



Liver X receptors regulate adrenal cholesterol balance

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Cholesterol is the obligate precursor to adrenal steroids but is cytotoxic at high concentrations. Here, we show the role of the liver X receptors (LXR α and LXR β) in preventing accumulation of free cholesterol in mouse adrenal glands by controlling expression of genes involved in all aspects of cholesterol utilization, including the steroidogenic acute regulatory protein, StAR, a novel LXR target. Under chronic dietary stress, adrenal glands from *Lxr α β ^{-/-}* mice accumulated free cholesterol. In contrast, wild-type animals maintained cholesterol homeostasis through basal expression of genes involved in cholesterol efflux and storage (ABC transporter A1 [ABCA1], apoE, SREBP-1c) while preventing steroidogenic gene (StAR) expression. Upon treatment with an LXR agonist that mimics activation by oxysterols, expression of these target genes was increased. Basally, *Lxr α β ^{-/-}* mice exhibited a marked decrease in ABCA1 and a derepression of StAR expression, causing a net decrease in cholesterol efflux and an increase in steroidogenesis. These changes occurred under conditions that prevented the acute stress response and resulted in a phenotype more specific to the loss of LXR α , including hypercorticosteronemia, cholesterol ester accumulation, and adrenomegaly. These results imply LXR α provides a safety valve to limit free cholesterol levels as a basal protective mechanism in the adrenal gland, where cholesterol is under constant flux.

Introduction

The adrenal cortex is responsible for synthesizing glucocorticoid hormones that are essential for survival under stress. This endocrine pathway is acutely regulated by the hypothalamic-pituitary-adrenal axis in response to stress through the release of ACTH from the anterior pituitary. ACTH signals the adrenal gland to increase the expression of a cascade of enzymes required for the conversion of cholesterol into biologically active glucocorticoids. The initial and rate-limiting step in this cascade is mediated by the steroidogenic acute regulatory protein (StAR) that transfers cholesterol from the outer to the inner mitochondrial membrane (1, 2). Inside the mitochondria, cytochrome P450 11A1 (CYP11A1) cleaves the cholesterol side chain to form pregnenolone (3), which can be further converted by a series of enzymes (e.g., type I β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 -isomerase) to all steroid hormones produced by the adrenal cortex. Because the stress response is intended to be of limited duration, tight regulation of this system is maintained by the negative feedback of circulating glucocorticoids on the hypothalamus and pituitary that decreases ACTH secretion and ultimately turn off glucocorticoid production (4, 5).

Nonstandard abbreviations used: ABCA1, ABC transporter A1; ACAT, acyl-CoA:cholesterol acyltransferase; ChIP, chromatin IP; CYP11A1, cytochrome P450 11A1; DEX, dexamethasone; HPA, hypothalamic-pituitary-adrenal; HSD3B1, hydroxysteroid dehydrogenase 3B1; HSL, hormone-sensitive lipase; ITS, insulin-transferrin-sodium selenite; LXR, liver X receptor; LXRE, LXR response element; m, mouse; QPCR, real-time quantitative RT-PCR; RXR, retinoid X receptor; SF-1, steroidogenic factor-1; SHP, small heterodimer partner; SR-B1, scavenger receptor-B1; StAR, steroidogenic acute regulatory protein; T1317, LXR agonist T0901317; TK, thymidine kinase.

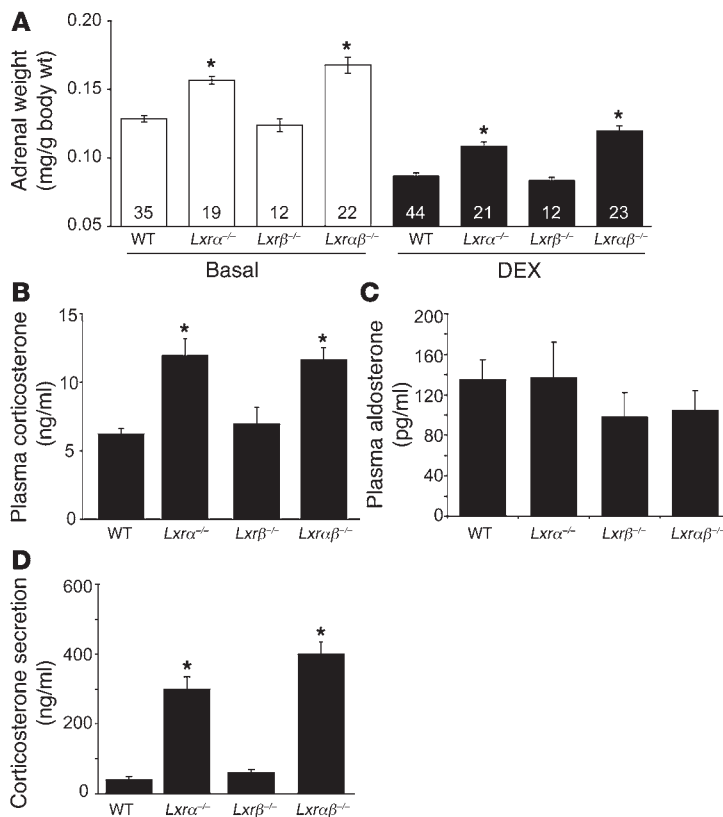
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Cholesterol is the precursor to all steroid hormones, and therefore a constant supply must be available to the adrenal gland. There are 3 ways of obtaining adrenal cholesterol for steroidogenesis: (a) lipoprotein-derived uptake, (b) hydrolysis of intracellular cholesterol esters, and (c) de novo synthesis. Of the 3 methods, lipoprotein-derived uptake is the most important, accounting for more than 80% of adrenal cholesterol (6, 7). In mice, adrenal cholesterol is obtained mainly from circulating HDL that is taken up via the interaction of apoA1 with scavenger receptor-B1 (SR-B1) in the selective uptake pathway (8, 9).

The direction of cholesterol flux in the adrenal gland depends upon the state of the organism. In the basal or resting state, cholesterol storage and efflux predominate. During an acute stress response, the immediate need for cholesterol substrate in the mitochondria is accomplished by the rapid mobilization of intracellular cholesterol stores. Chronic stress, environmental or dietary, results in the sustained import of cholesterol into the cell and mitochondria. Eventually, at the end of the stress response, the flux of adrenal cholesterol must be switched back to storage and efflux. The concentration of free cholesterol is tightly regulated due to its toxic effects at high levels. Basal storage of cholesterol in the form of cholesterol esters is maintained primarily through the action of acyl-CoA:cholesterol acyltransferase (ACAT) (10). Chemical inhibition of ACAT has been shown to cause cellular toxicity through the accumulation of free cholesterol (11). The ability of the adrenal gland to handle large changes in cholesterol flux is a feature that is also common to the liver. Although the liver is controlled by different input signals, parallel pathways of cholesterol utilization are present, including cholesterol storage, efflux, and metabolism (to bile acids).

Regulation of hepatic cholesterol homeostasis has previously been shown to be mediated by the liver X receptors (LXR α and

**Figure 1**

Adrenomegaly and primary hypercorticosteronemia are LXR α specific. (A) Adrenal pairs were weighed and normalized for body weight. Where indicated, 75 μ g DEX was administered twice a day for 5 days. Number of animals per group is indicated in bars. (B) Plasma corticosterone from wild-type and various *Lxr*^{-/-} mice ($n = 10$, except *Lxrβ*^{-/-}, where $n = 5$). (C) Plasma aldosterone from wild-type and various *Lxr*^{-/-} mice ($n = 8-9$). (D) Corticosterone secreted from supernatant of wild-type and various *Lxr*^{-/-} primary adrenal cells after 3 days in culture ($n = 4$). Data represent the mean \pm SEM. * $P < 0.05$, significantly different between genotypes for basal and DEX treated.

revealed adrenomegaly, with 41% heavier adrenal glands in *Lxrαβ*^{-/-} mice than in age-matched wild-type mice ($P < 0.05$) when corrected for body weight (Figure 1A). Adrenomegaly was specific to the loss of LXR α (35% increase in adrenal weight/body weight in *Lxrα*^{-/-} versus wild-type, $P < 0.05$) since adrenal glands of LXR β -deficient mice appeared normal. All genotypes exhibited adrenal atrophy upon treatment with dexamethasone (DEX) (Figure 1A), indicating that the central negative feedback through the hypothalamic-pituitary-adrenal axis was intact in mice lacking LXRs. Furthermore, in 2 models of acute stress (induced by repeated ACTH injections or 30-minute restraint stress), we observed no differences in either corticosterone or ACTH secretion between wild-type and *Lxrαβ*^{-/-} mice (data not shown), indicating no defect in the acute stress response.

Plasma corticosterone levels were measured in the LXR-deficient animals and found to be 2-fold higher ($P < 0.05$) in the *Lxrα*^{-/-} and *Lxrαβ*^{-/-} mice compared with wild-type and *Lxrβ*^{-/-} mice (Figure 1B), in agreement with similar findings in *Lxrαβ*^{-/-} mice (23). No difference in circulating aldosterone was observed, demonstrating a specific effect on glucocorticoids (Figure 1C). ACTH levels were measured to determine if both the adrenomegaly and corticosterone increases found in the LXR α -null mice could be explained by the trophic effects of this hormone. However, plasma ACTH levels were unchanged across all genotypes (wild-type, 61 ± 4 pg/ml, $n = 11$; *Lxrα*^{-/-}, 53 ± 3 pg/ml, $n = 5$; *Lxrβ*^{-/-}, 60 ± 9 pg/ml, $n = 7$; *Lxrαβ*^{-/-}, 67 ± 5 pg/ml, $n = 9$), suggesting no dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis. To further ensure that the elevated basal corticosterone seen in the LXR α -null mice was independent of any central defect, the secretion of corticosterone was measured in primary adrenal cultures (Figure 1D). Corticosterone concentrations in the media were 7.5-fold higher from *Lxrα*^{-/-} adrenal cells and 10-fold higher from *Lxrαβ*^{-/-} adrenal cells compared with wild-type and *Lxrβ*^{-/-} adrenal cells, supporting the primary role of the adrenal gland in the hypersecretion of corticosterone.

LXRα-null mice have elevated adrenal cholesterol esters. Since LXRs are known to play prominent roles in governing lipid metabolism in other tissues, we examined whether there were any differences in adrenal lipid content between wild-type and LXR-null mice. Histological analysis using oil red O staining demonstrated that LXR α -null mice had increased numbers of vacuoles that were associated with neutral lipids (Figure 2A). The increase in the number and size of the lipid-laden vacuoles was most notable in the *Lxrαβ*^{-/-} mice. Since *Lxrβ*^{-/-} mice appeared to have only a small increase in the accumulation of adrenal lipids compared with *Lxrα*^{-/-} mice,

LXR β) (12). The LXRs are members of the nuclear hormone receptor superfamily and are activated by oxysterols, which are endogenous metabolites of cholesterol (13). These receptors form obligate heterodimers with retinoid X receptors (RXRs) to govern gene transcription (14). LXR target genes include ABC transporters (ABCA1, ABCG1, and ABCG5/ABCG8) (15–18), SREBP-1c (17), apoE (19), and cholesterol 7 α -hydroxylase (CYP7A1), the rate-limiting step in the catabolism of hepatic cholesterol to bile acids (20, 21). The adrenal gland expresses abundant quantities of both LXR α and LXR β (12) as well as the known LXR agonist 22(R)-OH cholesterol, an intermediate in the conversion of cholesterol to pregnenolone (22). Previous work has shown that *Lxrαβ*^{-/-} mice have elevated corticosterone levels and, paradoxically, that long-term administration of an LXR agonist also increases plasma corticosterone in wild-type mice (23). However, despite microarray analysis, no molecular mechanism or physiologic explanation has been identified that could explain these findings.

In this paper, we demonstrate that, similarly to its role in other tissues, LXR α functions in the adrenal gland to limit the concentration of free cholesterol by coordinately regulating expression of genes involved in cholesterol efflux (ABCA1), storage (apoE, SREBP-1c), and metabolism to steroids (StAR). In doing so, the adrenal gland is able to maintain appropriate compartmentalization of cholesterol under the dynamic range of conditions that exist in this organ during the resting state and under chronic dietary stress.

Results

Loss of LXR α results in adrenomegaly and hypercorticosteronemia. Gross morphological examination of adrenal glands from *Lxrαβ*^{-/-} mice

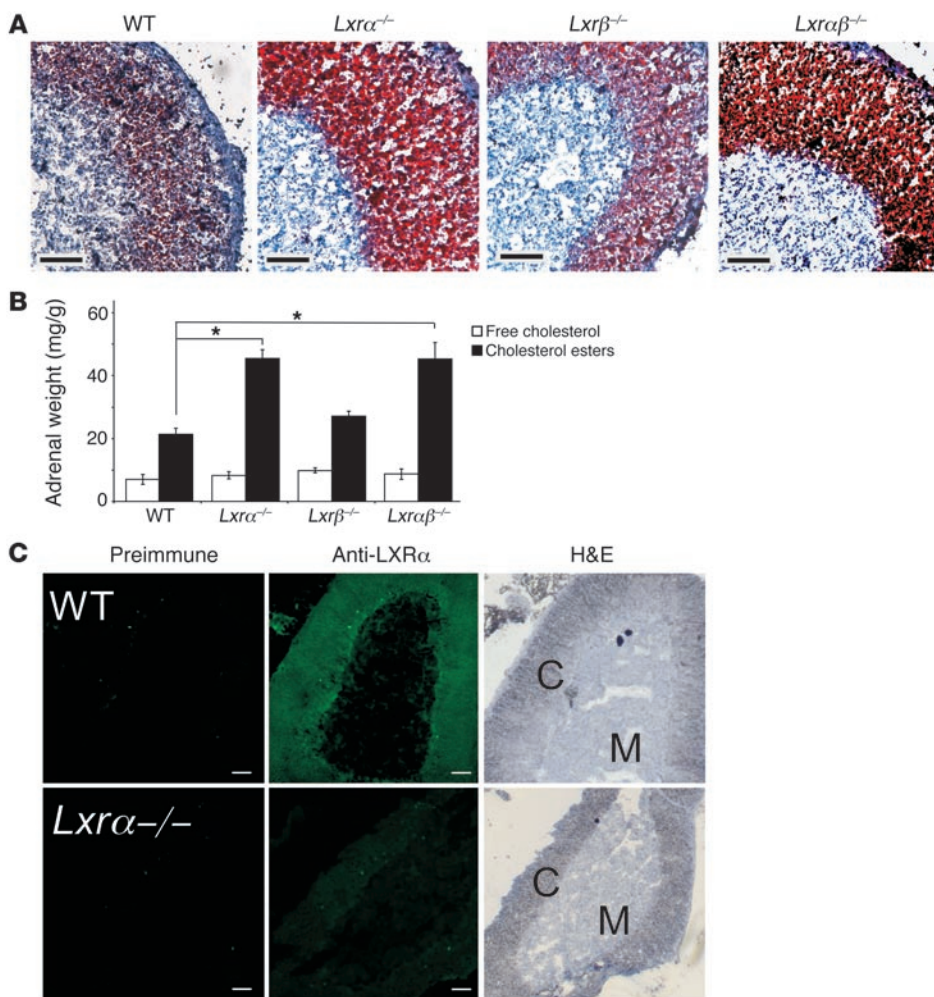


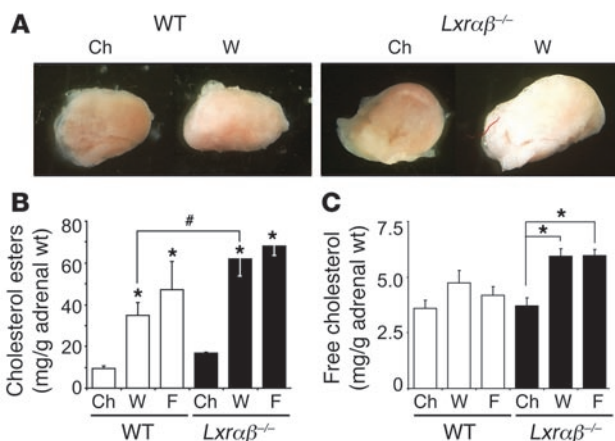
Figure 2 Analysis of lipid content and expression of LXR α in adrenal glands of wild-type and LXR knockout mice. (A) Oil red O staining of neutral lipids from cryosections of male adrenal glands. (B) Adrenal lipid content ($n = 4$). Data represent the mean \pm SEM. * $P < 0.05$, significantly different from wild type. (C) Immunohistochemistry of the adrenal cortex using an LXR α -specific antibody. Counterstaining was performed using H&E. C, cortex; M, medulla. Scale bars: 100 μ m.

we concluded the phenotype was due to the absence of LXR α . No significant difference in plasma cholesterol was observed between the genotypes; however, plasma triglycerides were lower in *Lxrα*^{-/-} and *Lxrαβ*^{-/-} mice (data not shown) in agreement with previously published reports (21, 25). Analysis of adrenal gland lipid extracts found that only the fraction containing cholesterol esters was significantly increased after normalizing to adrenal weight (2.1-fold, $P < 0.05$), and again this was restricted to the *Lxrα*^{-/-} and *Lxrαβ*^{-/-} mice (Figure 2B). No differences were observed between genotypes in adrenal phospholipid content (data not shown). Together, these data suggest the increase in the oil red O staining observed in the *Lxrα*^{-/-} and *Lxrαβ*^{-/-} adrenal glands was due to the accumulation of cholesterol esters. Since there was no upregulation of the trophic hormone ACTH and no histological evidence of adrenal hyperplasia, we conclude that the adrenomegaly observed in LXR α -null mice was primarily due to the accumulation of cholesterol esters.

The results described above suggested that LXR-dependent changes in adrenal cholesterol homeostasis were driven primarily by LXR α . The presence of LXR α in mouse adrenal glands was confirmed by immunohistochemistry using an LXR α -specific antibody (26). Strong staining was observed throughout the adrenal cortex but not the medulla of the wild-type mice (Figure 2C). However, the resolution on the cryosections prevented us from determining if there was specific staining in the narrow outer glo-

merulosa layer. The specificity of the LXR α antibody was verified by the absence of signal in the *Lxrα*^{-/-} adrenal sections and the negligible binding observed using preimmune serum.

LXR is critical for cholesterol homeostasis under chronic dietary stress. Previous work has demonstrated the importance of LXRs in governing lipid metabolism in response to dietary stress (21, 27). Since increased dietary fat intake is a known model for chronic stress (i.e., development of elevated basal corticosterone and an enhanced HPA response to stress; ref. 28), we next tested the effect of a Western (high-fat/high-cholesterol) diet on adrenal function in LXR-null animals. After 7 weeks on a Western diet, *Lxrαβ*^{-/-} mice developed severely blanched adrenal glands with a tendency toward increased size relative to wild-type mice (Figure 3A). Analysis of the lipid content from Folch-extracted adrenal glands found that mice fed either high-fat/high-cholesterol or high-fat only diets had significantly elevated cholesterol esters relative to chow-fed mice (Figure 3B). However, *Lxrαβ*^{-/-} mice accumulated more cholesterol esters than their wild-type counterparts on the Western diet, a trend that was also seen on the high-fat only diet. Intriguingly, free cholesterol was also increased specifically in the *Lxrαβ*^{-/-} mice fed either diet, suggesting a crucial role for LXR in maintaining free cholesterol levels in the adrenal gland (Figure 3C). No differences were observed in adrenal triglyceride levels with the various diets (data not shown).

**Figure 3**

Role of LXRs in cholesterol homeostasis under chronic dietary stress. (A) Gross appearance of adrenal glands removed from mice fed a chow (Ch) or Western (W) diet for 7 weeks. Cholesterol ester (B) and free cholesterol (C) quantitation from mice fed a Western diet or a high-fat (F) diet for 7 weeks. Individual adrenal pairs were processed for extraction. Data shown represent the mean \pm SEM ($n = 4$). * $P < 0.05$, significantly different from chow for that genotype; # $P < 0.05$, significantly different between genotypes.

LXR-dependent expression of genes controlling adrenal cholesterol homeostasis. To explore the underlying mechanisms that might account for free cholesterol accumulation in chronically challenged *Lxraβ^{-/-}* mice and the cholesterol ester accumulation, adrenomegaly, and hypercorticosteronemia observed in *LXRα*-null mice, gene expression was examined by real-time quantitative RT-PCR (QPCR) from adrenal glands of wild-type and *Lxraβ^{-/-}* animals treated with the potent synthetic LXR agonist T0901317 (T1317) (Figure 4A). *Lxraβ^{-/-}* mice were used as negative controls because they are refractory to treatment with the LXR agonist and they exhibit an adrenal phenotype identical to that of *Lxra^{-/-}* mice. *LXRα* itself was increased significantly by T1317 (1.7-fold, $P < 0.05$), indicating that the autoregulatory loop previously described for *LXRα* in adipose tissue is also present in the adrenal gland (29). Expression of *LXRβ* and *RXRα*, the predominant *RXR* heterodimeric partner expressed in adrenal gland, remained unchanged (Figure 4A and data not shown). Likewise, no differences were observed in the expression of the orphan receptor steroidogenic factor-1 (SF-1), which is required for expression of numerous genes involved in steroidogenesis (30), or the small heterodimer partner (SHP), which has been shown to repress selective LXR and farnesoid X receptor (FXR) target genes in the liver (31).

As expected, expression of several LXR target genes involved in lipid homeostasis was induced by T1317 treatment in wild-type but not *Lxraβ^{-/-}* adrenal glands. These included genes encoding the cholesterol efflux transporters, *ABCA1* (4.3-fold over vehicle-treated wild-type, $P < 0.05$) and *ABCG1* (4.4-fold, $P < 0.05$); the master regulator of fatty acid metabolism, *SREBP-1c* (5.7-fold, $P < 0.05$); and the lipoprotein apoE (2.4-fold, $P < 0.05$). In addition, a reproducible decrease in the basal mRNA levels of *ABCA1*, apoE, and *SREBP-1c* were observed in the *Lxraβ^{-/-}* mice. This correlated with a marked decrease in *ABCA1* protein in *Lxraβ^{-/-}* mice (2.5-fold lower than in wild-type mice; Figure 4B). These results are consistent with the conclusion that loss of LXR-dependent regu-

lation of cholesterol efflux may be sufficient to cause cholesterol accumulation and adrenomegaly over time.

In mice, adrenal sterols are mainly obtained via selective uptake involving SR-B1 and, to a lesser extent, endogenous adrenal synthesis (7). However, no changes in expression of either SR-B1 or HMGCoA reductase were observed in response to agonist or in *Lxraβ^{-/-}* mice (Figure 4A). Likewise, no significant changes were seen in expression of mRNA for hormone-sensitive lipase (HSL) or ACAT, the main enzymes responsible for hydrolyzing and synthesizing cholesterol esters, respectively. These results support the idea that the buildup of cholesterol esters in the *Lxraβ^{-/-}* mice was not due to increased uptake, increased synthesis, or decreased hydrolysis, but instead to decreased efflux, resulting in more substrate available for esterification.

LXR-dependent expression of genes controlling adrenal steroidogenesis. In most animal models, an increase in adrenal cholesterol ester depots signifies a shift from synthesis of steroid hormones toward the storage of cholesterol. However, in the *Lxraβ^{-/-}* model, we found that the increase in intracellular cholesterol esters was accompanied by an increase in corticosterone levels. To help explain this unusual phenotype, we measured the expression of genes involved in the steroidogenic pathway, including *StAR*, *CYP11A1*, and type I 3β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 -isomerase (HSD3B1). Initially, no differences in the expression of *StAR*, *CYP11A1*, and HSD3B1 in wild-type and *Lxraβ^{-/-}* mice could be observed under basal conditions (data not shown). However, in most species, including mice, the regulation of this pathway is extraordinarily sensitive to stress even under the best experimental conditions. Therefore, to limit the experimental variability caused by the fear response and show that this regulation is independent of the acute stress response, steroidogenic enzymes were measured in mice that were pretreated with DEX to suppress the hypothalamic-pituitary pathway. Under these conditions, QPCR analysis of wild-type and *Lxraβ^{-/-}* mice revealed significant differences (2-fold relative to wild-type) in the levels of the 3 steroidogenic enzymes, *StAR*, *CYP11A1*, and HSD3B1 (Figure 4C). Importantly, there was also a notable trend in increased expression of all 3 genes (*StAR*, 1.7-fold; *CYP11A1*, 1.5-fold, $P < 0.05$; HSD3B1, 1.6-fold) upon T1317 treatment in wild-type mice that was not observed in *Lxraβ^{-/-}* mice treated with T1317 (Figure 4C). The paradoxical increase in expression of these genes that was observed basally in *Lxraβ^{-/-}* mice and after T1317 treatment in wild-type mice is in agreement with the finding that loss of LXRs results in derepression of certain target genes (32). No elevation in ACTH receptor (MC2R) was observed in our studies (data not shown), consistent with the conclusion that LXR regulates expression of steroidogenic genes independently of an increase in ACTH receptor-mediated signaling.

Western blot analysis confirmed that *StAR* protein was also increased 3.3-fold basally in *Lxraβ^{-/-}* mice versus wild-type and 3.7-fold in wild-type mice treated with T1317 (Figure 4D). Consistent with the observed changes in steroidogenic enzyme gene expression, plasma corticosterone levels were increased significantly in the T1317-treated wild-type versus *LXR*-null mice and in untreated *Lxra^{-/-}* and *Lxraβ^{-/-}* versus wild-type mice. Corticosterone levels were markedly reduced in all genotypes upon treatment with DEX (compare y axis in Figures 4E and 1B), supporting the presence of an intact hypothalamic-pituitary axis in *LXR*-null mice.

LXR acts directly on mouse and human adrenal cells. To confirm that LXR-dependent effects on adrenal function were at the level of the

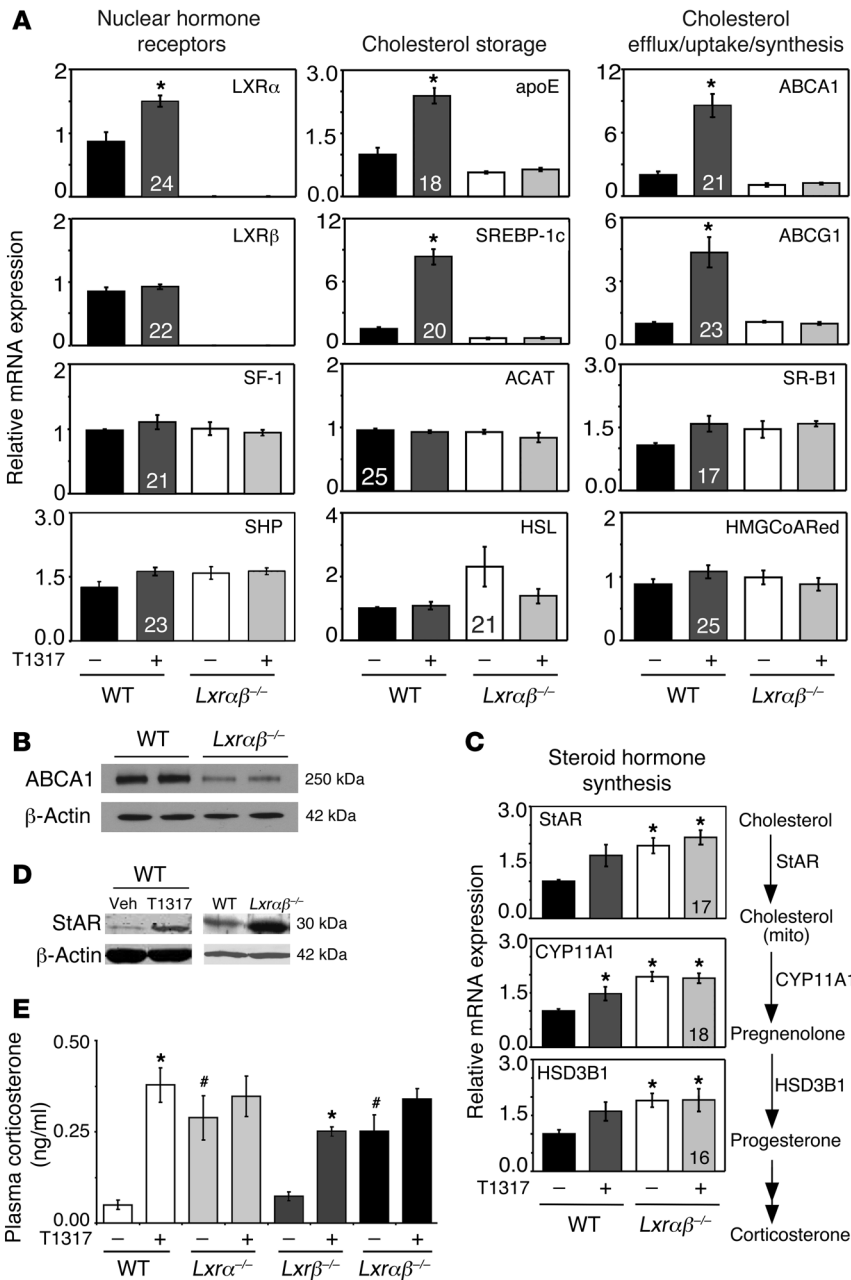


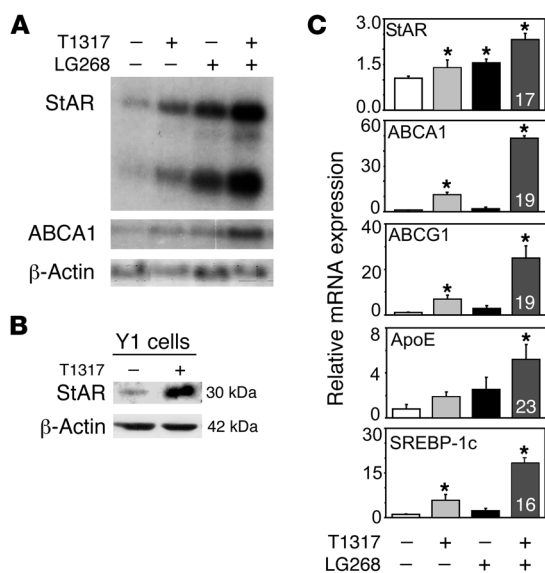
Figure 4

LXR-dependent regulation of genes involved in cholesterol homeostasis and steroidogenesis. Expression of genes involved in (A) cholesterol homeostasis ($n = 3-6$) and (C) steroid hormone synthesis ($n = 4-6$). For C, mice were injected twice a day for 5 days with 75 μg DEX to suppress acute stress-induced responses. Animals were treated with or without LXR agonist (T1317, 50 mpk) for 12 hours, and RNA was prepared from individual adrenal pairs and subjected to QPCR analysis. mRNA values were normalized to cyclophilin and plotted relative to wild-type, untreated controls. Cycle times for the highest-expressing group for each gene are shown in their corresponding bar. For comparison, cyclophilin cycle time = 19. * $P < 0.05$, significantly different from wild-type vehicle treated. (B) Western blot of ABCA1 from adrenal membrane lysates (40 μg) obtained from wild-type and *Lxra* β ^{-/-} mice under basal conditions. (D) Western blots of StAR in adrenal lysates (20 μg) from DEX-treated wild-type and *Lxra* β ^{-/-} mice (2 adrenal glands pooled per condition, $n = 3$). Samples were normalized to β -actin and fold induction calculated relative to wild type. (E) Corticosterone levels from lipid extracts of plasma from DEX-treated wild-type, and various *Lxr*^{-/-} mice with and without T1317 treatment ($n = 4-5$). Data represent the mean \pm SEM. * $P < 0.05$, significantly different from vehicle treated for that genotype. # $P < 0.05$, significantly different from wild-type vehicle treated. mHMGCoARed, mHMGCoA reductase; mito, mitochondria.

adrenal gland and independent of the hypothalamic-pituitary axis, we analyzed the effect of T1317 on cultured Y1 (mouse) and H295R (human) adrenal cells. Both cell lines were found to express LXR α and LXR β (data not shown). In Y1 cells, a classical RXR/LXR induction pattern was observed for mRNA expression of StAR and the known LXR target ABCA1 (Figure 5A). A synergistic increase was observed when both RXR and LXR ligands were present. Western blot analysis demonstrated that StAR protein was also increased 4.3-fold in Y1 cells treated with T1317 (Figure 5B). To determine whether LXRs might play a similar role in human adrenal cells, human H295R cells were treated with LXR and RXR agonists and analyzed using QPCR. StAR expression was increased 2.2-fold and apoE increased 6.4-fold in H295R cells treated with T1317 plus RXR agonist LG268 (Figure 5C). SREBP-1c and the cholesterol transport-

ers ABCA1 and ABCG1 were all strongly induced in response to LXR and RXR agonists (17-, 46-, and 23-fold, respectively).

Transcriptional regulation of StAR by LXR α . Inspection of the mouse StAR promoter revealed an LXR response element-like (LXRE-like) sequence containing a direct hexanucleotide repeat separated by 4 base pairs (Figure 6A). To determine the functionality of this element, HEK293 cells were cotransfected with mouse LXR α (mLXR α) and mRXR α and the region of the StAR promoter (mStARp254-Luc) containing the putative LXRE. A significant receptor- and ligand-dependent activation of the StAR promoter was observed (Figure 6A). To confirm that this region was important for activation, mutation, and deletion, constructs were generated and tested. Promoter activation of the LXRE mutant was indistinguishable from the mStARp65-Luc

**Figure 5**

LXR effects in mouse and human adrenal cells. (A) Northern blot analysis of ABCA1 and StAR from mouse Y1 cells treated with vehicle, LXR agonist T1317 (1 μ M), or RXR agonist LG268 (1 μ M) for 6 hours. Quantitation was performed by densitometry using the largest transcript for StAR normalized to β -actin. (B) Western blot analysis of StAR from Y1 cells treated with or without 1 μ M LXR agonist T1317 (20 μ g per lane, $n = 4$) for 24 hours. (C) QPCR analysis from human H295R cells treated with vehicle, T1317 (1 μ M), LG268 (1 μ M), or both ligands for 24 hours. Data represent the mean \pm SD ($n = 3$). Cycle times for the highest expressing group for each gene are shown in the corresponding bar. * $P < 0.05$, significantly different from control.

minimal promoter construct, marking this as the basal activation (Figure 6B). To verify the ability of LXRE to independently act as an LXR response element, 2 copies of the minimal element were tested in a heterologous thymidine kinase-luciferase (TK-luciferase) reporter plasmid. Cotransfection of LXR α and RXR α in the presence of T1317 plus LG268 resulted in a 10-fold increase in transactivation of the LXRE2-TK-Luc reporter relative to vehicle-treated cells (Figure 6B).

Direct interaction of the LXRE with the RXR/LXR heterodimer was confirmed by electromobility shift assays (Figure 6C). A significant band shift was observed when both LXR α and RXR α were added, which was specifically competed away by a 200-fold molar excess of the StAR LXRE or the lipoprotein lipase DR4.2, a previously characterized LXRE from the lipoprotein lipase promoter (33). The specificity of this interaction was demonstrated by the lack of competition by the mutated StAR LXRE and the ability of an mLXR α antibody, but not a preimmune antibody, to abolish heterodimer binding.

The in vivo function of the StAR LXR response element was demonstrated by chromatin IP (ChIP) using an anti-LXR polyclonal antibody. Specific enrichment of LXR at the StAR LXRE was found in wild-type and Lxr $\beta^{-/-}$ but not Lxr $\alpha^{-/-}$ and Lxr $\alpha\beta^{-/-}$ adrenal glands (Figure 6D). This effect was specific to the region of the LXRE and was not seen in a 2.3-kb upstream control region. Because LXR β is also expressed in the adrenal gland, we examined its ability to activate StAR transcription. Although LXR β bound to the StAR promoter when overexpressed in vitro (data not shown), there was no evidence for in vivo binding of LXR β to the StAR

promoter by ChIP analysis (Figure 6D). These findings provide further evidence that LXR α is the predominant LXR subtype governing StAR activity.

Discussion

In this report, we detail the discovery of LXR α as an important regulator of adrenal cholesterol homeostasis through its ability to modulate transcription of genes that govern the 3 major pathways of adrenal cholesterol utilization. These pathways include cholesterol efflux (ABCA1, ABCG1), storage (apoE, SREBP-1c), and conversion to steroid hormones (StAR). In the adrenal gland, where the flux of cholesterol is highly dynamic, LXR α appears to provide a cholesterol safety valve that operates on a chronic time scale and independently of the hypothalamic-pituitary axis. Thus, as in other tissues, we propose that adrenal LXR α functions as a sterol sensor and thereby maintains the concentration of free cholesterol below toxic levels under a variety of physiologic conditions, including chronic dietary stress.

In the resting state, the majority of adrenal cholesterol is directed toward storage, and any excess is eliminated by efflux. LXR α appears to facilitate this process by maintaining the basal expression of apoE and SREBP-1c for storage and ABCA1 for efflux while keeping the steroidogenic pathway turned off by basally repressing expression of StAR (Figure 7). Under stress, the flux of cholesterol changes dramatically due to enhanced hydrolysis of stored cholesterol esters, increased uptake of plasma cholesterol, and transport of free cholesterol into the mitochondria for production of steroid hormones. Under these conditions, the majority of free cholesterol is diverted to the mitochondria for steroidogenesis in response to ACTH. An increase in intracellular free cholesterol would also be expected to activate LXR α and in turn upregulate expression of its target genes, including apoE, SREBP-1c, and ABCA1, as well as provide a further boost in expression of StAR and the steroidogenic enzymes. The finding that the proteins encoded by these target genes govern all pathways for lowering free intracellular cholesterol supports the notion that LXR α functions as a safety mechanism for preventing overaccumulation of free cholesterol. Such a mechanism may be particularly important during chronic dietary stress, as evidenced by the buildup of free cholesterol in Lxr $\alpha\beta^{-/-}$ mice after 7 weeks on a high-fat or Western diet. It is of interest to speculate that this protective mechanism may also have a role at the end of an acute stress response in helping to remove excess cholesterol and restore the adrenal gland to its resting state.

The model presented in Figure 7 is supported by both the gain and loss of LXR function experiments detailed in this work. Pharmacologic activation of adrenal LXR by a potent agonist resulted in increased expression of the target genes described above and increased steroid hormone production. Interestingly, Lxr $\alpha\beta^{-/-}$ mice also exhibited a basal increase in expression of StAR and the steroidogenic enzymes CYP11A1 and HSD3B1 and a correspondingly higher level of corticosterone relative to that of wild-type mice. The derepression of LXR target genes in receptor-null mice is not uncommon (34) and has been shown to be due to the ability of LXRs to recruit corepressors to the target gene's promoter in the nonliganded state (32, 35). In the absence of LXR, these genes were derepressed, resulting in increased basal expression. In contrast, the basal expression of the cholesterol efflux transporter ABCA1 was decreased in Lxr $\alpha\beta^{-/-}$ mice. Selective repression of certain hepatic LXR target genes was reported previously for SHP (31), which is also expressed in the adrenal gland. Thus, it

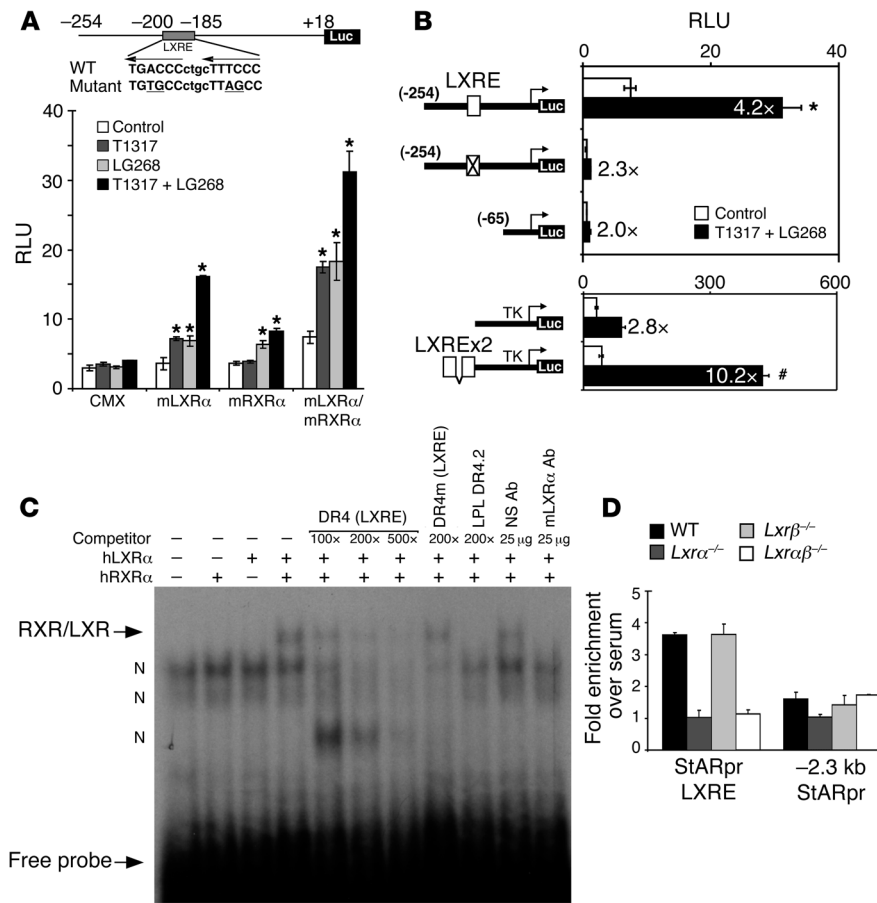


Figure 6 The RXR/LXR heterodimer directly binds and activates the mouse StAR promoter. **(A)** HEK293 cells were transfected with CMX alone, CMX-mLXR α , CMX-mRXR α , or both receptor plasmids in the presence of the -254 to +18 mouse StAR promoter (StARpr) linked to luciferase. Cells were treated with T1317 (1 μ M) and/or LG268 (0.1 μ M). The location, sequence, and mutation of the putative LXR response element (LXRE) from the mouse StAR promoter are shown. **P* < 0.05, significantly different from vehicle for that condition (CMX, LXR α , RXR α , or both). **(B)** Mutation and deletion constructs of the StAR promoter were tested to determine the location of the LXR response. A heterologous TK-Luc vector with or without 2 copies of the LXRE (-200) was also tested. Cotransfections contained the reporter and both mLXR α and mRXR α expression plasmids in the presence or absence of 1 μ M T1317 and 0.1 μ M LG268. **P* < 0.05, significantly different relative to mStARp65-Luc. #*P* < 0.05, significantly different relative to TK-Luc. **(C)** Electromobility shift assays were performed with [³²P]-labeled LXRE (-200) and in vitro-transcribed FLAG-hLXR α and/or human RXR α (hRXR α). Competitions were performed using unlabeled nucleotides at 100-fold, 200-fold, or 500-fold molar excess, as indicated. For antibody-binding reactions, 25 μ g of preimmune or mLXR α -specific antibody were included. LPL, lipoprotein lipase; N, nonspecific band. **(D)** ChIP of LXR from wild-type, *Lxr α ^{-/-}*, *Lxr β ^{-/-}*, or *Lxr α ^{-/-} Lxr β ^{-/-}* adrenal glands. Quantitation was performed by QPCR using the standard curve method, using serial dilutions of the input as standards. Results are expressed as fold enrichment over serum control and represent PCR amplification variability (mean \pm SD, *n* = 3).

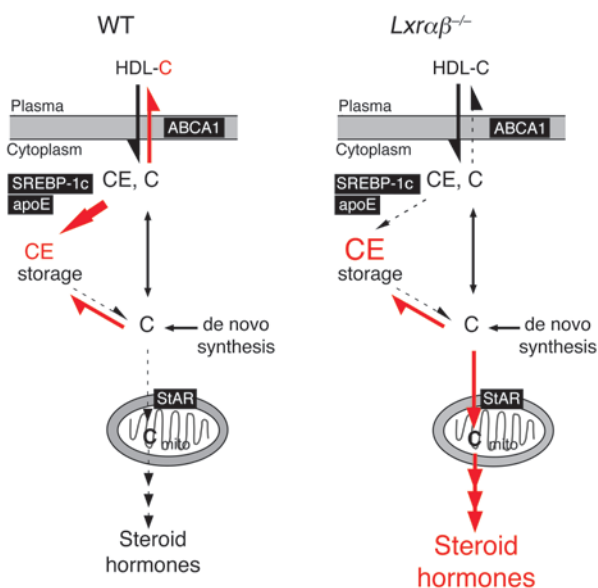
is possible that repression by SHP may account for some of the LXR promoter-specific effects we observed. Taken together, these changes in gene expression would explain the increased movement of cholesterol toward the steroidogenic pathway and cholesterol storage seen in LXR-null mice (Figure 7). Increased cholesterol esters generally correlate with a decrease in steroidogenesis. However, because LXR plays a role in both pathways, its absence leads to an uncoupling of these naturally opposing processes and generates the unique phenotype of the *Lxr α ^{-/-}* mice: elevated plasma

corticosterone in the presence of increased cholesterol esters.

In addition to the work reported here, several lines of evidence support the role of LXR in modulating expression of the genes discussed above. For example, LXRs have been shown to upregulate expression of apoE, ABCA1, and ABCG1 in macrophages as a means of preventing cholesterol accumulation (18, 19, 34). In the adrenal gland, apoE is localized within cells where it is believed to enhance storage of lipoprotein-derived cholesterol esters (36). Lack of apoE results in increased plasma corticosterone and reduced adrenal cholesterol esters (36, 37) while overexpression of adrenal apoE results in increased cholesterol esters and lower steroid production (38–40). In the liver, SREBP-1c is thought to aid in cholesterol storage by providing the fatty acid cosubstrate required for forming cholesterol esters (17). Although a definitive role for ABCA1 and ABCG1 in adrenal cholesterol efflux has not been shown explicitly, the LXR-dependent effects of these transporters in other tissues support our model (34, 41, 42). In particular, the importance of ABCA1 in mediating adrenal cholesterol efflux has emerged from a recent study in *Abcg5/g8^{-/-}* mice, which have 10-fold higher adrenal ABCA1 protein levels and a profound depletion of adrenal cholesterol (43).

The finding that StAR, which encodes the rate-limiting step in steroidogenesis, is also an LXR target gene is intriguing. The StAR promoter is under complex regulatory control involving numerous transcription factors, including AP-1, C/EBP, DAX-1, GATA, SF-1, Sp1, and SREBP-1a (reviewed in ref. 44). Oxysterols and lipoproteins have also been identified as chronic regulators of StAR gene and protein expression (45–47). Using an in vitro reporter assay, King et al. (46) concluded that oxysterols regulate StAR in a process thought to be independent of new gene transcription. In contrast, our data generated both in vivo and in vitro and utilizing a number of independent analyses demonstrate that LXR activation directly medi-

ates expression of StAR mRNA and protein. Whether this activation is due to 22(R)-OH cholesterol or other oxysterols generated during steroidogenesis remains to be established (3). Upregulation of StAR by LXR also provides a direct molecular mechanism for explaining the observation by us and others (23) that circulating corticosterone levels are increased in wild-type mice by LXR agonist treatment. Furthermore, the basal elevation in corticosterone we and others (23) observed in *Lxr α ^{-/-}* mice may also be explained in our model through derepression of StAR.

**Figure 7**

Model of LXR function in adrenal gland cholesterol homeostasis. This model summarizes the results obtained in this study and illustrates the role of LXR in maintaining basal adrenal cholesterol homeostasis. The flux of cholesterol is indicated in red and its relative magnitude is indicated by arrow thickness. LXR target genes are shown in black boxes. Dashed lines indicate repression or inactivation of a pathway. C, cholesterol; CE, cholesterol esters. See text for details.

In summary, we have demonstrated the role of LXR α in the basal maintenance of adrenal cholesterol homeostasis. LXR achieves this fine-tuning of intracellular cholesterol by the regulation of genes involved in all paths of cholesterol utilization in the adrenal gland. These findings complement the known functions of LXRs as master regulators of cholesterol balance in liver and other tissues, where excess cholesterol is processed through a parallel set of pathways (i.e., metabolism, storage, and efflux). The presence of an LXR regulatory pathway in the adrenal gland is particularly important under chronic dietary stress to prevent the buildup of potentially toxic levels of intracellular cholesterol.

Methods

Materials. T1317 was purchased from Cayman Chemical or Sigma-Aldrich, and DEX was from Sigma-Aldrich. LG268 was from Ligand Pharmaceuticals. β -actin antibody was obtained from Sigma-Aldrich. StAR and ABCA1 antibodies were gifts provided by Douglas Stocco (Texas Tech University Health Sciences Center, Lubbock, Texas, USA) and Michael Fitzgerald and Mason Freeman (Massachusetts General Hospital, Boston, Massachusetts, USA), respectively.

Animal studies. LXR-knockout mice (21) and age/sex-matched wild-type controls were maintained on a mixed strain background (C57BL/6:129Sv) and housed in a temperature-controlled room with a 12-hour light/dark cycle. All experiments were performed on age-matched male mice between 3 and 8 months of age. For dietary studies, animals were fed 7 weeks and treated as described (27). Animals raised on Harlan diet 7001 (Harlan Teklad), containing 25% protein, 4% fat, and 45% carbohydrate, had a later onset of adrenomegaly (~6 months) than animals raised on Harlan diet 2016S (Harlan Teklad), containing 16% protein, 4% fat, and 60% carbohydrates (onset of adrenomegaly as early as 3 months). Unless otherwise indi-

cated, mice were fed ad libitum the 2016S diet from Harlan Teklad. Mice were sacrificed within 1 minute of initial handling by decapitation and within 1.5 hours after the start of the light cycle. Animals treated with DEX (2.5 mg/ml sesame oil) were given 2.5 mpk twice daily by subcutaneous injection for 5 days. LXR agonist T1317 was dosed at 50 mpk (5 mg/ml in 1% methylcellulose) by oral gavage. All animal experiments were approved by the Regional Ethics Committee of the Université Blaise Pascal (Clermont-Ferrand, France) and the Institutional Animal Care and Research Advisory Committee at the University of Texas Southwestern Medical Center (Dallas, Texas, USA).

Measurement of plasma hormones. Trunk blood was collected in EDTA tubes on ice, and plasma was stored at -80°C . ACTH, corticosterone, and aldosterone levels were measured by RIA (MP Biomedicals). For DEX-treated samples, corticosterone was first extracted from 200 μl of plasma with 10 volumes of dichloromethane. For quantitation of corticosterone from primary cultures, 200 μl of media was assayed directly.

Primary culture of adrenal cells. Adrenals were digested with 1 mg/ml collagenase at 37°C for 10 minutes. After gravity sedimentation, supernatants were pelleted and submitted to a repeated collagenase digestion (5 to 6 times) until digestion was complete. Supernatants of viable cells were pooled and seeded at a density of 150,000 cells/well in 24-well plates and maintained at 37°C , 5% CO_2 in DMEM containing 100 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin supplemented with 2.5% fetal calf serum, 2.5% horse serum, and 1% insulin-transferrin-sodium selenite (ITS; Sigma-Aldrich) for 2 days. On the third day, cells were placed in serum-free media for 24 hours. Media were then stored at -20°C until corticosterone assay.

Oil red O staining. Lipid staining was performed on cryosections obtained from 3- to 4-month-old mice incubated in 1,2-propanediol for 1 minute and in oil red O (Sigma-Aldrich) at 60°C for 7 minutes. Specimen integrity was verified by staining with Harris hematoxylin (Sigma-Aldrich) according to the manufacturer's instructions.

Immunohistochemistry. Cryosections were fixed with 4% paraformaldehyde for 10 minutes and washed 3 times with PBS. Cell permeability was enhanced by incubation with 0.1% Triton-X 100 (Fischer Scientific) for 10 minutes. Slides were blocked for 1 hour in 5% skim milk and 2% goat serum in PBS and incubated overnight at 4°C with the mLXR α antiserum (26) at a dilution of 1:150. The slides were washed 3 times in PBS, incubated for 1 hour with rabbit anti-IgG-fluorescein (Sanofi Pasteur) at a 1:150 dilution, and washed 3 times with PBS prior to analysis.

Cholesterol ester quantitation. Adrenal lipids were extracted as described (48). High performance thin-layer chromatography (HPTLC) plates (Silica Gel 60; Merck) were used after prewashing with a mixture of methanol/chloroform (1:1, v/v) followed by heating at 125°C for 5 minutes. Plates were developed using hexanes, diethyl ether, and glacial acetic acid (80:20:2, v/v), then sprayed with 10% (w/v) copper sulfate in 8% (v/v) phosphoric acid solution and heated at 180°C for 15 minutes to stain the lipids. Cholesterol and cholesterol esters were quantified against standards by densitometry (Sigma Scan Pro; Sigma-Aldrich). For Figure 3, cholesterol esters were fractionated from free cholesterol using solid-phase extraction over silica columns (49). Free cholesterol was assayed using reverse-phase high-performance liquid chromatography coupled to a triple quadrupole mass spectrometer (Applied Biosystems). External calibration curves were constructed for quantitation of cholesterol relative to the internal standard 25-OH-cholesterol- d_3 (Avanti Polar Lipids Inc.) added at the beginning of the extraction. Cholesterol esters were measured using a kit from Roche Diagnostics.

Real-time PCR. Total RNA was isolated using RNeasyStat60 (Tel-Test Inc.) and processed for real-time PCR on an ABI Prism 7900 HT system (Applied Biosystems) as described (50). QPCR primers are shown in Table 1.

Western blot analysis. Protein extracts were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated



Table 1
 QPCR primer sequences

Gene	Accession Number	Primers	NT Location (exon)
<i>mAbca1</i>	NM_013454	5'-TCCTCATCCTCGTCATTCAAA-3' 5'-GGACTTGGTAGGACGGAACT-3'	5855-5998 (42)
<i>mAbcg1</i>	NM_009593	5'-GCTGTGCGTTTTGTGCTGTT-3' 5'-TGCAGCTCCAATCAGTAGCACTAA-3'	1634-1717 (13)
<i>mAcat</i>	NM_009230	5'-TGGCTTGTCCAGCTCTTAACTG-3' 5'-GTTGCTCCCATGAAGTGAAGAAA-3'	520-596 (Xa) ^A
<i>mApoe</i>	NM_009696	5'-TCTGACCAGGTCCAGGAAGAG-3' 5'-CATCAGTGCCGTCAGTTCCTG-3'	205-267 (3-4)
<i>mCyclophilin</i>	NM_011149	5'-GGAGATGGCACAGGAGGAA-3' 5'-GCCCGTAGTGCTTCAGCT-3'	470-545 (3-4)
<i>mCyp11a1</i>	NM_019779	5'-TGAATGACCTGGTGCTTCGT-3' 5'-GGCAAAGCTAGCCACCTGTA-3'	1225-1292 (5)
<i>mHmgcr</i>	NM_008255	5'-CTTGTGGAATGCCTTGTGATTG-3' 5'-AGCCGAAGCAGCACATGAT-3'	578-653 (6-7)
<i>mHsd3b1^B</i>	NM_008293	5'-TCATCCCAGGCAGACCAT-3' 5'-CACAGGCCTCCAATAGTTCT-3'	402-467 (3-4)
<i>mHsl</i>	NM_010719	5'-GCTGGAGGAGTGTTTTTTGC-3' 5'-AGTTGAACCAAGCAGGTCACA-3'	1243-1306 (6)
<i>mLxrα</i>	NM_013839	5'-AGGAGTGTGACTTCGCAAA-3' 5'-CTCTTCTGCCGCTTCAGTTT-3'	635-735 (4-5)
<i>mLxrβ</i>	NM_009473	5'-AAGCAGGTGCCAGGGTTCT-3' 5'-TGCATTCTGTCTCGTGGTTGT-3'	1084-1204 (2-3)
<i>mSf-1</i>	NM_139051	5'-CCCTTATCCGGCTGAAATT-3' 5'-CCAGGTCCTCGTGTACGA-3'	121-196 (1-2)
<i>mShp</i>	NM_011850	5'-CGATCCTCTTCAACCAGATG-3' 5'-AGGGCTCCAAGACTTCACACA-3'	559-640 (1-2)
<i>mSr-b1</i>	NM_016741	5'-TCCCATGAAGTGTCTGTGAA-3' 5'-TGCCCGATGCCCTTGA-3'	1331-1397 (11-12)
<i>mSrebp-1c</i>	NM_011480	5'-GGAGCCATGATTGCACATT-3' 5'-GGCCCGGGAAGTCACTGT-3'	nd-198 (1c-2)
<i>mStar</i>	NM_011485	5'-CGGAGCAGAGTGGTGTATC-3' 5'-TGAGTTTAGTCTTGAGGGACTTC-3'	682-771 (5-6)
<i>hCYCLOPHILIN</i>	NM_000942	5'-GGAGATGGCACAGGAGGAA-3' 5'-GCCCGTAGTGTCTCAGTTT-3'	497-572 (3-4)
<i>hABCA1</i>	NM_005502	5'-CCAGGCCAGTACGGAATTC-3' 5'-CCTCGCCAACCCAGTAGGA-3'	2852-2918 (20-21)
<i>hABCG1^C</i>	NM_207630	5'-CCCAGCAGATTTTGTCTATGGA-3' 5'-CCAGCCGACTGTTCTGATCA-3'	1546-1607 (17-18)
<i>hAPOE</i>	NM_000041	5'-TGGGTTGCTGGTCAATTC-3' 5'-TCTGTCTCCACCGTTGCT-3'	101-164 (3)
<i>hSTAR</i>	NM_000349	5'-CCCCAATGTCAAGGAGATCAA-3' 5'-CAGCTCGTGAGTAATGAATGATCTT-3'	708-774 (3-4)
<i>hSREBP-1c</i>	NM_004176	5'-GGCCGGGCGCAGATC-3' 5'-TTGTTGATAAGCTGAAGCATGTCT-3'	nd-307 (1c-2)

^A(55). ^BPrimers cross-react with HSD3B4. ^CPrimers detect all isoforms (1-7). nd, not determined.

overnight at 4°C with primary polyclonal antibodies against ABCA1 (1:1000), StAR (1:1000), or β -actin (1:2000), followed by a 1-hour incubation with a peroxidase-conjugated anti-rabbit IgG. Peroxidase activity was measured using the Western Lightning System (PerkinElmer) or ECL Plus (Amersham Biosciences). Quantitation was performed using Quantity One software version 4.2.1 from Bio-Rad.

Cell culture. Y1 cells were maintained at 37°C, 5% CO₂ in DMEM containing 100 U/ml penicillin and 100 μ g/ml streptomycin supplemented with 10% fetal calf serum. Cells were plated at 2.5 \times 10⁵ cells per well in 6-well plates. The next day, cells were placed in serum-free media and treated 24 hours later with 1 μ M T1317, 1 μ M LG268, or T1317 plus LG268 for 6 hours. H295R cells (NCI-H295R) from ATCC were maintained in DMEM:

F12 (Invitrogen) containing 100 U/ml penicillin, 100 μ g/ml streptomycin supplemented with 1% ITS plus Premix (BD Biosciences – Clontech), and 2.5% Nu-Serum (BD Biosciences) at 37°C, 5% CO₂. Cells were seeded at 5 \times 10⁵ cells/well in 6-well plates. After 4 days, the media were substituted with serum-free media (DMEM:F12 alone), and 24 hours later, T1317 (1 μ M) and LG268 (1 μ M) were added in fresh serum-free media and incubated 24 hours before harvesting RNA as described above.

Northern blots. Total RNA (20 μ g) was pooled, size fractionated on 1% agarose-formaldehyde gel, transferred to a nylon membrane (Amersham Biosciences), and hybridized with [³²P]-labelled cDNA probes as previously described (34).

Plasmids. The mStar promoter (p-254StAR/Luc) was a gift from K.M. Caron (Duke University, Durham, North Carolina, USA). Mutations in the LXRE half sites were introduced by splicing by overlapping extension PCR. The LXREx2-TK-Luc reporter was generated by ligating the StAR LXRE (-200) into the *Bam*HI site of TK-Luc. All constructs were verified by sequencing.

Cotransfection assays. HEK293 cells were transfected with calcium phosphate as described (51). StAR promoter-luciferase reporter constructs (50 ng) were added in combination with CMX-mLXR α (15 ng), CMX-mRXR α (15 ng), β -galactosidase (10 ng), and pGEM for a total of 150 ng/well. Ligands were added 6-8 hours later in delipidated media. Cells were harvested 14-16 hours later and assayed for luciferase and β -galactosidase activity. Luciferase values were normalized for transfection efficiency using β -galactosidase and expressed as RLU of triplicate assays (mean \pm SD).

Electrophoretic mobility shift assays. Gel shift assays were performed as described previously (52). For antibody-binding reactions, 25 μ g of preimmune or mLXR α -specific antibody (26) was added to the reaction for an additional 10 minutes on ice prior to loading onto the gel. Gels were autoradiographed with intensifying screens for 60 hours at -80°C.

ChIP. ChIP was carried out as described (53, 54). Pooled adrenal pairs (from 5 mice/genotype) were cross-linked in 1% formaldehyde containing PBS, 1 mM DTT, and 1 mM PMSF for 10 minutes at RT. Adrenal gland nuclei were recovered by dounce homogenization in a hypotonic

buffer followed by centrifugation. Chromatin was sheared to 200-1000 bp by sonication. After preclearing, 10 μ g of LXR α / β antibody (Santa Cruz Biotechnology Inc.) was added for overnight incubation. Protein G agarose (60 μ l) was used to recover the immune complexes. Washes and elutions were performed in accordance with the Upstate USA Inc. ChIP kit. DNA was reverse-crosslinked overnight at 65°C and purified using a spin column (QIAGEN) to a final volume of 50 μ l. QPCR was performed as described above, using 5 μ l of template DNA with the following primers: mouse StAR promoter (StARpr) LXRE forward, TCTA-CATTTACAACCTTAGAGAAGC, reverse, TAGGGGAAAGCAGGGGT-CACG; StARpr neg-2.3 kb forward, CAGCAAGAGTAAAGTAGTCATTG, reverse, GAACGCTGAGAGACATCCAGC.



Statistics. For comparison between 2 groups, the unpaired 2-tailed Student's *t* test was performed. One-way ANOVA followed by the Student-Newman-Keuls procedure was used to compare more than 2 groups. All tests were performed using the software program Primer of Biostatistics (version 4.02; McGraw-Hill).

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