mice (Table 1). In wild-type mice, this increase of plasma cholesterol was attributable mainly to an increased cholesterol content in HDL-sized fractions, whereas in *Lxra*^{-/-} mice, additional cholesterol was found predominantly in the intermediate density lipoprotein-sized fractions (data not shown). Upon cholesterol feeding, plasma TG levels were decreased by 44% and 70% in wild-type and *Lxra*^{+/-} mice, respectively, and to almost undetectable levels in *Lxra*^{-/-} mice. Fast-protein liquid chromatography analysis revealed extremely

TABLE 1. Animal characteristics and plasma lipid concentrations in chow- and cholesterol-fed wild-type and *Lxrα*^{-/-} mice

Characteristics	Chow Diet			One Percent Cholesterol Diet		
	Wild Type	<i>Lxrα</i> ^{+/-}	<i>Lxrα</i> ^{-/-}	Wild Type	<i>Lxrα</i> ^{+/-}	<i>Lxrα</i> ^{-/-}
Body weight (g)	27.7 ± 4.0	29.3 ± 2.9	29.0 ± 4.0	30.7 ± 3.1	30.0 ± 2.3	26.1 ± 5.9
Liver weight (% of body weight)	4.7 ± 0.4	4.8 ± 0.3	4.8 ± 0.2	4.3 ± 0.3	4.8 ± 0.3	6.0 ± 0.8 ^{a,b}
Plasma cholesterol (mM)	2.5 ± 0.3	2.6 ± 0.4	2.4 ± 0.4	3.5 ± 0.7 ^a	2.9 ± 0.4	3.4 ± 0.4 ^a
Plasma TGs (mM)	1.2 ± 0.7	1.2 ± 0.5	1.0 ± 0.3	0.7 ± 0.3	0.4 ± 0.2 ^a	0.03 ± 0.1 ^a
Plasma free fatty acids (μM)	548 ± 150	517 ± 216	490 ± 151	490 ± 138	425 ± 69	383 ± 139

Lxrα^{+/-}, mice heterozygous for liver X receptor α expression; *Lxrα*^{-/-}, mice homozygous for liver X receptor α expression; TG, triglyceride. Values are expressed as means ± SD (n = 6 in all groups).

^aSignificant difference between chow-fed and cholesterol-fed mice with the same genotype.

^bSignificant difference between genotypes fed the same diet.

low concentrations of VLDL-associated TGs in *Lxrα*^{-/-} mice (Fig. 1).

Under chow-fed conditions, hepatic TG levels were similar in all strains (Fig. 2A). Feeding a cholesterol-enriched diet led to increased hepatic TG levels in wild-type mice only. Hepatic PL contents were similar in all groups on either diet (Fig. 2B). Hepatic cholesterol levels were similar in wild-type, *Lxrα*^{+/-}, and *Lxrα*^{-/-} mice under chow-fed conditions. Upon feeding a cholesterol-enriched diet, *Lxrα*^{-/-} mice accumulated massive amounts of cholesterol, of which ~65% was esterified, resulting in severe hepatic steatosis (Fig. 2C, D). As expected, the high intake of dietary cholesterol led to a compensatory decrease in hepatic expression of *Hmgr*, the gene encoding the rate-controlling enzyme in cholesterol synthesis (Fig. 3A).

Fractional cholesterol absorption is higher in cholesterol-fed *Lxrα*^{-/-} mice than in wild-type controls

To assess the contribution of dietary cholesterol to hepatic CE accumulation, fractional cholesterol absorption was measured in wild-type and *Lxrα*^{-/-} mice fed either chow or the cholesterol-enriched diet. Intestinal expression of *Npc1l1*, encoding the recently identified cholesterol absorption protein (18), was similar in both strains and induced by the cholesterol diet in wild-type mice only (Fig. 4A). Expression of *Abcg5* and *Abcg8* was induced in wild-type mice upon cholesterol feeding, which did not occur in mice lacking LXRα (Fig. 4B, C), confirming previous studies by Repa et al. (19). Under chow-fed conditions, no difference in fractional cholesterol absorption between the genotypes was observed (Fig. 4D). When fed

a cholesterol-enriched diet, fractional cholesterol absorption was decreased in both genotypes compared with the chow-fed mice; however, this decrease was less pronounced in *Lxrα*^{-/-} mice, correlating with the prevailing expression levels of *Abcg5* and *Abcg8*. Under cholesterol-fed conditions, *Lxrα*^{-/-} mice showed a 70% higher fractional cholesterol absorption than their wild-type littermates.

Stimulation of bile salt synthesis and biliary cholesterol secretion is impaired in *Lxrα*^{-/-} mice

One of the major pathways for the liver to shed excess cholesterol is by its secretion into bile, either as free cholesterol or after conversion into bile salts. We measured hepatobiliary excretion of cholesterol, bile salts, and PLs and assessed the hepatic mRNA expression levels of several proteins involved in these processes.

In wild-type mice, physiological activation of LXRα by dietary cholesterol resulted in an increased expression of *Cyp7a1*, the gene encoding the key enzyme in the process of converting cholesterol into bile salts, whereas expression of this gene was decreased slightly in *Lxrα*^{-/-} mice upon cholesterol feeding (Fig. 3B). Nevertheless, hepatobiliary bile salt excretion rates were not different between the groups (Table 2), indicative of circulating bile salt pools of similar size in all groups. Because under steady-state conditions *Cyp7a1* expression reflects the hepatic bile acid synthesis rate, these data indicate that bile acid synthesis is not induced in cholesterol-fed *Lxrα*^{-/-} mice, which may contribute to hepatic cholesterol accumulation. As expected, dietary cholesterol upregulated the expression of both *Abcg5* and *Abcg8* in wild-type and

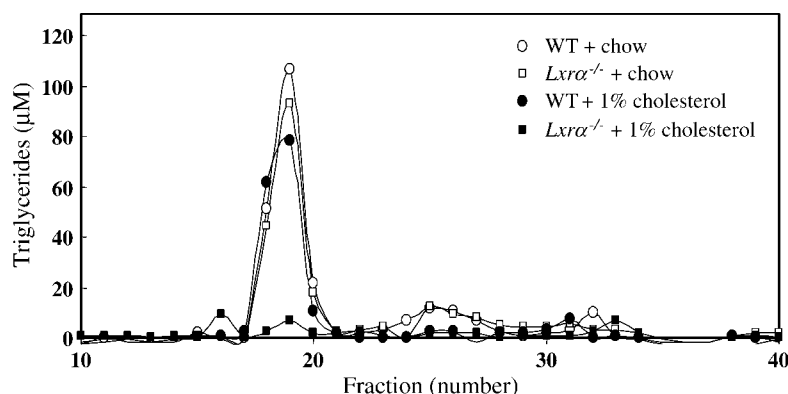


Fig. 1. Distribution of triglycerides (TGs) in plasma lipoprotein fractions of chow-fed and cholesterol-fed wild-type (WT) and liver X receptor α-homozygous (*Lxrα*^{-/-}) mice, separated using fast-protein liquid chromatography.

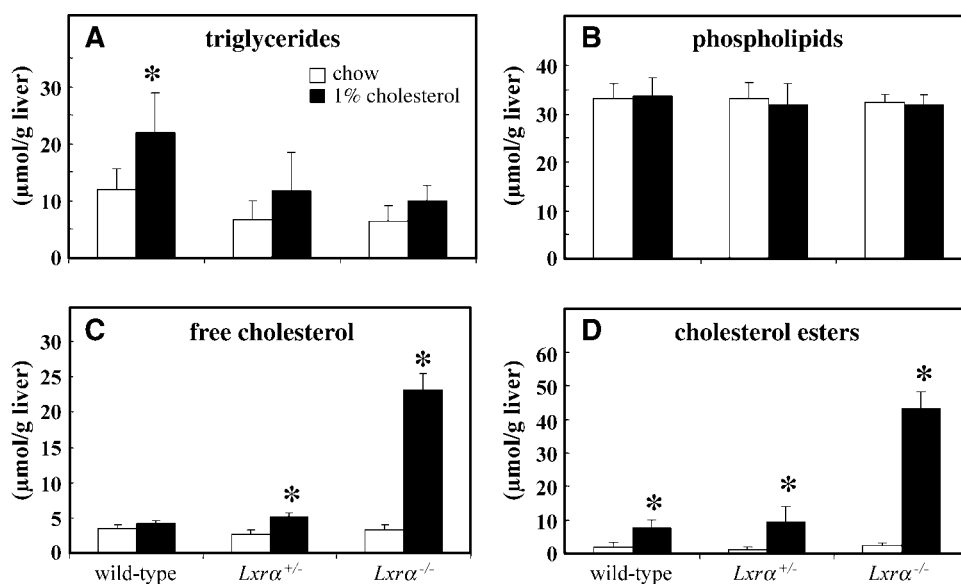


Fig. 2. Hepatic lipid levels in wild-type, heterozygous ($Lxra^{+/-}$), and $Lxra^{-/-}$ mice fed either chow or a diet containing 1% cholesterol. Panels A–D represent hepatic triglyceride, phospholipid, free cholesterol, and cholesterol ester levels, respectively. Data are presented as means of six animals \pm SD. * Significant differences between chow-fed and cholesterol-fed mice with the same genotype, determined using one-way ANOVA ($P < 0.05$).

$Lxra^{+/-}$ mice. As reported previously (19), mice deficient in LXR α were unable to upregulate the expression of these transporters that are crucial for biliary cholesterol secretion (Fig. 3C, D), which will have contributed to the massive hepatic accumulation of both CE and free cholesterol, the latter being a substrate for *Abcg5/8*. The highly increased hepatic free cholesterol levels in the $Lxra^{-/-}$ mice, however, were associated with the stimula-

tion of biliary cholesterol secretion to wild-type levels, suggestive of substrate-induced stimulation of transporter activity. Biliary PL secretion rates were slightly higher in $Lxra^{-/-}$ mice on both diets, although this difference was not statistically significant, and they were not affected by cholesterol feeding (Table 2). Analysis of biliary bile acid composition revealed a 30% higher cholic acid-muricholic acid ratio in $Lxra^{-/-}$ mice compared with wild-type mice.

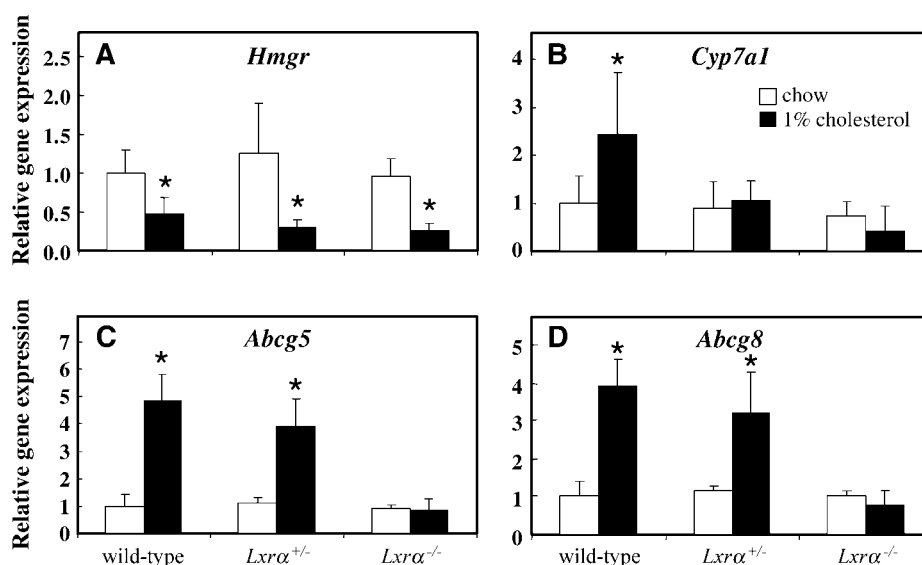


Fig. 3. Hepatic gene expression in wild-type, $Lxra^{+/-}$, and $Lxra^{-/-}$ mice fed either chow or a diet containing 1% cholesterol, measured by real-time PCR. Panels A–D show expression levels of *Hmgr*, *Cyp7a1*, *Abcg5*, and *Abcg8*, respectively. mRNA was prepared from individual mice ($n = 6$ per group), and data are presented as means of six animals tested in duplicate \pm SD. Expression values are normalized to β -actin, and expression in chow-fed wild-type mice was set at 1.00. * Significant differences between chow-fed and cholesterol-fed mice with the same genotype, determined using one-way ANOVA ($P < 0.05$). *Cyp7a1*, cholesterol 7 α -hydroxylase; *Hmgr*, 3-hydroxy-3-methylglutaryl-coenzyme A reductase.

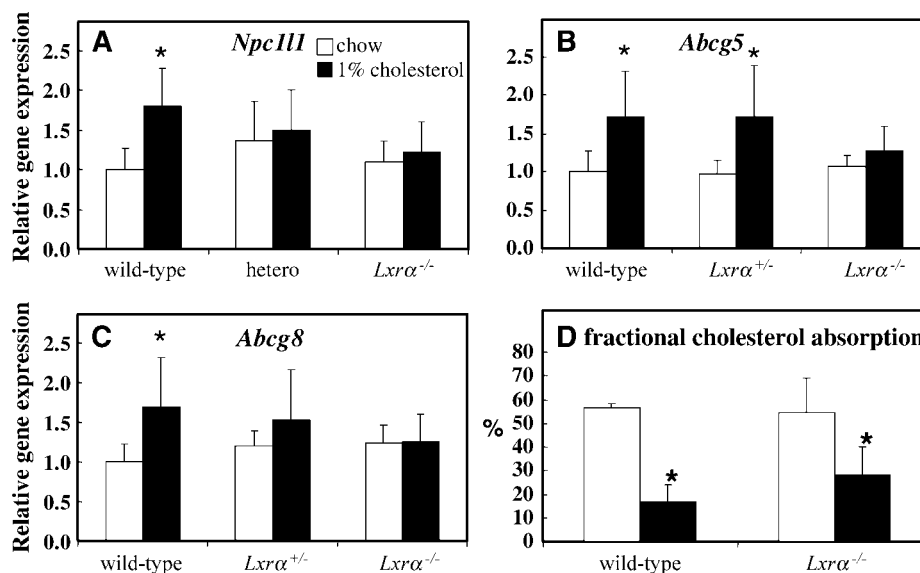


Fig. 4. A–C: Intestinal gene expression in wild-type, *Lxra*^{+/-}, and *Lxra*^{-/-} mice fed either chow or a diet containing 1% cholesterol, measured by real-time PCR. mRNA was prepared from individual mice (n = 6 per group), and data are presented as means of six animals tested in duplicate ± SD. Expression values are normalized to β -actin, and expression in chow-fed wild-type mice was set at 1.00. D: Fractional cholesterol absorption in chow-fed and cholesterol-fed wild-type and *Lxra*^{-/-} mice, measured by the fecal dual-isotope method described in Materials and Methods. Values represent means ± SD. * Significant differences between chow-fed and cholesterol-fed mice with the same genotype, determined using one-way ANOVA ($P < 0.05$). *Npc1l1*, Niemann-Pick C1 like-1.

Feeding cholesterol to wild-type mice resulted in a decrease of this ratio (from 1.00 ± 0.17 to 0.64 ± 0.17), whereas *Lxra*^{-/-} mice failed to respond to the cholesterol diet in this respect (data not shown), confirming the data from Peet et al. (7). These differences in cholic acid-muricholic acid ratios cannot be explained entirely by differences in hepatic Cyp8b1 expression levels between the groups (data not shown).

Strongly reduced VLDL-TG production in *Lxra*^{-/-} mice fed a cholesterol-enriched diet

To evaluate the causes of cholesterol-induced hypotriglyceridemia, we measured the hepatic expression of genes involved in lipogenesis (Fig. 5). Hepatic expression of *Srebp-1c*, the master switch controlling the expression of lipogenic genes (20), was more than doubled upon cholesterol feeding in wild-type mice. The increase was less pronounced in *Lxra*^{+/-} mice and did not occur in *Lxra*^{-/-} mice. Protein levels of the mature, nuclear form of SREBP-1c were not induced by the cholesterol diet in wild-type

mice and were clearly decreased in cholesterol-fed *Lxra*^{-/-} mice. Accordingly, expression of *Acc-1*, *Fas*, and *Gpat* was reduced in *Lxra*^{-/-} mice after feeding the cholesterol-enriched diet, as was that of *Pemt* and *Ctptc*, genes involved in PL synthesis (data not shown). In wild-type mice, the SREBP-1c target genes *Fas* and *Acc-1* were not induced, in agreement with unchanged nuclear SREBP-1 protein, whereas expression of *Scd1* was markedly stimulated. Expression of *Scd1*, the gene encoding the rate-controlling enzyme in the biosynthesis of monounsaturated fatty acids (21), was strongly reduced in *Lxra*^{-/-} mice on both chow and cholesterol diets compared with wild-type controls.

VLDL-TG production was not affected in *Lxra*^{-/-} mice under chow-fed conditions (Fig. 6A, B). However, *Lxra*^{-/-} mice fed a diet containing 1% cholesterol showed a 61% reduction in VLDL-TG production. The diameter of the VLDL particles in these mice was reduced by 30% compared with that in the cholesterol-fed wild-type controls (65.3 ± 7.9 nm vs. 94.2 ± 13.8 nm, respectively) (Table 3). Analysis of VLDL composition showed that

TABLE 2. Bile flow and biliary secretion rates in chow- and cholesterol-fed wild-type and *Lxra*^{-/-} mice

Variable	Chow Diet			One Percent Cholesterol Diet		
	Wild Type	<i>Lxra</i> ^{+/-}	<i>Lxra</i> ^{-/-}	Wild Type	<i>Lxra</i> ^{+/-}	<i>Lxra</i> ^{-/-}
Bile flow (μ l/min/100 g body weight)	4.98 ± 0.78	4.84 ± 0.62	4.80 ± 0.84	3.49 ± 1.04	3.67 ± 0.79	5.12 ± 1.11^a
Bile salts (nmol/min/100 g body weight)	226 ± 102	247 ± 75	221 ± 88	151 ± 67	186 ± 47	202 ± 141
Cholesterol (nmol/min/100 g body weight)	3.1 ± 1.8	3.1 ± 0.6	3.2 ± 1.3	6.8 ± 4.7	8.5 ± 2.9^b	6.3 ± 2.3^b
PLs (nmol/min/100 g body weight)	27.7 ± 10.4	33.3 ± 6.4	41.2 ± 11.0	22.8 ± 7.9	26.6 ± 6.7	42.3 ± 18.1

PL, phospholipid. Values are expressed as means ± SD (n = 6 in all groups).

^aSignificant difference between genotypes fed the same diet.

^bSignificant difference between chow-fed and cholesterol-fed mice with the same genotype.

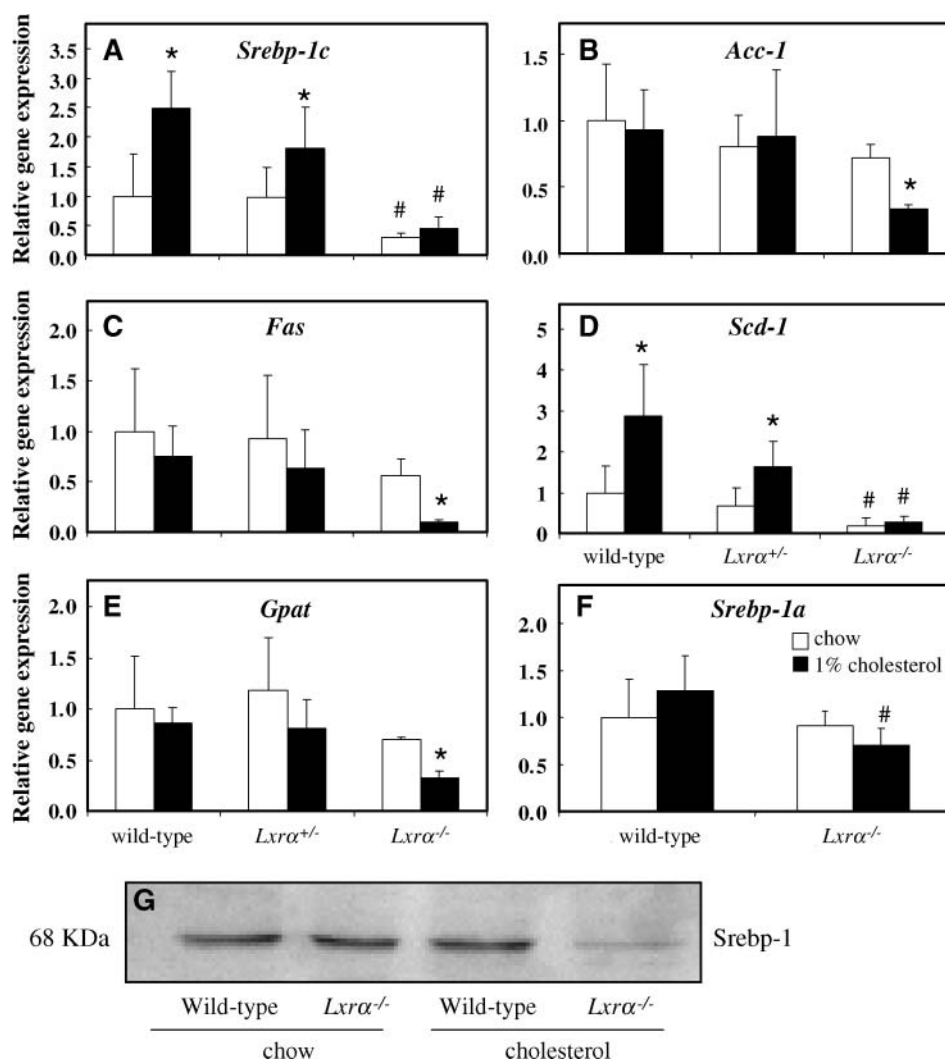


Fig. 5. A–F: Hepatic gene expression in wild-type, *Lxrα*^{+/-}, and *Lxrα*^{-/-} mice fed either chow or a diet containing 1% cholesterol, measured by real-time PCR. mRNA was prepared from individual mice (n = 6 per group), and data are presented as means of six animals tested in duplicate ± SD. Expression values are normalized to *β-actin*, and expression in chow-fed wild-type mice was set at 1.00. * Significant differences between chow-fed and cholesterol-fed mice with the same genotype; # significant differences versus wild-type mice fed the same diet, determined using one-way ANOVA ($P < 0.05$). *Acc-1*, acetyl-coenzyme A carboxylase; *Gpat*, glycerol-3-phosphate acyltransferase; *Scd1*, stearyl coenzyme A desaturase 1; *Srebp-1c*, sterol-regulatory element binding protein 1c. G: Western blot analysis of nuclear SREBP-1 protein in wild-type and *Lxrα*^{-/-} mice fed either chow or a diet containing 1% cholesterol. Each lane represents a pool of nuclear extracts from six animals per group.

VLDL particles of cholesterol-fed *Lxrα*^{-/-} mice contained relatively less TG (52% vs. 77%) and more cholesterol (43% vs. 11%) (Table 3). The apolipoprotein B content of the VLDL fraction did not differ between the groups, indicating a similar number of VLDL particles being produced by the liver (Fig. 6C). This conclusion is supported by the fact that the reduction of VLDL-TG secretion calculated on the basis of particle size and relative TG content (–69%) is of the same order of magnitude as the actually measured reduction (–61%). Cholesterol feeding did not affect hepatic VLDL-TG production in wild-type mice, whereas activation of LXR with the pharmacological agonist T0901317 induced VLDL-TG production by 2.2-fold (Fig. 6B), as described previously (1). The fact that

cholesterol feeding did not induce VLDL-TG production in wild-type mice indicates that diet-induced LXR activation does not have the same metabolic consequences as pharmacological activation. In *Lxrα*^{-/-} mice, treatment with T0901317 slightly induced VLDL-TG production, indicating that the reduced TG production upon cholesterol feeding is probably not the result of activation of the LXRβ isoform.

Fatty acid profiles

Fatty acid profiles in both liver and VLDL were measured to assess whether the absence of LXRα, and hence the low expression levels of *Scd1*, differentially affected fatty acid composition. Relative amounts of the most promi-

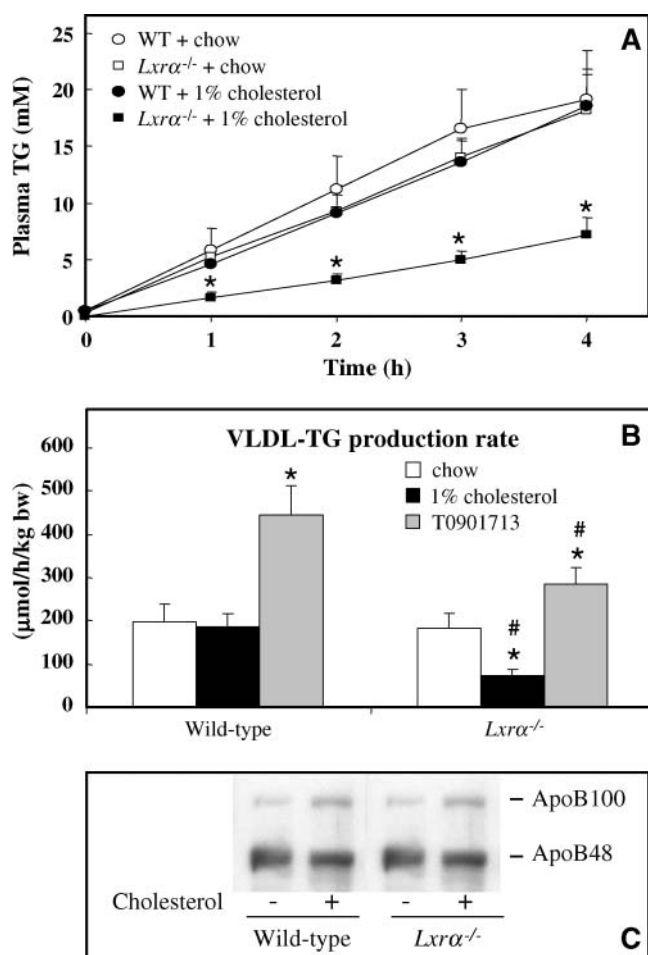


Fig. 6. A: Plasma TG concentrations in chow-fed and cholesterol-fed wild-type (WT) and *Lxrα*^{-/-} mice after injection of Triton WR-1339. B: VLDL-TG production rate in μmol/kg/h, calculated from the plasma TG versus time curve, including wild-type and *Lxrα*^{-/-} mice treated with T0901317. Values represent means of six animals ± SD. * Significant differences between chow-fed and cholesterol-fed mice or between chow-fed and T0901317-treated mice with the same genotype; # significant differences versus wild-type mice fed the same diet, determined using one-way ANOVA ($P < 0.05$). C: Western blot of apolipoprotein B (ApoB) contents in nascent VLDL particles of chow- and cholesterol-fed wild-type and *Lxrα*^{-/-} mice ($n = 3$ per group). Each lane represents VLDL from an individual representative mouse.

nant fatty acid species in the liver are shown in **Fig. 7A**. Please note that fatty acid profiles of chow- and cholesterol-fed mice are not directly comparable because of slightly different fatty acid compositions in the diets. This figure shows small but statistically significant differences between wild-type and *Lxrα*^{-/-} mice: under chow-fed conditions, *Lxrα*^{-/-} mice had less 18:1ω9 fatty acids and more saturated 18:0 fatty acids in their livers compared with wild-type controls, which is probably related to the reduced expression of *Scd1*. When fed the cholesterol-enriched diet, *Lxrα*^{-/-} mice showed a reduction in 16:0 only compared with wild-type controls. Figure 7B shows the fatty acid profiles of total VLDL fractions. In VLDL, small differences were observed between genotypes. Under both dietary conditions, *Lxrα*^{-/-} mice showed reduced

amounts of 18:1ω9 and 16:0, which is partly compensated for by 18:0 and 18:2ω6 in the cholesterol-fed mice. Thus, the changes observed in VLDL largely correlate with those observed in total hepatic fatty acid content. However, the physiological relevance thereof is not known.

Figure 7C shows that, in absolute terms, excess hepatic CEs in livers of cholesterol-fed *Lxrα*^{-/-} mice preferentially contained 18:1ω9 and 18:2ω6 (i.e., species much less abundant in hepatic TGs) (Fig. 7D). As expected, the fatty acid composition of hepatic PLs reflected that of whole liver (Fig. 7E).

DISCUSSION

LXRα is an essential regulatory factor in the maintenance of hepatic cholesterol homeostasis whose function cannot be fully compensated for by other “control systems” during dietary cholesterol overload in mice (7). Besides its role in cellular cholesterol homeostasis, LXRα also controls several steps of fatty acid metabolism. To evaluate the physiological relevance of this coordinated regulation of cholesterol and fatty acid metabolism, we fed *Lxrα*^{-/-} mice and their wild-type littermates a diet enriched with 1% cholesterol to provide a physiological means for LXR activation and measured the relevant fluxes of cholesterol and their impact on hepatic VLDL production.

LXRα-deficient mice fed the high-cholesterol diet accumulated massive amounts of CEs, as described previously by Peet et al. (7). The ABC transporter heterodimer ABCG5/ABCG8 has been identified as an exporter of sterols at the apical membranes of small intestinal epithelial cells and of hepatocytes (22, 23). LXR is known to regulate the expression of these genes and therefore is involved in the control of both intestinal cholesterol absorption and hepatobiliary cholesterol excretion. In contrast to wild-type mice, mice lacking LXRα were unable to induce the intestinal expression of *Abcg5* and *Abcg8* upon cholesterol challenge, which resulted in a 70% higher fractional cholesterol absorption in *Lxrα*^{-/-} mice during cholesterol feeding. As absorbed cholesterol is delivered directly to the liver, this caused a significantly increased flux of cholesterol to the livers of *Lxrα*^{-/-} mice and, consequently, a reduction of hepatic cholesterol synthesis, as evident from the reduced expression of *Hmgcr*, the gene encoding the rate-controlling enzyme in this process. *Lxrα*^{-/-} mice were incapable of inducing hepatic *Abcg5/8* expression in response to increased cholesterol influx. As a consequence, large amounts of free cholesterol and CEs accumulated in the livers of these mice. Under most conditions, expression of *Abcg5/8* appears to be not rate-limiting for biliary cholesterol secretion in mice (24). Apparently, the capacity of the *Abcg5/8* transporter in *Lxrα*^{-/-} mice is just sufficient to accommodate biliary cholesterol secretion at wild-type levels, albeit at much higher intracellular cholesterol concentrations, probably reflecting “substrate compensation” (i.e., higher activity of the transporter complex in the presence

TABLE 3. Composition and size of nascent VLDL particles of wild-type and *Lxrx*^{-/-} mice fed either chow or the 1% cholesterol diet

Variable	Chow Diet		One Percent Cholesterol Diet	
	Wild Type	<i>Lxrx</i> ^{-/-}	Wild Type	<i>Lxrx</i> ^{-/-}
Cholesterol (%)	7.9 ± 1.1	10.5 ± 1.8 ^a	11.1 ± 2.7 ^a	34.3 ± 5.4 ^{a,b}
PLs (%)	11.6 ± 3.0	12.8 ± 2.7	12.0 ± 2.8	14.0 ± 1.9
TGs (%)	80.5 ± 3.0	78.8 ± 5.2	76.9 ± 5.0	51.7 ± 6.6 ^{a,b}
TG/PL ratio	7.4 ± 2.1	6.3 ± 1.7	6.8 ± 2.3	3.8 ± 0.9 ^{a,b}
Diameter (nm)	85.6 ± 12.6	95.3 ± 9.1	85.7 ± 10.4	66.4 ± 7.5 ^{a,b}
Volume (10 ⁵ nm ³)	3.5 ± 1.5	4.6 ± 1.3	3.4 ± 1.2	1.6 ± 0.5 ^{a,b}

Values are expressed as means ± SD (n = 6 in all groups).

^aSignificant difference between genotypes fed the same diet.

^bSignificant difference between chow-fed and cholesterol-fed mice with the same genotype.

of excess substrate). In addition, *Abcg5/8* independent pathway(s) for biliary cholesterol secretion may have been stimulated. As described previously by Peet et al. (7), the capacity to eliminate excess cholesterol through accelerated conversion into bile salts and subsequent excretion via the feces was also impaired in cholesterol-fed *Lxrx*^{-/-} mice compared with wild-type mice, as indicated by an inability to induce hepatic *Cyp7a1* expression. Thus, the inability to induce biliary cholesterol secretion sufficiently, to stimulate bile salt synthesis, and to suppress intestinal cholesterol absorption ultimately leads to the hepatic accumulation of cholesterol in *Lxrx*^{-/-} mice, of which a large part is esterified to buffer its toxic effects, leading to “CE steatosis.”

We observed that the hepatic free cholesterol and CE accumulation in cholesterol-fed *Lxrx*^{-/-} mice was accompanied by extremely low plasma levels of TGs. Phar-

macological activation of LXR by T0901317 leads to hypertriglyceridemia, particularly in mouse models with defective clearance of TG-rich lipoproteins. LXR activation was associated with the production of large, TG-rich VLDL particles (1). Unlike the effects of T0901317, dietary cholesterol-induced activation of LXR did not result in increased VLDL-TG production in wild-type mice. Additionally, the absence of LXRα did not influence VLDL-TG production under chow-fed conditions, whereas under cholesterol-fed conditions, the VLDL-TG production rate was strongly reduced only in mice lacking LXRα. A frequently reported cause of increased VLDL-TG production is enhanced hepatic de novo lipogenesis (25), although recent studies by our group (26, 27) do not support this general idea. The key regulator of lipogenesis is SREBP-1c, the transcription of which is under the control of LXR (8, 28). As in T0901317-treated mice, *Srebp-1c* gene expression

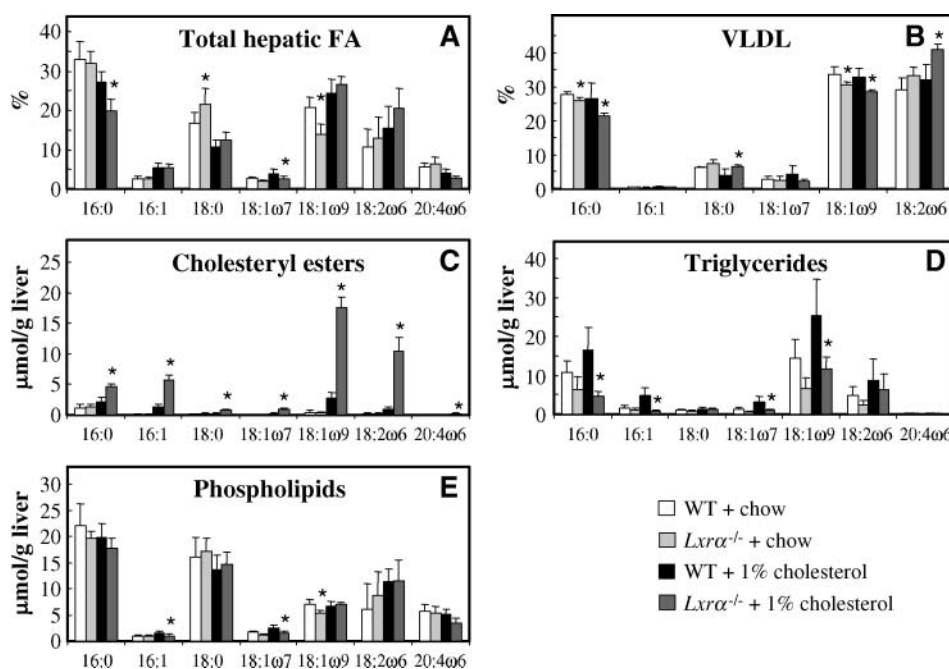


Fig. 7. A, B: Relative concentrations of the most prominent fatty acid species in livers (A) and VLDL particles (B) of chow-fed and cholesterol-fed wild-type (WT) and *Lxrx*^{-/-} mice. C–E: Absolute concentrations of the various fatty acids in hepatic cholesteryl esters, TGs, and phospholipid fractions, respectively. Values represent means of six animals ± SD. * Significant differences between chow-fed and cholesterol-fed mice with the same genotype, determined using one-way ANOVA ($P < 0.05$).

was induced in cholesterol-fed wild-type mice. However, the lipogenic target genes downstream of SREBP-1c were not induced upon cholesterol feeding. SREBP-1c requires translocation from the endoplasmic reticulum (ER) to the Golgi, a step controlled by sterol-regulatory element binding protein cleavage-activating protein (SCAP), and subsequent cleavage to its mature, nuclear form to activate gene expression. These processes have been shown to be enhanced by pharmacological LXR agonists (10). Our study shows that cholesterol feeding led to increased mRNA levels of SREBP-1c in wild-type mice but did not induce its maturation to the active, nuclear form, resulting in unaffected expression of the SREBP-1c target genes *Fas*, *Acc1*, and *Gpat*. Comparable results have been shown by Lehrke et al. (11), who showed that hepatic overexpression of LXR α induced gene expression but not the maturation of SREBP-1c. Thus, actual lipogenesis was not induced in cholesterol-fed wild-type mice.

In mice deficient for LXR α , expression of SREBP-1c was lower compared with that in their wild-type littermates under chow-fed conditions. Upon cholesterol feeding, *Srebp-1c* expression was not induced in these mice, in contrast to data reported previously (28). To test whether this discrepancy may be attributable to differential expression of *Srebp-1a* in our experiment, we also quantified hepatic *Srebp-1a* mRNA levels. Surprisingly, we also did not note any induction of *Srebp-1a* expression in cholesterol-fed Lxr α ^{-/-} mice. Consequently, we must assume that this discrepancy is related to mouse strain differences (C57Bl/6J Sv129/OlaHsd vs. A129/C57Bl6), which may affect hepatic LXR β expression, the dose and duration of cholesterol feeding [2 weeks, 1% (w/w) vs. 1 week, 2% (w/w)], or other factors. Apparently, our Lxr α ^{-/-} mice are unable to maintain basal expression of this key lipogenic gene. In addition, hepatocytes of cholesterol-fed Lxr α ^{-/-} mice experience high intracellular sterol levels. When sterols are present at high concentrations in a cell, the SCAP/SREBP complex is likely retained in the ER. Cholesterol alters SCAP conformation, leading to SCAP retention in the ER (29), which subsequently leads to reduced levels of active nuclear SREBP-1c and lower expression of its target genes. Repa et al. (28) previously reported a reduction of the SREBP-1c target genes *Fas* and *Acc-1* in cholesterol-fed Lxr α ^{-/-} mice, which was accompanied by a 75% decrease in hepatic fatty acid synthesis.

The absence of Lxr α was associated with strongly reduced *Scd1* expression levels under both dietary conditions. SCD1 deficiency in mice is associated with severe hypotriglyceridemia (21, 30), and SCD1 activity correlates with plasma TG levels in several mouse strains (30). Yet, livers of *Scd1*^{-/-} mice were shown to be deficient in CE and TG (31), which was clearly not the case in Lxr α ^{-/-} mice. Furthermore, low hepatic *Scd1* expression in Lxr α ^{-/-} mice was associated with low plasma TG only after cholesterol feeding, indicating that low *Scd1* expression alone is not responsible.

We checked whether the low expression of *Scd1* in Lxr α ^{-/-} mice influenced the fatty acid profile in liver and VLDL particles of these mice. We observed that the

relative amounts of the various FA species in liver and VLDL were slightly different between the groups, but these differences were small and the physiological relevance thereof is uncertain. The distribution of the fatty acids among the various lipid fractions clearly differed between Lxr α ^{-/-} and wild-type mice fed cholesterol. The monounsaturated fatty acids present in the livers of cholesterol-fed Lxr α ^{-/-} mice were preferentially incorporated into CEs, probably to buffer the toxic effects of free cholesterol, rather than being used for TG production. Because these mice are unable to increase the endogenous production of monounsaturated fatty acids as substrates for GPAT activity and subsequent TG synthesis, this resulted in a reduced availability of TG for secretion into VLDL. It has been shown that intrahepatic CEs can compete with TG for incorporation into the VLDL hydrophobic core, and increasing the amount of cholesterol within the liver cell will alter the composition of VLDL core lipids but will not increase the number of VLDL particles that are assembled and secreted (32, 33). In cholesterol-fed Lxr α ^{-/-} mice, the low availability of TG for VLDL production is compensated for by CEs that are excessively abundant in their livers; therefore, the VLDL particle composition reflects the hepatic lipid composition.

Thus, this study confirms that LXR α has a major role in the routing of dietary cholesterol by controlling its intestinal absorption, catabolism, and biliary secretion in mice. The lack of LXR α leads to massive accumulation of free cholesterol and CEs in the liver upon excessive cholesterol intake, which is caused by the combination of increased cholesterol absorption and impaired catabolism and an inability to induce biliary secretion sufficiently. The enhanced use of fatty acids for incorporation into CEs, together with the inability to induce lipogenesis, results in decreased VLDL-TG production and the formation of atherogenic, cholesterol-enriched VLDL particles. The unaffected VLDL-TG production in Lxr α ^{-/-} mice under chow-fed conditions indicates that LXR has a direct effect in controlling VLDL composition only under conditions of hepatocellular cholesterol overload. Furthermore, this study underscores the fact that dietary cholesterol-induced LXR activation has metabolic consequences that are strikingly different from those of pharmacological activation, probably as a consequence of altered intracellular membrane composition. ■

The authors thank Drs. Bei Shan and Margrit Schwarz (formerly at Tularik, Inc., San Francisco, CA) for making available the Lxr α ^{-/-} mouse strain. This work was supported by Grant 912-02-063 from the Netherlands Organization for Scientific Research.

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