FXR-mediated down-regulation of CYP7A1 dominates LXRα in long-term cholesterol-fed NZW rabbits

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Abstract We investigated how cholesterol feeding regulates cholesterol 7α-hydroxylase (CYP7A1) via the nuclear receptors farnesoid X receptor (FXR) and liver X receptor α (LXRα) in New Zealand white rabbits. After 1 day of 2% cholesterol feeding, when the bile acid pool size had not expanded, mRNA levels of the FXR target genes short-heterodimer partner (SHP) and sterol 12α-hydroxylase (CYP8B) were unchanged, indicating that FXR activation remained constant. In contrast, the mRNA levels of the LXRα target genes ATP binding cassette transporter A1 (ABCA1) and cholesteryl ester transfer protein (CETP) increased 5-fold and 2.3-fold, respectively, associated with significant increases in hepatic concentrations of oxysterols. Activity and mRNA levels of CYP7A1 increased 2.4 times and 2.2 times, respectively. After 10 days of cholesterol feeding, the bile acid pool size increased nearly 2-fold. SHP mRNA levels increased 4.1-fold while CYP8B declined 64%. ABCA1 mRNA rose 8-fold and CETP mRNA remained elevated. Activity and mRNA of CYP7A1 decreased 60% and 90%, respectively. Feeding cholesterol for 1 day did not enlarge the ligand pool size or change FXR activation, while LXRα was activated highly secondary to increased hepatic oxysterols. As a result, CYP7A1 was up-regulated. After 10 days of cholesterol feeding, the bile acid (FXR ligand) pool size increased, which activated FXR and inhibited CYP7A1 despite continued activation of LXRα. Thus, in rabbits, when FXR and LXRα are activated simultaneously, the inhibitory effect of FXR overrides the stimulatory effect of LXRα to suppress CYP7A1 mRNA expression.—Xu, G., H. Li, L-x. Pan, Q. Shang, A. Honda, M. Ananthanarayanan, S. K. Erickson, B. L. Shneider, S. Shefer, J. Bollineni, B. M. Forman, Y. Matsuzaki, F. J. Suchy, G. S. Tint, and G. Salen. **FXR-mediated down-regulation of CYP7A1 dominates LXRα in long-term cholesterol-fed NZW rabbits. *J. Lipid Res.* 2003.** 44: **1956–1962.**

Supplementary key words dietary cholesterol • oxysterol • short-heterodimer partner • ATP binding cassette transporter A1

It is well established that feeding cholesterol to rats up-regulates cholesterol 7α-hydroxylase (CYP7A1), the rate-limiting enzyme for classic bile acid synthesis (1–4). As a result, hepatic cholesterol destined for circulating LDL is diverted to bile acid synthesis, and the accumulation of cholesterol in the plasma and liver is prevented. Unlike in rats, feeding cholesterol to New Zealand white (NZW) rabbits was associated with repressed CYP7A1 (5) and a tremendous increase in plasma cholesterol levels. Further studies suggested that expansion of the bile acid pool size was responsible for inhibition of CYP7A1 in the cholesterol-fed rabbits (6), and that the inhibition was not directly related to the accumulated cholesterol (7). However, the molecular mechanism(s) for these findings remains unknown.

Recent studies have shown that the orphan nuclear receptors farnesoid X receptor (FXR) (8–10) and liver X receptor α (LXRα) (11–13) are negative and positive regulators of CYP7A1 transcription. The potent ligands for FXR activation are the bile acids chenodeoxycholic, deoxycholic, and lithocholic acid (8), while the ligands for LXRα activation are oxysterols (14, 15). It was reported that 24*S*,25-epoxycholesterol (24*S*,25E), 24*S*-hydroxycholesterol (24*S*-OH), 22*R*-hydroxycholesterol (22*R*-OH) (16, 17), and 27-hydroxycholesterol (27-OH) (18) are potent activating ligands for LXRα. Further studies demonstrated that short-heterodimer partner (SHP) (19–21) and bile salt export pump (BSEP) (22) are positively regulated and sterol 12α-hydroxylase (CYP8B) (19, 23) is a negatively regulated target gene for FXR. ATP binding cassette transporter A1 (ABCA1) (24) and cholesteryl ester transfer protein (CETP) (25) are target genes positively regulated by LXRα. Chiang et al. found (26) that FXR/RXR did not directly bind to the promoter

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region of human *CYP7A1* and suggested that bile acid-activated FXR inhibited *CYP7A1* transcription by an indirect mechanism. Recently, two groups suggested (20, 21) that in mice, activated FXR repressed CYP7A1 through an FXR-SHP-LRH-1 cascade. More recently, it has been shown that feeding cholic acid to *SHP* knock-out mice could still inhibit CYP7A1 expression (27, 28). This report suggests that other pathways might be involved in negative regulation of CYP7A1 transcription independent of the FXR-SHP-LRH-1 cascade. For example, induction of cytokines (29) or activation of the c-Jun N-terminal kinase pathway (30) by bile acids could also result in repression of CYP7A1 transcription. In addition, the pregnane X receptor activated by lithocholic acid might play a role in down-regulation of CYP7A1 (31). However, in FXR knock-out mice, feeding cholic acid did not suppress *Cyp7a1* mRNA levels as seen in the cholic acid-fed wild-type mice (19). This observation suggested that in general, FXR-mediated regulation plays the major role in bile acid-mediated regulation of CYP7A1.

The focus of the present study is on the effects of dietary cholesterol on FXR and LXRα activation and their role in the regulation of CYP7A1 in NZW rabbits. As there is no method available to measure directly the activation of FXR and LXRα in whole-animal models, we measured the mRNA expression of regulated target genes for FXR (SHP, BSEP, and CYP8B) and LXRα (ABCA1 and CETP) to indicate the activation state of the nuclear receptors. We report that after 1 day of cholesterol feeding, LXRα but not FXR was activated and thus, CYP7A1 was up-regulated substantially. After 10 days of cholesterol feeding, both FXR and LXRα were simultaneously activated, but CYP7A1 was inhibited markedly. These observations suggest that in cholesterol-fed NZW rabbits, the inhibitory effect of activated FXR on CYP7A1 transcription overrides the stimulatory effect of activated LXRα.

MATERIALS AND METHODS

Animal experimental design

Male NZW rabbits ($n = 36$) weighing 2.5–2.75 kg (Convance, Denver, PA) were used in this study. The rabbits were divided into three groups: controls fed regular rabbit chow ($n = 12$), rabbits fed regular chow containing 2% cholesterol (Purina Mills Inc., St. Louis, MO) for 1 day ($n = 12$), and rabbits fed regular chow containing 2% cholesterol (3 g/day) for 10 days ($n = 12$). After completion of the treatments, bile fistulas were constructed in six rabbits from each group, as described previously (7). The biliary bile acid outputs (mg/h) that represented the hepatic bile acid fluxes were measured in bile collected within the first 0.5 h immediately after cannulation of the common bile duct. The bile drainage was continued for 5 days to recover deoxycholic acid pool for calculation of the bile acid pool size, as described previously (6). The remaining six rabbits in each group were sacrificed to collect blood and liver specimens. Blood samples were used for measurements of plasma cholesterol levels. The liver tissues were immediately frozen for measurements of mRNA levels of FXR and FXR target genes (SHP, BSEP, CYP8B, and NTCP), LXRα, and LXRα target genes ABCA1 and CETP, concentration of oxysterols [24*S*,25E, 24*S*-OH, 22*R*-OH, 25-hydroxycholesterol (25-OH), and 27-OH], nuclear protein of FXR/RXR, and CYP7A1 mRNA levels and activity.

The animal protocol was approved by the Institutional Animal Care and Use Committee at Veteran's Administration Medical Center, East Orange, NJ and the Institutional Animal Care and Use Committee at University of Medicine and Dentistry of New Jersey Medical School, Newark, NJ.

Biochemical analyses

The electrophoretic mobility shift assay was performed as described previously (32). Briefly, after nuclei were isolated, nuclear extracts were prepared in a lysis buffer with a high concentration of NaCl. The nuclear receptor proteins FXR, LXRα, and retinoid X receptor (RXR), were synthesized in vitro by transcription and translation (TNT)-coupled reticulocyte lysate system from Promega (Madison, WI) with PCMX·hFXR (a plasmid containing CMV promoter and hFXR gene open reading frame), PCMX·hLXRα, and PCMX·hRXR. The synthesized FXR/RXR and LXRα/RXR are used as a standard to locate the specific binding band for the biological FXR/RXR or LXRα/RXR protein isolated from the experimental rabbit liver specimens. The specific probe for FXR was a double-stranded oligonucleotide containing the sequences 5′-AAGGTCAATGACCTTA-3′ and 5′-TAAGGTCATTGACCTT-3′ and that of LXRα, 5′-TGGACGCCCGCTAGTAACCCCGGT-3′ and 5′-ACCGGGGTTACTAGCGGGCGTCCA-3′. The sequences of mutant probe for FXR were: 5′-AAGAACAATGTTCTTA-3′ and 5′-TAAGAACATTGTTCTT-3′, while those of mutant probe for LXRα were: (top) 5′-TAGAGGCCCGCTAGTAATCCCGGT-3′ and (bottom) 5′-ACCGGGATTACTAGCGGGCCTCTA-3′. The unlabeled specific and mutant probes for FXR or LXRα were added as the positive and negative competitors to compete with the specific probe labeled with P^{32} to identify the specific binding to the synthesized standard FXR/RXR or LXRα/RXR protein. Competitor (cold or mutated probe) was added in a 100-fold excess and was preincubated with the extracted nuclear proteins (10 μg) in a binding reaction solution (20 μl) containing 2 μg poly dI-dC, 20 mM HEPES (pH 7.5), 1.5 mM $MgCl_2$, 1 mM DTT, 2 mM EDTA, 50 mM KCl, and 3% glycerol on ice for 30 min before adding labeled probe. After another hour of incubation with P^{32}-labeled specific probe (0.06 pmol, 25,000 cpm) on ice, the protein-DNA complexes were analyzed by low ionic strength system electrophoresis on 8% polyacrylamide gel in 0.375 × Tris-borate-EDTA buffer. The gel was then dried and subjected to PhosphorImager and Imagequant software (Molecular Dynamics, CA) to quantify the abundance of FXR/RXR and LXRα/RXR protein.

Northern blotting analyses

Probe preparation. Rabbit cDNA probes for CYP7A1, FXR, SHP, BSEP, CYP8B, cyclophilin, LXRα, and CETP were prepared by PCR with degenerate primer as previously described (32). In addition, primers for ABCA1 were as follows: 5′ AGGAGGTGATGTTTCGAC-3′/5′AGCTCCATGGACTTGTTGA-3′. All rabbit probes were [α-^{32}P]dCTP-labeled DNA.

RNA isolation. Total RNA was isolated from frozen rabbit liver tissue using the single-step RNA isolation method with TRIZOL Reagent (Invitrogen Life Technologies, Carlsbad, CA), as described by Chomozynski and Sacchi (33). Poly(A)$^+$ RNA was isolated from 2 mg total RNA by oligo dT cellulose using the Fast-Track 2.0 mRNA isolation Kit (Invitrogen Life Technologies) described by Biesecker, Gottschalk, and Emerson (34).

Hybridization. Northern blot hybridization was performed as previously described by Thomas (35). Briefly, 10 μg poly(A)$^+$ RNA was electrophoresed on a formaldehyde-agarose (1.0%) gel and transferred to a nylon membrane (Nytran supercharge nylon transfer membrane, Schleicher and Schuell). The membrane was baked for 2 h at 80°C and hybridized to a ^{32}P-labeled DNA probe for 16 h at 42°C. The membrane was washed at 55°C

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in 0.2 × SSC, 0.1% SDS for 30 min. Relative expression levels were quantified using a PhosphorImager (Molecular Dynamics) and standardized against cyclophilin controls.

Oxysterol analysis

Oxysterols were determined based on the method described by Dzeletovic et al. (36) with some modifications.

Alkaline hydrolysis and extraction. Liver specimen (100 mg wet weight) was homogenized in 2.5 ml of distilled water. [$^2H_7$]27-OH (32 ng) as internal recovery standard, 1 ml 0.5 N ethanolic KOH, and 5 μg butylated hydroxytoluene were added to 100 μl of the homogenate, and alkaline hydrolysis was allowed to proceed at 37°C for 1 h (37). After the addition of 0.4 ml of distilled water and extraction twice with 2 ml of n-hexane, the extract was evaporated to dryness under nitrogen.

Purification by disposable silica cartridge. The residue was dissolved in 1 ml of toluene and applied to a Bond Elut SI cartridge (100 mg). After washing with 1 ml of n-hexane, cholesterol was eliminated with 8 ml of n-hexane-2-propanol (99.5:0.5; v/v). Oxysterols were then eluted with 5 ml of n-hexane-2-propanol (7:3; v/v).

Analysis by high-resolution gas liquid chromatography-mass spectrometry with selected-ion monitoring. After removal of the solvent under a gentle stream of nitrogen, the oxysterols were converted into trimethylsilyl (TMS) ether derivatives with 100 μl of TMSI-H (GL Sciences, Tokyo, Japan) for 15 min at 55°C. Gas liquid chromatography-mass spectrometry (GC-MS) with selected-ion monitoring (SIM) was performed using a JMS-SX102 instrument equipped with a JMA DA-6000 data-processing system (JEOL, Tokyo, Japan). The accelerating voltage was 10 kV, the ionization energy was 70 eV, the trap current was 300 μA, and the mass spectral resolution was about 10,000 (38). An Ultra Performance capillary column (25 m × 0.32 mm id) coated with methylsilicone (Agilent Technologies, DE) was used at a flow rate of helium carrier gas of 1.0 ml/min. The column oven was programmed to change from 100°C to 270°C at 30°C/min after a 1 min delay from the start time. The multiple ion detector was focused on *m/z* 173.1360 for 22*R*-OH, *m/z* 343.3000 for 24*S*,25E, *m/z* 413.3239 for 24*S*-OH, *m/z* 456.3787 for 25-OH and 27-OH, and *m/z* 463.4226 for [$^2H_7$]27-OH.

Assays for activities of CYP7A1

Hepatic microsomes were prepared by differential ultracentrifugation (39). Protein was determined according to Lowry et al. (40). CYP7A1 activity was measured in hepatic microsomes by the isotope incorporation method of Shefer, Salen, and Batta (39).

Assay for bile acids

Bile acid concentration and composition were analyzed by capillary gas-liquid chromatography as previously described (7).

Statistical method

Data are shown as means ± SD and were compared statistically by Student's *t*-test (unpaired). The BMDP Statistical Software (BMDP Statistical Software, Inc., Los Angeles, CA) was used for statistical evaluations.

RESULTS

Plasma cholesterol levels increased 5-fold from 25 ± 5 mg/dl to 125 ± 17 mg/dl ($P < 0.0001$) in rabbits fed 2% cholesterol for 1 day and 38-fold to 958 ± 126 mg/dl ($P < 0.0001$) after 10 days. Hepatic cholesterol concentrations also rose 64% from 2.1 ± 0.3 to 3.5 ± 1.3 mg/g ($P < 0.05$) in rabbits fed 2% cholesterol for 1 day and 3.6-fold to 17.0 ± 2.5 mg/g ($P < 0.001$) after 10 days.

The bile acid pool in these rabbits was composed mainly of deoxycholic acid (87.5 ± 4.3%), a potent activating ligand for FXR (8). After 1 day of 2% cholesterol feeding, the bile acid pool size and hepatic flux did not change significantly (**Fig. 1**). However, after 10 days, the bile acid pool size enlarged 86% from 240 ± 62 to 446 ± 69 mg ($P < 0.01$), and the hepatic bile acid flux increased 2-fold from 30 ± 10 to 68 ± 19 mg/h ($P < 0.01$) (Fig.1).

Hepatic concentrations of oxysterols, 24*S*,25E, 24*S*-OH, 22*R*-OH, 25-OH, and 27-OH increased significantly after both 1 day and 10 days of 2% cholesterol feeding (**Fig. 2**). 24*S*,25E was not detected in the liver of rabbits fed regular chow (baseline control), but rose to trace amounts (<0.10 nmol/g) after 1 day of 2% cholesterol feeding and increased more than 3.6-fold to 0.36 ± 0.19 nmol/g after 10 days of the feeding. Hepatic 24*S*-OH concentrations increased 4-fold, from 0.10 ± 0.04 to 0.40 ± 0.18 nmol/g ($P < 0.01$), and 14.7-fold, to 1.47 ± 0.31 nmol/g ($P < 0.0001$), after 1 day and 10 days, respectively. 22*R*-OH concentration increased 33% from 0.012 ± 0.002 to 0.016 ± 0.002 nmol/g ($P < 0.05$) and 4.5-fold to 0.054 ± 0.015 nmol/g ($P < 0.001$) after 1 day and 10 days, respectively. 27-OH concentration increased more than 4-fold from trace (<0.10 nmol/g) to 0.41 ± 0.17 nmol/g and 10-fold to 1.13 ± 0.37 nmol/g after 1 day and 10 days, respectively. 25-OH concentration increased more than 3-fold from trace (<0.10 nmol/g) to 0.31 ± 0.09 nmol/g and 0.32 ± 0.06 nmol/g after 1 day and 10 days, respectively.

CYP7A1 mRNA level increased 2.2-fold from 0.38 ± 0.03 to 0.85 ± 0.22 units ($P < 0.05$) after 1 day of 2% cholesterol feeding and was accompanied by 2.4-fold increase in CYP7A1 activity from 26.9 ± 6.6 to 65.3 ± 20.6 pmol/mg/min ($P < 0.01$) (**Fig. 3**). In contrast, after 10 days of cholesterol feeding, CYP7A1 mRNA declined 90% to 0.04 ± 0.01 units ($P < 0.0001$), and activity decreased 60% to 10.6 ± 2.3 pmol/mg/min ($P < 0.001$) as compared with control baseline values (Fig. 3 and **Fig. 4**).

mRNA levels of the FXR target genes SHP, BSEP, and CYP8B did not change after 1 day of cholesterol feeding (Fig. 4). In contrast, after 10 days of cholesterol feeding when the bile acid pool size and hepatic flux had doubled, mRNA levels of SHP increased 4.1-fold from 0.30 ±

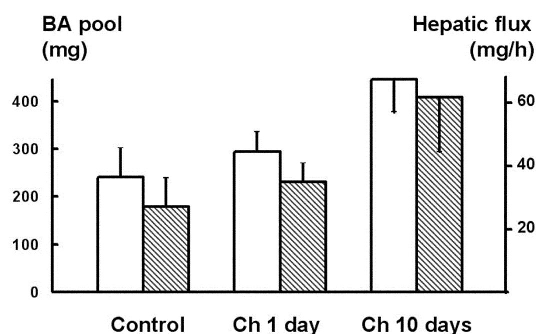

Fig. 1. The bile acid (BA) pool size (open bar) and hepatic flux (hatched bar) in rabbits fed regular chow (control, n = 5), and 2% cholesterol for 1 day (Ch 1 day, n = 5) and 10 days (Ch 10 days, n = 5). Error bars indicate ± SD.

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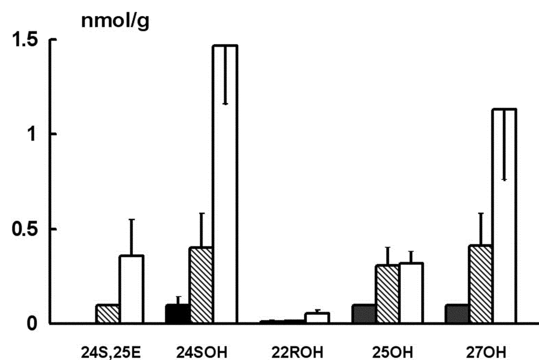

Fig. 2. Hepatic oxysterol concentrations in rabbits fed regular chow (solid bar, n = 5) or fed 2% cholesterol for 1 day (hatched bar, n = 4) or 10 days (open bar, n = 4). 24*S*,25E, 24*S*,25-epoxycholesterol; 24*S*OH, 24*S*-hydroxycholesterol; 22*R*OH, 22*R*-hydroxycholesterol; 25OH, 25-hydroxycholesterol; 27-OH, 27-hydroxycholesterol. Error bars indicate ± SD.

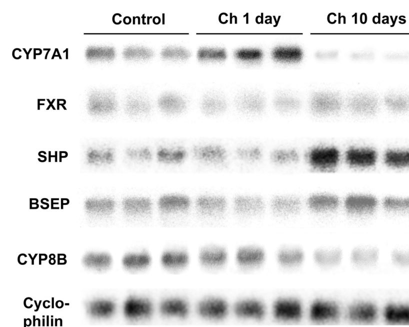

Fig. 4. Northern blotting analysis for hepatic mRNA expression of farnesoid X receptor (FXR) and FXR target genes in rabbits fed regular chow (control) and 2% cholesterol for 1 day (Ch 1 day) or 10 days (Ch 10 days). Cyclophilin is used as an internal standard. SHP, short heterodimer partner; BSEP, bile salt export pump; CYP8B, sterol 12α-hydroxylase.

0.08 to 1.24 ± 0.41 units ($P < 0.05$), but BSEP mRNA levels did not change (0.35 ± 0.04 vs. 0.43 ± 0.05 units ($P = 0.11$). The expression of CYP8B, a target gene negatively regulated by FXR, decreased 64% from 0.58 ± 0.07 to 0.21 ± 0.04 units ($P < 0.01$) after 10 days. FXR mRNA levels did not show significant change after either 1 day (0.34 ± 0.08 units to 0.21 ± 0.05 units) or 10 days (0.32 ± 0.06 units) of 2% cholesterol feeding (Fig. 4). FXR/RXR protein levels measured by electrophoretic mobility shift assay (**Fig. 5**) also did not change after 1 day (51.9 ± 5.0 units to 55.4 ± 5.0 units, n = 4) and did not show significant increase (65.6 ± 11.5 units, n = 5, $P = 0.064$) after 10 days of 2% cholesterol feeding.

Unlike FXR target genes, the mRNA expression of LXRα target genes, ABCA1 and CETP, increased 5-fold from 0.17 ± 0.04 to 0.85 ± 0.18 units ($P < 0.01$) and 2.3-fold from 0.23 ± 0.04 to 0.53 ± 0.12 units ($P < 0.05$), respectively, after 1 day of cholesterol feeding (**Fig. 6**). After 10 days of cholesterol feeding, ABCA1 mRNA levels rose further to 1.37 ± 0.15 units (8-fold, $P < 0.001$), while CETP levels remained elevated (0.65 ± 0.07 units, $P < 0.001$ as compared with controls). LXRα mRNA levels did not change significantly after 1 day of 2% cholesterol feeding from 0.18 ± 0.07 to 0.23 ± 0.05 units, but increased 94% to 0.35 ± 0.04 units ($P < 0.05$) after 10 days of 2% cholesterol feeding. Similarly, LXRα/RXR protein remained unchanged after 1 day of cholesterol feeding (12.7 ± 4.3 vs. 14.7 ± 5.0 units, n = 4), but rose 2-fold (26.5 ± 7.0 units, n = 4, $P < 0.05$) after 10 days of treatment (**Fig. 7**).

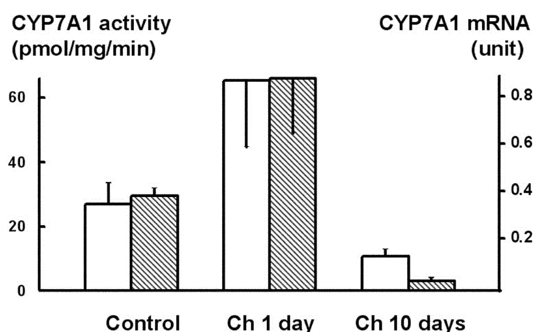

Fig. 3. Cholesterol 7α-hydroxylase (CYP7A1) activity (open bar, n = 6) and mRNA levels (hatched bar, n = 3) in rabbits fed regular chow (control) and 2% cholesterol for 1 day (Ch 1 day) or 10 days (Ch 10 days). Error bars indicate ± SD.

DISCUSSION

The results of this investigation demonstrate the different effects of 1 day and 10 days of 2% cholesterol feeding

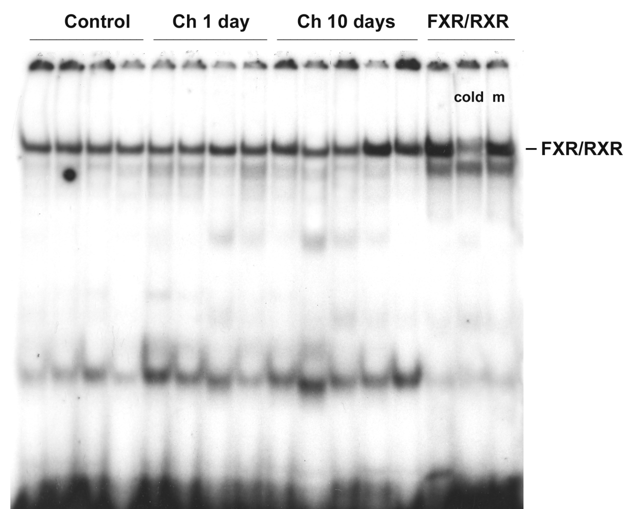

Fig. 5. Electrophoretic mobility shift assay (EMSA) for hepatic farnesoid X receptor (FXR)/retinoid X receptor (RXR) protein levels. FXR/RXR protein synthesized by the transcription and translation (TNT) system was applied as a standard on the first three lanes from the right (FXR/RXR). The unlabeled specific (cold) and mutant (m) probes for FXR were added to the second and first lanes from the right, respectively, as the positive and negative competitors that compete with the FXR-specific probe labeled with P^{32} to identify the specific binding to FXR/RXR protein in the samples of synthesized standard (FXR/RXR). Control (n = 4); Ch 1 day, 2% cholesterol fed for 1 day (n = 4); Ch 10 days, 2% cholesterol fed for 10 days (n = 5).

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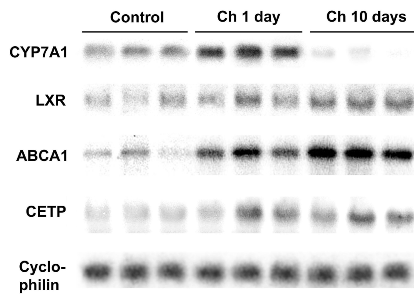

Fig. 6. Northern blotting analysis for hepatic mRNA expression in liver X receptor (LXR) and LXR target genes in rabbits fed regular chow (control) and 2% cholesterol for 1 day (Ch 1 day) or 10 days (Ch 10 days). Cyclophilin is used as an internal standard.

on the activation of LXRα and FXR and the regulation of CYP7A1 in rabbits. After 1 day of cholesterol feeding, hepatic concentrations of oxysterols, the activating ligands for LXRα, rose significantly such that LXRα became activated, as evidenced by the rise in mRNA levels of the target genes ABCA1 and CETP in the liver. In contrast, mRNA levels of positively and negatively regulated FXR target genes SHP, BSEP, and CYP8B did not change, which indicated that FXR activation remained unchanged. After 1 day of cholesterol feeding, the bile acid pool and hepatic flux, which contained 87% deoxycholic acid, an activating ligand for FXR, did not increase, providing an explanation for why further FXR activation did not occur. CYP7A1, the target gene of both FXR and LXRα, was up-regulated in response to the enhanced activation of LXRα secondary to the increase in its high-affinity oxysterol ligands. After 10 days of cholesterol feeding, the bile acid (FXR ligand) pool size and hepatic flux increased significantly, leading to increased FXR activation, as indicated by increased mRNA levels of SHP, positively regulated by FXR, and diminished mRNA levels of CYP8B, negatively regulated by FXR (Fig. 4). Both activity and mRNA of CYP7A1 were decreased despite the increased expression of LXRα mRNA and LXRα/RXR protein, and continued activation of LXRα, as evidenced by further increase in ABCA1 mRNA level and sustained increase of CETP mRNA (Fig. 6). This finding suggests that CYP7A1 transcription was increased after 1 day of cholesterol feeding because of LXRα activation when FXR activation had remained unchanged. In contrast, after 10 days of cholesterol feeding, when both FXR and LXRα were simultaneously activated, CYP7A1 was down-regulated. These results suggest that in rabbits, the inhibitory effect of FXR is dominant over the stimulatory effect of LXRα in regulation of CYP7A1 under these conditions.

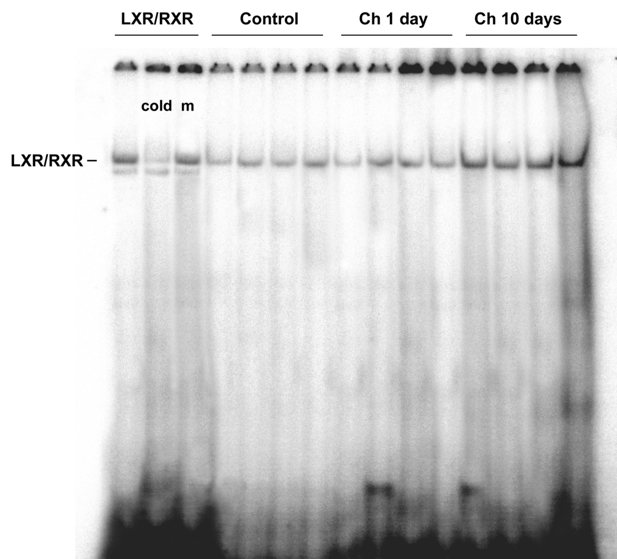

Fig. 7. EMSA for hepatic LXRα/RXR protein levels. LXRα/RXR protein synthesized by the TNT system was applied as a standard on the first three lanes from the left (LXRα/RXR). The unlabeled specific (cold) and mutant (m) probes for LXRα were added to the second and third lanes from the left, respectively, as the positive and negative competitor to compete with the LXRα-specific probe labeled with P^{32} to identify the specific binding to LXRα/RXR protein in the samples of synthesized standard (LXRα/RXR). Control (n = 4); Ch 1 day, 2% cholesterol fed for 1 day (n = 4); Ch 10 days, 2% cholesterol fed for 10 days (n = 4).

We recognize that other pathways independent of FXR/SHP cascade might also be activated by the increased bile acid flux and contribute to negatively regulate CYP7A1 transcription. However, it seems likely that FXR plays the major role in the bile acid feedback regulation of CYP7A1, because in FXR knock-out mice, feeding cholic acid did not repress transcription of *Cyp7a1* (19).

The results of this study also shed light on the effects of dietary cholesterol on CYP7A1 in NZW rabbits at the molecular level. Similar to rats, rabbit CYP7A1 responds positively to activated LXRα after cholesterol feeding. In rabbits fed cholesterol for only 1 day, when FXR activation remained unchanged, increased formation of oxysterols, some of which are high-affinity ligands for LXRα, induced CYP7A1 (Fig. 6). However, CYP7A1 was inhibited after 10 days of cholesterol feeding because FXR became activated by the expanded bile acid (FXR ligand) pool, although LXRα continued to be strongly activated simultaneously. Therefore, it is unlikely that inhibition of CYP7A1 after 10 days of cholesterol feeding in rabbits was due to a direct effect from dietary cholesterol. Rather, the expanded bile acid (FXR ligand) pool led to increased FXR activation that resulted in decreased CYP7A1 mRNA and activity.

The results from this study are different from those of the rabbit studies reported by Overturf et al. (41) and Poorman et al. (42), in which the experiments were carried out in hypercholesterolemia-resistant rabbits, a special colony of NZW rabbits obtained by selective bleeding. In these rabbits, baseline CYP7A1 mRNA levels are 7-fold higher than in ordinary New Zealand rabbits and remain elevated after cholesterol feeding. Following cholesterol feeding, fecal bile acid outputs were 2-fold higher as compared with ordinary rabbits. Therefore, bile acid synthesis rates were higher, but the bile acids reabsorbed through the intestine were less in these cholesterol-fed hypercholesterolemia-resistant rabbits as compared with ordinary NZW rabbits. Thus, in the hypercholesterolemia-resistant rabbits, cholesterol feeding did not increase the func-

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tional bile acid (FXR ligand) pool size as seen in the rabbits of the present study, because increased amounts of bile acids were lost in the feces but not reabsorbed via the ileum to circulate through the liver. As a result, in these rabbits FXR was not activated after cholesterol feeding and CYP7A1 was not down-regulated. Obviously, different responses of CYP7A1 to dietary cholesterol exist not only between species but also among the individuals in the same species. However, at least in rabbits challenged with dietary cholesterol, activation of FXR seems dominant in determining the down-regulation of CYP7A1.

In the present study, we report a significant increase in hepatic oxysterols (24*S*,25E, 24*S*-OH, 22*R*-OH, 25-OH, and 27-OH) concentrations in rabbits fed 2% cholesterol for both 1 day and 10 days. Among these oxysterols, 24*S*-OH and 27-OH concentrations increased the most. Janowski et al. comprehensively studied the effect of various naturally produced oxysterols on LXR activation in vitro (16). They suggested that 24*S*,25E, 24*S*-OH, and 22*R*-OH were all highly effective ligands for LXRα. Further, Zhang et al. (17) found that in rats fed an atherogenic diet, increased concentrations of hepatic 24(*S*),25E and 24(*S*)-OH were detected in hepatocyte nuclei. Thus, they suggested that these two oxysterols are the physiologic high-affinity ligands for LXRα. More recently, it was reported that 27-OH may also be an endogenous ligand for LXRα in monohepatocytes (18). The increased hepatic concentrations of endogenous LXR ligands in combination with higher expression of LXR mRNA and its target genes support the idea that LXR was activated in rabbits fed 2% cholesterol for both 1 day and 10 days.

It was reported that 24*S*-OH is only produced in the brain (43). Thus, it is not clear why this oxysterol increased 14.7-fold in the liver after cholesterol feeding if synthesis is limited only to the brain. It is well established that plasma cholesterol does not pass the blood barrier to enter the brain. The tremendous increase in plasma cholesterol should not penetrate into the brain to produce additional amounts of 24*S*-OH. Therefore, we postulate that the increased hepatic concentration of 24*S*-OH may be also produced in the liver.

In summary, this experiment shows that 1 day of cholesterol feeding activated LXRα by increasing hepatic oxysterol concentrations but did not increase FXR activation because the bile acid pool size remained unchanged. As a result, CYP7A1 mRNA and activity increased. In contrast, after 10 days of cholesterol feeding, FXR was activated by expanding the bile acid (FXR ligand) pool size. While LXRα remained activated, CYP7A1 mRNA and activity were decreased. Thus, in the longer-term cholesterol-fed NZW rabbits, the inhibitory effect of FXR appears to override the stimulatory effect of LXRα, resulting in a net suppression of CYP7A1 mRNA expression.

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