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Regulation of the Aldo-Keto Reductase Gene *akr1b7* by the Nuclear Oxysterol Receptor LXRα (Liver X Receptor-α) in the Mouse Intestine: Putative Role of LXRs in Lipid Detoxification Processes

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Liver X receptors (LXRs) regulate the expression of a number of genes involved in cholesterol and lipid metabolism after activation by their cognate oxysterol ligands. AKR1-B7 (aldo-keto reductase 1-B7) is expressed in LXR target tissues such as intestine, and because of its known role in detoxifying lipid peroxides, we investigated whether the AKR1-B7 detoxification pathway was regulated by LXRs. Here we show that synthetic LXR agonists increase the accumulation of AKR1-B7 mRNA and protein levels in mouse intestine in wild-type but not *lxr*$^{-/-}$ mice. Regulation of *akr1b7* by retinoic X receptor/LXR heterodimers is dependent on three response elements in the proximal murine *akr1b7* promoter. Two of these *cis*-acting elements are specific for regulation by the LXRα isoform. In addition, in duodenum of wild-type mice fed a synthetic LXR agonist, we observed an LXR-dependent decrease in lipid peroxidation. Our results demonstrate that *akr1b7* is a direct target of LXRs throughout the small intestine, and that LXR activation plays a protective role by decreasing the deleterious effects of lipid peroxides in duodenum. Taken together, these data suggest a new role for LXRs in lipid detoxification. (*Molecular Endocrinology* 18: 888–898, 2004)

MOUSE VAS DEFERENS protein/aldo-keto reductase 1-B7 (AKR1-B7) encodes an aldose-reductase (1) involved in detoxification processes. It is the major enzyme responsible for the reduction of isocaproaldehyde formed from the side-chain cleavage of cholesterol in the first step of murine steroidogenesis (2). Transcriptional regulation of *akr1b7* has been well defined in two of the major organs in which it is expressed. In mouse adrenal cortex, its expression is acutely regulated by ACTH control (3, 4), whereas in the mouse vas deferens a strong androgen-dependent accumulation has been described (1). Lau *et al.* (5) have shown by RNase protection that *akr1b7* is also expressed in mouse kidney, eye, intestine, and, to a very low extent, in liver. The link between AKR1-B7 and the production of hormones in adrenal glands has been well documented (2); however, its role in intestine is still unresolved. Lefrançois-Martinez *et al.* (2) have shown that AKR1-B7 is able to detoxify 4-hydroxynonenal (4-HNE), a cytotoxic α,β-unsaturated acyl aldehyde. This product is a natural result of lipid peroxidation and cleavage that occurs in response to oxidative stress and aging (6). Hence, we hypothesized that AKR1-B7 could be one of the several enzymes involved in the detoxification of lipid peroxides in intestine.

Lipids are vital nutrients that, among their many roles, can have a major impact on gene expression. Intracellular concentrations of cholesterol and fatty acids are controlled by mechanisms involving transcriptional regulation (7). Several nuclear receptors are known to regulate lipid absorption, storage, and utilization. Liver X receptors, LXRα (NR1H3) and LXRβ (NR1H2) (8), belong to a subclass of nuclear receptors that form obligate heterodimers with retinoid receptors RXRs (retinoic X receptors) and are bound and activated by a class of naturally occurring oxysterols (9, 10). RXR/LXR heterodimers activate their target genes

Abbreviations: AKR1-B7, Aldo-keto reductase 1-B7; 9cRA, 9 *cis*-retinoic acid; 4-HNE, 4-hydroxynonenal; LXR, liver X receptor; LXRE, LXR response element; *lxr*$\alpha^{-/-}$, *lxr*α knockout mouse; *lxr*$\beta^{-/-}$, *lxr*β knockout mouse; *lxr*$\alpha;\beta^{-/-}$, *lxr*α and *lxr*β knockout mouse; mpk, mg/kg; PPAR, peroxisome proliferator-activated receptor; PUFA, polyunsaturated fatty acid. RXR, retinoic X receptor; 22(R)OH, 22(R)-hydroxycholesterol; 22(S)OH, 22(S)-hydroxycholesterol; T1317, T0901317 compound.

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by binding to specific response elements termed LXREs (LXR response elements) that contain a hexanucleotide direct repeat separated by four nucleotides (8). During the last few years, LXRs have been shown to act as major sensors of intracellular sterol concentrations (11). Use of *lxr*-deficient mice has also helped to elucidate the role of LXRs in fatty acid metabolism (12, 13), insulin mediation of fatty acid and cholesterol biosynthesis (14, 15), glucose homeostasis (16), and oxysterol stimulation of epidermal differentiation (17). Consistent with this observation, the LXR-regulated genes described thus far include cholesterol 7α-hydroxylase (*cyp7a1*) (18), the rate-limiting step in the conversion of cholesterol to bile acids, cholesterol ester transfer protein, which translocates cholesteryl esters between lipoprotein fractions (19), and several ATP-binding cassette transporters including ABCA1 (20, 21), ABCG1 (22), and ABCG5 and ABCG8 (23). The role of LXRs in the intestine has been well documented, especially in the regulation of cholesterol absorption and excretion by the ATP-binding cassette transporters (21, 23–25).

Here we show that mice fed receptor-selective agonists for LXRs and RXRs exhibit increased *akr1b7* mRNA levels in the intestine. Analysis of the mouse *akr1b7* promoter revealed the presence of three RXR/LXR binding sites. *In vivo* and *in vitro* experiments suggest that *akr1b7* regulation is mediated predominantly by LXRα. Together, these data suggest a new mechanism for regulating the expression of *akr1b7* by oxysterols in the intestine and further expand the role of the LXRs in maintaining normal lipid homeostasis.

RESULTS

Expression of the Intestine aldo-keto Reductase *akr1b7* Gene Is Stimulated by Synthetic Agonists of RXRs and LXRs

To test the regulation of *akr1b7* by LXRs and RXRs in the intestine, wild-type and *lxr*$\alpha;\beta^{-/-}$ mice were treated with potent LXR or RXR agonists [T0901317 compound (T1317) or LG268] for 12 h or 10 d (Fig. 1A, *left and right panels*, respectively). Both agonists resulted in an increase of *akr1b7* mRNA accumulation in the duodenum of wild-type mice. A lack of increase of *akr1b7* mRNA accumulation by T1317 was observed in the *lxr*$\alpha;\beta^{-/-}$ mice, demonstrating that this gene may be a direct target of LXRs. The RXR agonist LG268 induced an increase of *akr1b7* accumulation in both wild-type and *lxr*$\alpha;\beta^{-/-}$mice, suggesting the possible role of other RXR partners in *akr1b7* expression.

We next determined whether the increase of *akr1b7* transcription resulted in accumulation of the protein (Fig. 1B). In wild-type mice fed T1317, AKR1-B7 protein was elevated 3.6-fold above untreated wild-type mice. In *lxr*$\alpha;\beta^{-/-}$ mice no increase in protein levels was observed. However, the basal level of AKR1-B7 protein was higher (3.5-fold). Because duodenum exhibited the highest fold changes, Western blot analyses were performed on this intestinal segment in both *lxr*$\alpha^{-/-}$ and *lxr*$\beta^{-/-}$ single knock-out mice after treatment with T1317 for 12 h (Fig. 1C). Analysis showed a 2.5-fold increase in *lxr*$\beta^{-/-}$ mice similar to that seen in wild-type mice (3.1-fold increase), whereas no increase was seen in the *lxr*$\alpha^{-/-}$ mice suggesting that *akr1b7* is regulated mainly by LXRα *in vivo.* As observed with the *lxr*$\alpha;\beta^{-/-}$ mice, an elevation of the AKR1-B7 basal level was present in the *lxr*$\alpha^{-/-}$ mice (2-fold compared with the wild type).

To further investigate the LXRα-selective regulation of *akr1b7*, we studied the expression pattern of the two LXR isoforms in the intestine. The results showed that whereas both LXRs were expressed throughout the intestine (Fig. 1D), expression of LXRα was significantly higher than that of LXRβ in the proximal parts (duodenum and beginning of the jejunum) compared with the distal part (ileum). These data suggested that the gradient of expression observed for *akr1b7* could be due, in part, to a similar pattern of expression for LXRα, as well as other transcription factors.

Identification of Binding Sites for RXR-LXR in the Promoter of *akr1b7*

To identify the region responsible for the LXR-mediated regulation of *akr1b7*, we used progressive deletions of the promoter. CV1 cells were transfected with pCMX-mRXRα and pCMX-mLXRα or pCMX-mLXRβ (Fig. 2A). The *akr1b7* promoter construct including -510 bp to $+41$ bp (0.5 *akr1b7*-luc) was the most responsive to the natural ligands 22(R)-hydroxycholesterol [22(R)OH] and 9-*cis*-retinoic acid (9cRA) (13-fold with LXRα and 6.2-fold with LXRβ). The 0.5 *akr1b7*-luc reporter gene showed the same inducibility as the known $(LXRE)_3$-tk-luc reporter gene (Fig. 2B), derived from the sequence of the mouse *cyp7a1*-LXRE, which has also been shown to be more inducible by LXRα than LXRβ (18). Note that the $-249/+41$ bp luciferase construct was still inducible by LXRα with a 6.8-fold increase, whereas LXRβ failed to transactivate the reporter gene (Fig. 2A), suggesting that specific sequences were involved for the LXRα response. The response of the 0.5 *akr1b7*-luc reporter gene to the synthetic ligands LG268 and T1317 was in the same range as those observed with the natural ligands 22(R)OH and 9cRA (Fig. 2C). Treatment with both synthetic ligands resulted in a more than additive effect as expected. In addition, LXRα was more efficient than LXRβ (27.0- *vs.* 11.2-fold) at trans-activating the promoter, consistent with the observed effects in the single-knockout mice reported above. To examine the LXR-mediated transcriptional regulation of this construct in an intestinal cell system, transient transfections with 0.5 *akr1b7*-luc were performed in CaCo2/clone 7 cells. As expected, the reporter gene was similarly responsive, giving a 19.6- and 10.8-fold increase with pCMX-mLXRα and pCMX-mLXRβ, respectively (Fig. 2D).

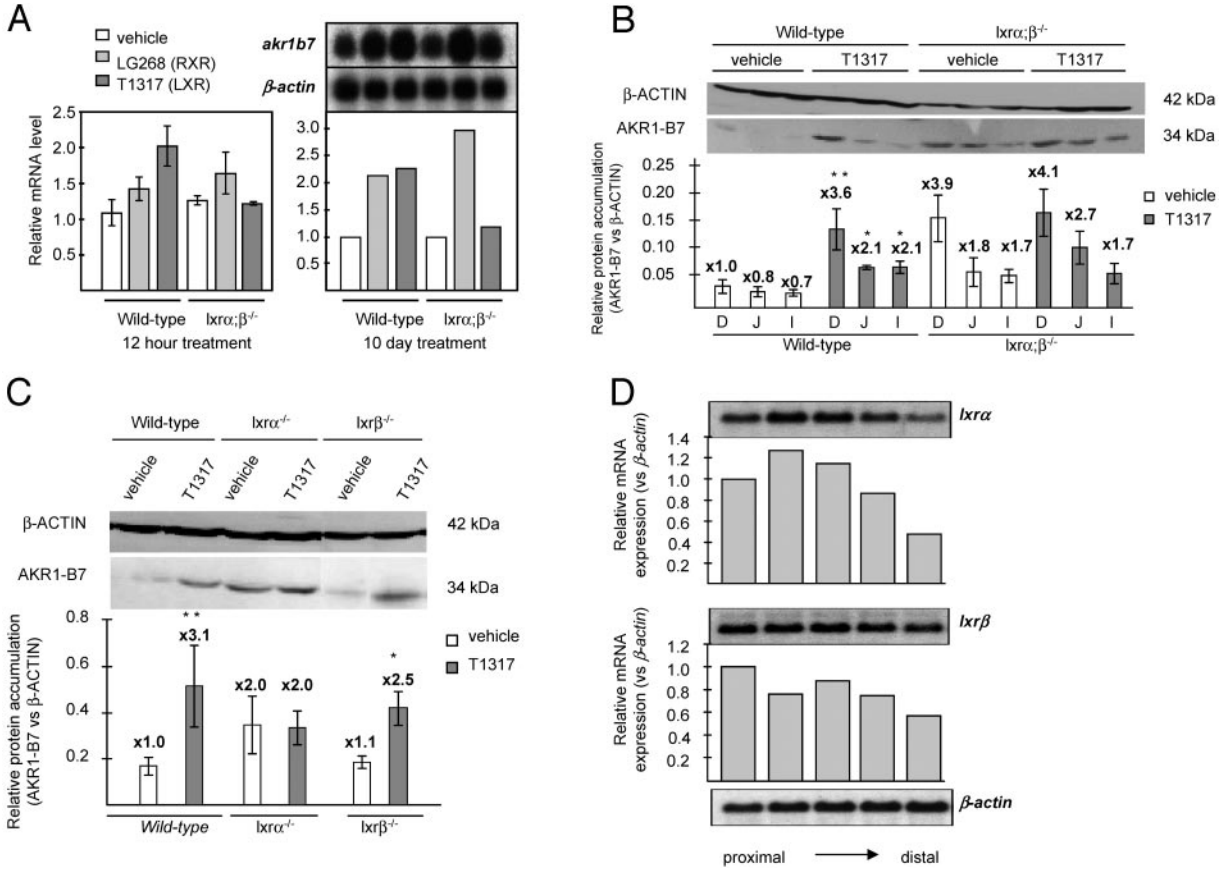

Fig. 1. Intestine Levels of *akr1b7* mRNA (A) and Protein (B and C), and LXR mRNA (D)

Three-month-old male mixed-strain mice of various LXR genotypes were fed *ad libitum* diets containing 0.02% cholesterol plus vehicle, 30 mpk LG268, or 50 mpk T1317 for 12 h or 10 d. A, Relative levels of akr1b7 mRNA in mouse duodenum as measured by quantitative real-time PCR (*left panel*) or Northern analysis (*right panel*). B and C, For Western blot analysis, 3-month-old male mixed strains of various LXR genotypes were fed *ad libitum* diets containing 50 mpk T1317 for 12 h. Representative Western blots of duodenum (D), jejunum (J), and ileum (I) are shown in pooled samples of two mice (B), and of the duodenum from an individual mouse (C). *Graphics* show the fold induction of the AKR1-B7 protein standardized by β-actin; mean ± SEM of three to four independent experiments. D, Northern blot analysis was performed on pooled samples of three to four animals per group; small intestine mRNA was isolated from mucosa and dissociated in five portions from duodenum (the most proximal) to ileum (the most distal). Student's *t* test: *, $P < 0.05$; **, $P < 0.005$.

The search for LXREs revealed three potential sequences upstream from the initiation start site (Fig. 3A) and arbitrarily named LXRE3 (−259 to −244), LXRE1 (−236 to −221), and LXRE2 (−153 to −168). To test the ability of these LXREs to bind RXR/LXR, EMSAs were performed with nuclear extracts obtained from CaCo2/TC7 cells or livers from the four genotypes. To confirm the binding of LXRα, a polyclonal antibody developed against the N-terminal domain of the protein was used. As shown in Fig. 3B, this antiserum was specific for LXRα. With the nuclear extracts from the CaCo2/TC7 cells, two complexes were observed (Fig. 3C). A 20-fold excess of unlabeled *abca1* LXRE (Table 1) was able to compete for the binding of both complexes, whereas a 100-fold excess of an Sp1-binding sequence did not compete with the radiolabeled probe. Furthermore, inclusion of a specific LXRα antiserum eliminated only the highest migrating complex, suggesting that this band contains RXR/LXRα heterodimer. The ability of the LXRE1 and LXRE2 oligos to bind the RXR/LXR heterodimer was demonstrated by using unlabeled probes to compete for binding to the radiolabeled LXRE3 probe. At 20-fold excess, both LXREs were able to compete for RXR/LXR binding. To determine the relative affinity of the three *akr1b7*-LXREs for LXRs, we used a labeled *abca1*-LXRE probe and competed with unlabeled *akr1b7* LXRE1, LXRE2, and LXRE3 and a nonspecific SP1 binding sequence (Fig. 2D). LXRE1, -2, and -3 were able to compete both complexes at 20- and 50-fold excess, whereas the SP1 oligo was not. Moreover, LXRE1 seemed to be the most efficient competitor in this assay. To test the relative affinity of the *akr1b7*-LXREs for both LXR isoforms, we used liver nuclear extracts from wild-type, *lxrα*$^{-/-}$, *lxrβ*$^{-/-}$, or *lxrα;β*$^{-/-}$ mice. *Abca1*-LXRE was used as control. Figure 3E showed that no

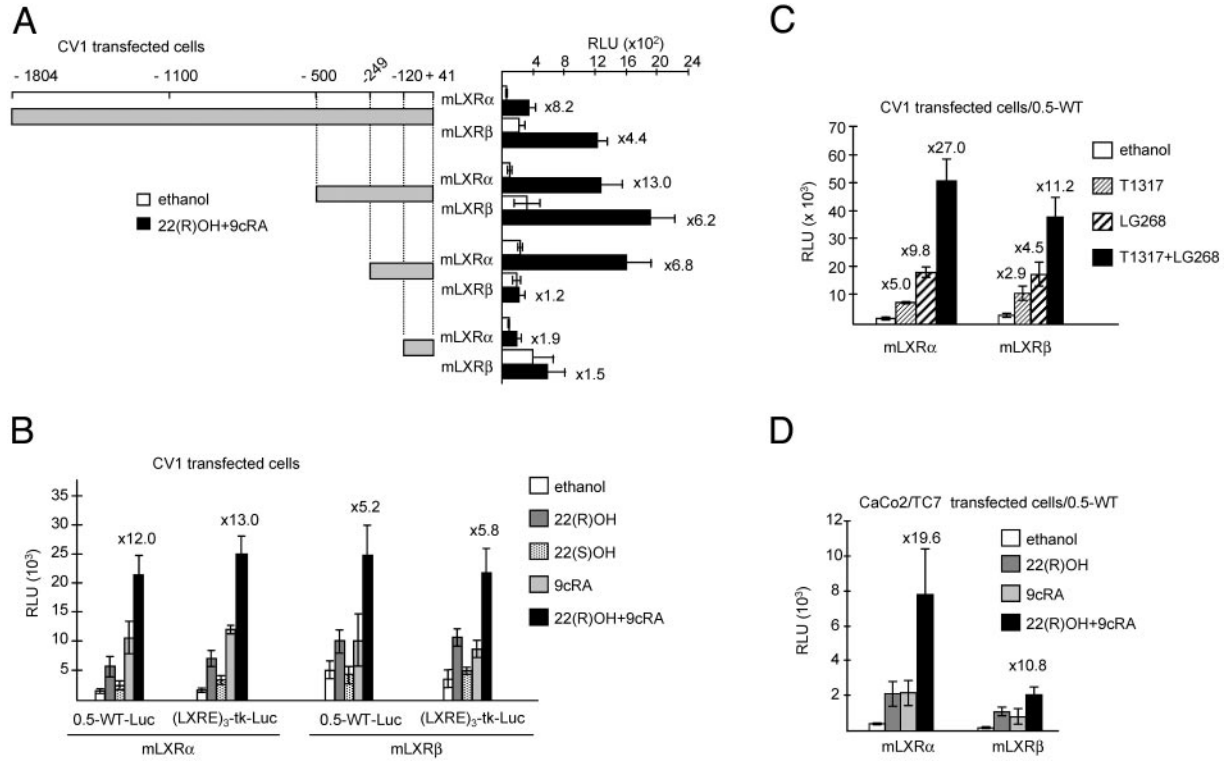

Fig. 2. Identification of Regulatory Regions Responsible for Oxysterol-Induced Expression of *akr1b7* through LXRs

A, Restriction fragments from *akr1b7* 5′-flanking region were cloned upstream from the luciferase reporter gene in pGL3 plasmid. CV1 cells were cotransfected with 200 ng *akr1b7*-luc constructs, 50 ng pCMX-mLXRα or pCMX-mLXRβ, and 50 ng of pCMX-mRXRα and completed to a final amount of DNA with 200 ng pCMX and 2 ng *Renilla* plasmid (for normalization). Twenty hours after transfection, cells were treated with vehicle (ethanol), 9cRA (1 μM), 22(R)OH (10 μM), 22(S)OH (10 μM), or 9cRA (1 μM) plus 22(R)OH (10 μM). Luciferase activity was normalized with the *Renilla* system. Values are means ± SEM of four to seven independent transfections in triplicate. *Numbers* indicate the positions from the initiation start site +1. B, The −510;+41 bp region of *akr1b7*-luc has been compared with $(LXRE)_3$tk-luc reporter gene, derived from the LXRE sequence of *cyp7a1*. CV1 cells were transfected and treated as described for panel A. Luciferase activity was normalized with the *Renilla* system (Yelen). Values are means ± SEM of at least four independent transfections in triplicate. C, CV1 transfections were performed as described above. Cells were treated with vehicle (ethanol), T1317 (1 μM), LG268 (1 μM), or T1317 (1 μM) plus LG268 (1 μM). Luciferase activity was normalized with the *Renilla* system. Values are means ± SEM of four independent transfections in triplicate. D, The −510;+41 bp region of *akr1b7*-luc was transfected in human intestinal CaCo2/clone 7 cells as described for CV1, treated with LXR and/or RXR ligands, and assayed for luciferase activity as above. Values are means ± SEM of at least four independent experiments in triplicate.

specific bands were present when liver nuclear extracts from *lxr*$\alpha;\beta^{-/-}$ mice were used. In contrast, nuclear extracts from wild-type and the single-knockout mice produced specific bands that contained LXR protein complexes bound to the LXRE probes. However, no differences in the intensity of the bands representing binding to the different LXREs was observed. Thus, LXRE1, -2, and -3 seemed to bind RXR/LXRβ with the same intensity as RXR/LXRα. These results suggest that the specific activity of LXRα on the 0.5-kb promoter of *akr1b7* promoter observed *in vivo* and in transient transfections might involve additional specific factors.

Functional Analysis of the Three LXREs

The *akr1b7* LXREs were tested for activity in transient transfections in CV-1 cells. Wild-type and mutant sequences used in these assays are reported in Table I. Mutations of either LXRE1 (m1) or LXRE2 (m2) affected induction of the luciferase activity by LXRα, but not LXRβ, when compared with the wild-type reporter gene (Fig. 4). Conversely, mutation of the LXRE3 site (m3) affected both LXRα and LXRβ action. This suggested that the three LXREs conferred LXRα responsiveness, whereas only LXRE3 seemed to be involved in *akr1b7* induction by LXRβ. These results were consistent with the data obtained with the −249/+41 bp construct, which contained only LXRE1 and LXRE2, and was only responsive to LXRα (Fig. 2A). Lastly, the m1m2m3 mutant was no longer responsive to 22(R)OH, whereas 9cRA was still able to induce the luciferase expression, confirming the results shown above (Fig. 1A) in which *akr1b7* expression continued to be responsive to RXR in the absence of both LXRα and LXRβ.

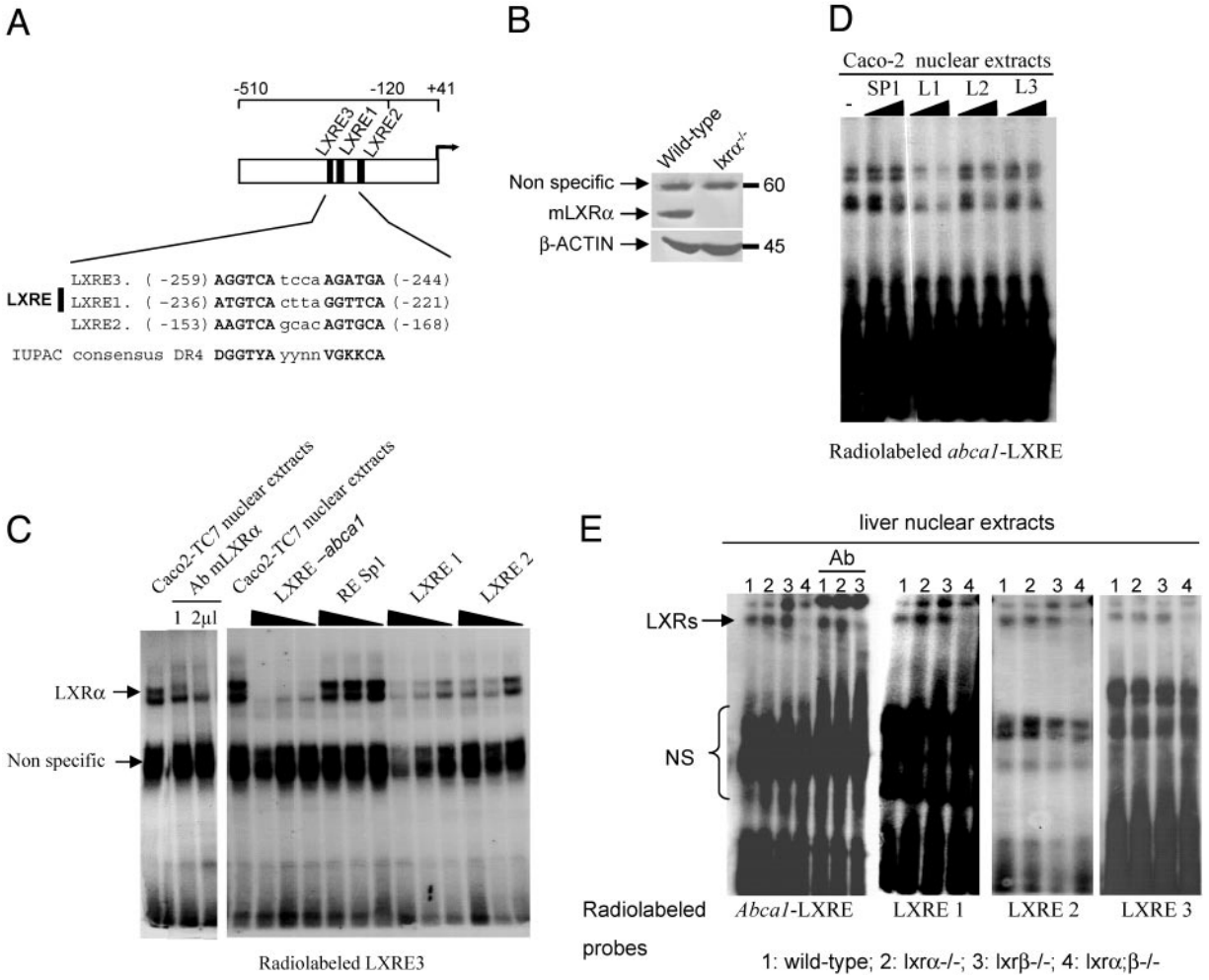

Fig. 3. Identification of Three Potential LXRs within the 5′-Flanking Region of *akr1b7* Gene

A, *Bold sequences* indicate the location of the hexanucleotide core elements of the putative LXREs compared with the IUPAC consensus sequence used for the screening. B, Western blots were performed with 30 μg of proteins from liver of wild-type and *lxr*$\alpha^{-/-}$ mice. C, EMSAs were performed as outlined in *Materials and Methods*. ^{32}P-labeled *akr1b7* LXRE3 oligonucleotide was incubated with 10 μg of nuclear extracts from CaCo2/TC7 cells. Competitions were performed using 100-, 50-, or 20-fold molar excess of unlabeled oligonucleotides for *abca1* LXRE, SP1 binding sequence, *akr1b7* LXRE1, or *akr1b7* LXRE2. Mouse antiserum against LXRα (2 μl) was used. D, ^{32}P-labeled *abca1* LXRE oligonucleotide was incubated with 10 μg of nuclear extracts from CaCo2/TC7 cells. Competitions were performed using 20 or 50 molar excess of unlabeled oligonucleotides for SP1 binding sequence, *akr1b7*-LXRE1 (L1), *akr1b7*-LXRE2 (L2), or *akr1b7*-LXRE3 (L3). E, ^{32}P-labeled *akr1b7* LXRE1, *akr1b7* LXRE2, *akr1b7* LXRE3, or *abca1* LXRE oligonucleotide was incubated with 5 μg of nuclear extracts of liver from *wild-type* (lanes 1), *lxr*$\alpha^{-/-}$ (lanes 2), *lxr*$\beta^{-/-}$ (lanes 3), or *lxr*$\alpha;\beta^{-/-}$ mice (lanes 4). Mouse antiserum against LXRα (1 μl) was used. IUPAC, International Union of Pure and Applied Chemistry.

Putative Involvement of LXR in Lipid Detoxification Processes

Because AKR1-B7 in adrenals has been associated with detoxification of isocaproaldehyde, and *in vitro* in the transformation of the cytotoxic 4-HNE to less toxic catabolites (26, 27), we proposed that intestinal AKR1-B7 could protect intestinal cells from toxic lipids. To date, no *akr1b7*-deficient mice have been reported to test this hypothesis; however, our data showing *akr1b7* is a target gene of LXRs led us to study whether these nuclear receptors could govern protection against lipid peroxidation. For that purpose we analyzed the status of lipid peroxidation in the various portions of the intestine by measuring malondialdehyde levels (a byproduct of polyunsaturated fatty acid peroxidation) in wild-type, *lxr*$\alpha^{-/-}$ and *lxr*$\beta^{-/-}$ mice fed T1317 (Fig. 5). In duodenum a significant decrease in the levels of malondialdehyde were seen when wild-type mice were fed T1317 compared with vehicle control ($P < 0.05$). The same effect was observed in the *lxr*$\beta^{-/-}$ mice, but not in the *lxr*$\alpha^{-/-}$ mice (Fig. 5A). This finding further supports the hypothesis that LXRα plays a predominant role in this process. Note that in *lxr*$\alpha^{-/-}$ mice the basal level of malondialdehyde was lower than in the wild-type mice ($P < 0.05$), probably due to the increased level of the basal expres-

Table 1. Sequences of Synthetic Oligonucleotides Used in EMSAs and Directed Mutagenesis

Oligonucleotides	Sequences	Positions
LXRE1	5′-CAACC**ATGTCA**CTTA**GGTTCA**CAAAC-3′	−236 to −221
m1-LXRE1	5′-CAACC**AT<u>A</u>T<u>A</u>A**CTTA**GGTCA**CAAAC-3′	
LXRE2	5′-GATTA**AAGTCA**GCAC**AGTGCA**AAGGC-3′	−153 to −168
m2-LXRE2	5′-GATTA**AAG<u>CA</u>A**GCAC**AGTGCA**AAGGG-3′	
LXRE3	5′-TTGAA**AGGTCA**TCCA**AGATGA**ACTGG-3′	−259 to −244
m3-LXRE3	5′-TTGAA**AGGTCA**TCCA**AGG<u>T</u>TG<u>G</u>**ACTGG-3′	
ABCA1	5′-CAGGCTTTGACCGATAGTAACCTCTGCGCT-3′	
SP1	5′-GGATAAAGGAGGCGGGCTCTTACC-3′	

The mutant LXREs failed to bind the RXR/LXR heterodimer in EMSAs (data not shown). *Bold letters* indicate the putative LXR and RXR binding sites. *Underlined letters* represent the mutations introduced in the respective oligonucleotides.

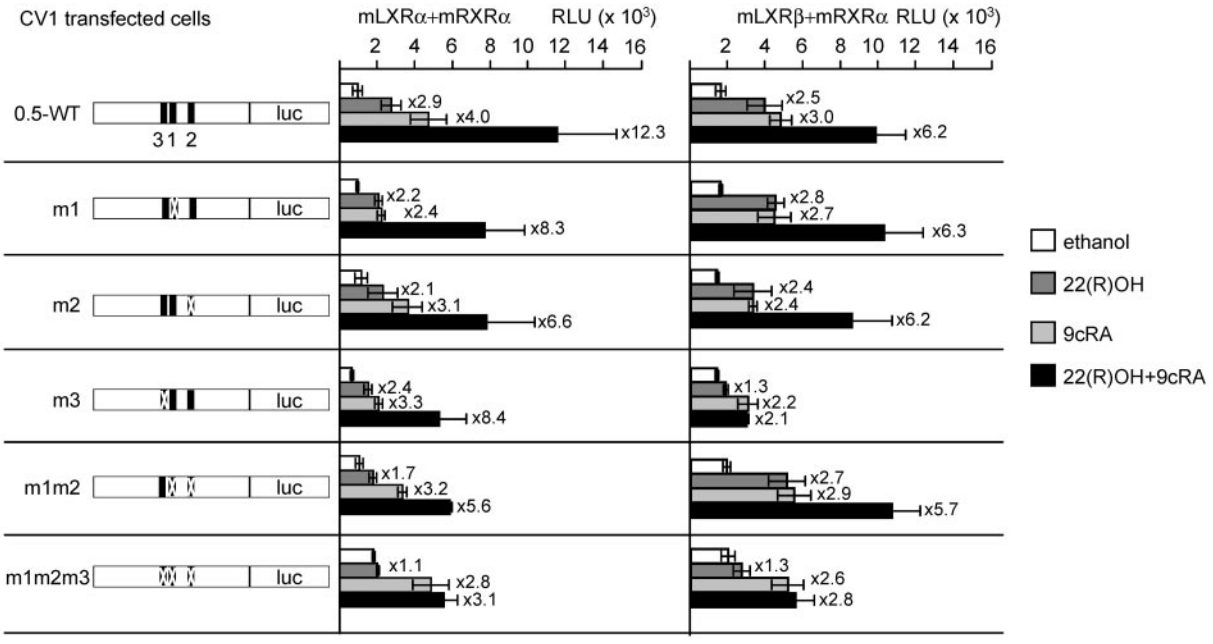

Fig. 4. Functional Characterization of LXREs in the *akr1b7* Promoter

Mutations in the region −510/+41 bp of *akr1b7* were generated by site-directed mutagenesis. CV1 cells were cotransfected with 200 ng of *akr1b7*-luc construct, with 50 ng of pCMX-mLXRα or pCMX-mLXRβ and 50 ng of pCMX-mRXRα and adjusted to a final amount of DNA with 500 ng of pCMX. Results were normalized with the *Renilla* system. Values are means ± SEM of at least four to seven independent transfections in triplicate.

sion of target genes such as *akr1b7*. Interestingly, in ileum T1317 had no effect on the malondialdehyde concentration regardless of the genotype used in the experiment (Fig. 5B). Altogether, these data suggested that LXRs may play a role in alleviating lipid peroxide accumulation in the proximal portion of the small bowel.

DISCUSSION

In this paper we have identified *akr1b7* as a new LXR target gene *in vivo*. The use of the LXRα and/or β knockout mice demonstrated that LXRα is the predominant regulator of *akr1b7* in the intestine. This conclusion was supported further by the finding that the *akr1b7* promoter contains three LXREs, two of which are selective for activation by LXRα/RXR heterodimers. As is usually observed for target genes of permissive RXR heterodimeric partners, *akr1b7* gene expression is responsive to either ligand of the heterodimer and exhibits a more than additive response when both ligands are present. As would be expected of a direct target gene, LXR ligand-dependent transcription of *akr1b7* is rapid; after 12 h of treatment with synthetic ligands, expression for *akr1b7* mRNA is sig-

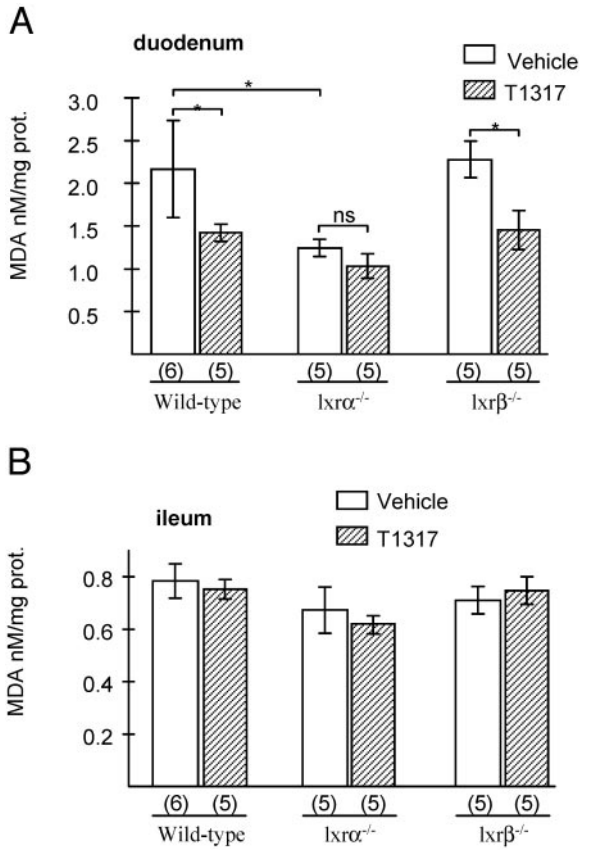

Fig. 5. Antioxidant Status of Duodenum and Ileum in Wild-Type, *lxr*$\alpha^{-/-}$, and *lxr*$\beta^{-/-}$ Mice

Malondialdehyde was determined in duodenum and ileum homogenates after lipid peroxidation induction. Measurements were performed on wild-type, *lxr*$\alpha^{-/-}$, and *lxr*$\beta^{-/-}$ mice fed T1317 for 12 h in duodenum (A) or ileum (B). All mice were 4 months old, and the number used for each measurement is indicated in *brackets*. Student's *t* test: *, $P < 0.05$.

nificantly increased in duodenum, and this is followed by a strong up-regulation of AKR1-B7 protein levels (3.1-fold, Fig. 1C).

Various factors have been shown to govern *akr1b7* expression including ACTH in adrenals (4) and androgens in vas deferens (1). In adrenals, AKR1-B7 is believed to be involved in the detoxification of isocaproic aldehyde, a highly toxic lipid byproduct of steroidogenesis (2). In intestine, especially in duodenum where lipid absorption is very high, our finding that *akr1b7* is regulated by LXRs would be consistent with an expanded role for this enzyme in intestinal lipid detoxification. In keeping with this hypothesis, we note that *akr1b7* has the same intestinal expression pattern (5) as other known LXR target genes such as *abca1*, *abcg5*, and *abcg8* (21, 23). Western blot analyses have revealed a decreasing gradient of *akr1b7* expression from duodenum to ileum (Fig. 1B). This expression pattern mirrors the gradient of cholesterol and lipid absorption (28) as well as the mRNA expression of LXR (Fig. 1D) along the small intestine.

Although the diet is an indispensable source of nutrient lipids, at high concentrations these lipids (especially cholesterol) become toxic to cells (7). Because of their increased exposure to high dietary concentrations of lipids, enterocytes have evolved several protective mechanisms to limit their exposure and uptake of lipids. One major class of lipid sensors that sits at the top of this protective pathway are the LXRs. For example, one way that LXRs regulate cholesterol levels is to accelerate cholesterol efflux out of the cell by transcriptional activation of genes encoding ATP-binding cassette proteins, such as ABCA1 or ABCG5/ABCG8 (21, 23). In addition to free cholesterol accumulation, lipid peroxidation represents another important source of toxic lipid byproducts in the intestine (29), and 4-HNE has been described as one such molecule (30). 4-HNE is a relatively stable, long-lived, and diffusible lipid that is generally considered to be cytotoxic (31). Several mechanisms (26, 27) have been developed to reduce 4-HNE to a less toxic compound, and among them AKR1-B7 has been shown to be effective *in vitro* (2). Given the importance of AKR1-B7 in other tissues, it is not surprising, therefore, that a regulatory cascade governing its expression has evolved in the intestine. In addition to the LXRs, it is of interest to note that other nuclear receptor lipid sensors may also be involved in modulating *akr1b7* transcription. Indeed, we demonstrated (Fig. 1A) that RXR agonists could still induce *akr1b7* expression in the absence of LXRs, suggesting that another RXR heterodimeric partner may regulate its expression as described for *abca1* (21).

The intriguing finding that malondialdehyde levels in duodenum of mice fed T1317 (Fig. 5A) or 2% cholesterol (data not shown) provided *in vivo* evidence suggesting that LXRs are involved in protection against lipid peroxidation. The low level of peroxidation observed in the *lxr*$\alpha^{-/-}$-deficient mice (Fig. 5A), as well as in the *lxr*$\alpha;\beta^{-/-}$ mice (data not shown), may be explained by the fact that in intestine of these animals there was an increase in the basal expression of the LXR target *akr1b7*. It has been shown that in the absence of ligands, RXR/LXR interacts with corepressors, such as nuclear receptor corepressor (32) and therefore, in the knockout mice, where this interaction is absent, the basal level of some target genes is increased. This phenomenon has previously been shown for *abca1* (21). Hence, the high expression level of *akr1b7*, *abca1*, and other LXR target genes in both *lxr*$\alpha^{-/-}$ and *lxr*$\alpha;\beta^{-/-}$ mice could result in greater protection against lipid peroxidation as observed in the knockout mice. This physiological role of LXRs in mice seems to be limited to the duodenum (Fig. 5B), where lipid absorption is highest.

Intestinal enterocytes have evolved an intricate nuclear receptor-mediated mechanism for protecting themselves from lipid overload (11). In the vasculature, polyunsaturated fatty acids (PUFAs) have been proposed to be involved in promoting atheromas, because they can serve as substrates for lipid

peroxidation (33). PUFAs and other oxidized lipids are known to bind and activate peroxisome proliferator-activated receptors (PPARs), which, in turn, increase expression of the sterol sensor, LXRα. Interestingly, 4-HNE has been shown to have a synergistic effect on the PPARγ ligand-dependent activity in erythrocytes (34). Even though such effects have not yet been demonstrated in enterocytes, we hypothesize the existence of a feed-forward regulatory loop in the intestine (Fig. 6), where one of the end products of lipid peroxidation, potentially 4-HNE, may act indirectly through PPARs to induce LXRα expression. Increased levels of LXRα and its activation by oxysterols would, in turn, up-regulate the expression of detoxifying genes such as *akr1b7*, and ultimately reduce oxidative stress.

In conclusion, the present work further expands the role of the LXRs as key regulators of lipid metabolism and detoxification. Although the human ortholog of *akr1b7* has not yet been studied, it is tempting to speculate that LXR agonists could be effective in reducing the deleterious effects of lipid peroxidation, a recognized risk factor for atherosclerosis (35, 36).

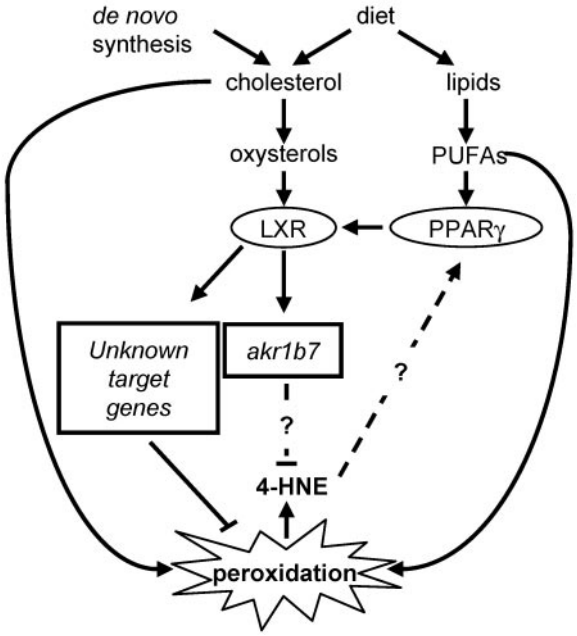

Fig. 6. Model for a Putative Role of LXRs in Detoxification of Lipid Peroxidation

Peroxidation could be initiated by PUFAs or cholesterol. One of the various toxic byproducts of peroxidation in cells is 4-HNE. Activation of RXR/LXR heterodimers has a protective effect against lipid peroxidation by increasing *akr1b7* expression to detoxify metabolites such as 4-HNE and/or through induction of unknown target genes. 4-HNE and PUFAs are known to synergistically activate PPARs, which may increase LXRα protein levels.

MATERIALS AND METHODS

Materials

22(R)OH, 22(S)OH, and 9cRA were purchased from Sigma-Aldrich (L'Isle D'Abeau, France). T0901317 (T1317) was a generous gift from Tularik (South San Francisco, CA) or purchased from Sigma-Aldrich. The natural ligands were diluted in ethanol. Plasmids used have been described elsewhere: pCMX-mLXRα, pCMX-mLXRβ, pCMX-mRXRα, pCMX (37).

Animal Studies

Lxr-knockout mice (*lxr*$\alpha^{-/-}$, *lxr*$\beta^{-/-}$, and *lxr*$\alpha;\beta^{-/-}$) (18, 21) were maintained on a mixed strain background (C57BL/6: 129Sv) and housed in a temperature-controlled room with 12-h light/12-h dark cycles. Mice were fed diets containing 0.02% cholesterol plus vehicle, 30 mg/kg (mpk) LG268 or 50 mpk T1317 for 12 h or 10 d; some animals have been gavaged with vehicle or 50 mpk T1317 for 12 h. After the dietary treatment, mice were anesthetized with 200 μl of avertin (0.02 mg/ml) or nembutal and exsanguinated before organ harvest. The small intestine was divided in three parts of equal lengths (duodenum, jejunum, and ileum), and the mucosa were gently scraped as previously described (21). Polyclonal mLXRα antibodies were generated by immunization of rabbits with a peptide comprising the 72 N-terminal amino acids of mLXRα fused to glutathione-*S*-transferase. Individually housed rabbits were injected sc with the recombinant protein and then reinjected 21 d later. After 2 wk, animals were anesthesized before exsanguination. After an overnight coagulation at 4 C, blood was centrifuged at 5000 rpm for 30 min, and the sera were collected, aliquoted, and stored at −80 C. Immunoprecipitation experiments (data not shown) and Western blot analyses (Fig. 3B) have demonstrated that the antibodies specifically recognize mLXRα. All the animals were maintained and handled according to the recommendations of the University Ethics Committee (Université Blaise Pascal) and the Institutional Animal Care and Research Advisory Committee (University of Texas Southwestern Medical Center).

RNA Measurements

Quantitative Real-Time PCR

Total RNA was isolated using RNAStat60 (Tel-Test, Inc.), treated with DNAse I (Roche Molecular Biochemicals, Indianapolis, IN) and reverse transcribed with random hexamers using the Superscript II First Strand Synthesis System (Invitrogen, San Diego, CA). Real-time PCR was performed on an ABI Prism 7900 HT system (ABI Advanced Technologies, Inc., Columbia, MD) as previously described (23) using 25 ng of template cDNA for each reaction. The following primers were designed to span exon junctions and specifically detect *akr1b7* (NM_009731) or cyclophilin (NM_011149), which was used as the internal control: *Akr1b7*-F, 5′-ccctcacgcatacaggagaa-3′ (819–838); *akr1b7*-R: 5′-gccatgtcctcctcactcaa-3′ (861–880); cyclophilin-F, 5′-ggagatggcacaggaggaa-3′ (470–488); cyclophilin-R, 5′-gcccgtagtgcttcagctt-3′ (527–545).

Northern Blot Analysis

Total RNA from four to six mice per treatment were pooled and mRNA was purified using oligo-dT cellulose columns (Amersham Pharmacia Biotech, Arlington Heights, IL). Poly(A+) RNA (5 μg) was run on a formaldehyde-denaturing agarose gel, transferred to membrane, and hybridized with ^{32}P-labeled cDNA probes for *akr1b7* or *β-actin* as previously described (21, 23). Results were quantified by phosphor imager and graphed relative to *β-actin* expression.

Western Blot Analysis

Tissue cellular extracts of the three intestinal portions (40 μg) were subjected to SDS-PAGE and transferred to nitrocellulose membrane (Amersham Pharmacia Biotech). Membranes were incubated overnight at 4 C with primary polyclonal antibodies raised against either AKR1-B7 (1:3,000), mLXRα(1:10,000), or actin (1:2,000), followed by a 1 h incubation with a peroxidase-conjugated antirabbit IgG (Roche Diagnostics, Meylan, France) at 1:10,000. Peroxidase activity was detected with the Western Lightning System (Perkin-Elmer Life Sciences, Courtaboeuf, France).

Cell Culture and Transfection Assays

CV-1 cells were maintained at 37 C in an atmosphere of 5% CO_2 DMEM containing 100 U/ml penicillin and 100 μg/ml streptomycin supplemented with 5% fetal calf serum. On d 0, CV-1 cells were seeded at 50×10^3 cells per well in 24-well plates and allowed to adhere overnight. The following day, cells were washed and transfected, in serum-free medium, with 500 ng/well of cDNA (reporter gene, 200 ng; pCMX-mLXRα or -β, 50 ng; pCMX-mRXR, 50 ng; pCMX, 200 ng) using Exgen500 (Euromedex, Mundolsheim, France). On d 2, cells were washed twice with 1$\times$ PBS, and the medium without fetal calf serum was applied with the various ligands or vehicles. 22(R)OH (10^{-5} M), 22(S)OH (10^{-5} M), 9cRA (10^{-6} M), T1317 (10^{-6} M), and LG268 (10^{-6} M) were added to cells as 1000$\times$ stock solutions in ethanol. After 24 h, cells were harvested for luciferase activity using commercial kits (Yelen, Ensues-la-Redonne, France). The *Renilla* system (Yelen) was used to normalize all the experiments, as recommended by the manufacturer.

The intestinal cell line, CaCo2-clone TC7 (38, 39) (Dr. M. Rousset, Institut National de la Santé et de la Recherche Médicale, Unité 170, Villejuif, France) was maintained at 37 C in an atmosphere of 5% CO_2 in DMEM containing 100 U/ml penicillin and 100 μg/ml streptomycin supplemented with 20% fetal calf serum. Transfections were performed in the same manner as described for CV-1 cells.

Identification of the Putative LXREs

Alignment of various identified LXREs within the target genes described in mouse (ApoE-ME1, ABCA1, SREBP-1c, CYP7A1, human cholesterol ester transfer protein, and chicken ACCα) allowed us to determine a consensus direct-repeat separated by four nucleotides (cDR4). The IUPAC (International Union of Pure and Applied Chemistry) consensus sequence (**DGGTYA**yynn**VGKKCA**) was searched within the 0.5-kb fragment upstream of the transcription start site. Three putative DR4 were found: LXRE1 (87.5% of identity with the cDR4), LXRE2 (93.7% of identity), and LXRE3 (87.5% of identity).

EMSA

EMSAs were performed as previously described (40). Sequences of *abca1*-LXRE, *akr1b7*-LXRE 1, -2, and -3, and SP1 are shown in Table 1. Experiments were done with nuclear extracts from CaCo2/TC7 proteins (10 μg) or from livers (5 μg) using the appropriate labeled probe. The specificity of binding by RXR-LXR was tested by competition with $\times$100, $\times$50, and $\times$20 excess of various unlabeled LXREs or Sp1, or by supershift using a LXRα-specific antibody. After electrophoresis, the gel was dried at 80 C for 1 h and autoradiographed with intensifying screen at $-$80 C overnight.

Antioxidant Status of the Various Portions of the Intestine

For lipid peroxidation, homogenates were prepared on ice using a ratio of 1 g wet tissue to 9 ml KCl (150 mmol/liter) using a Polytron homogenizer. Malondialdehyde as thiobarbituric acid-reactive substances) were measured, using a spectrophotometer (Uvikon 941 plus series, Kontron Instruments, St Quentin en Yvelines, France), in duodenum and ileum homogenates (41) after lipid peroxidation induced by $FeSO_4$ (2 μmol/liter)-ascorbate (50 μmol/liter) for 30 min in a 37 C water bath in an oxygen-free medium, using a standard of 1,1,3,3-tetraethoxypropane as previously described (42).

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