Control of cellular cholesterol efflux by the nuclear oxysterol receptor LXRα

Asha Venkateswaran*,†, Bryan A. Laffitte†‡, Sean B. Joseph§, Puiying A. Mak*, Damien C. Wilpitz‡, Peter A. Edwards*¶ and Peter Tontonoz†‡

*Departments of Biological Chemistry and Medicine, ‡Howard Hughes Medical Institute, §Department of Pathology and Laboratory Medicine, and ¶Molecular Biology Institute, University of California, Los Angeles, CA 90095

Communicated by Richard J. Havel, University of California, San Francisco, CA, August 4, 2000 (received for review June 16, 2000)

LXRα is a nuclear receptor that has previously been shown to regulate the metabolic conversion of cholesterol to bile acids. Here we define a role for this transcription factor in the control of cellular cholesterol efflux. We demonstrate that retroviral expression of LXRα in NIH 3T3 fibroblasts or RAW264.7 macrophages and/or treatment of these cells with oxysterol ligands of LXR results in 7- to 30-fold induction of the mRNA encoding the putative cholesterol/phospholipid transporter ATP-binding cassette (ABC)A1. In contrast, induction of ABCA1 mRNA in response to oxysterols is attenuated in cells that constitutively express dominant-negative forms of LXRα or LXRβ that lack the AF2 transcriptional activation domain. We further demonstrate that expression of LXRα in NIH 3T3 fibroblasts and/or treatment of these cells with oxysterols is sufficient to stimulate cholesterol efflux to extracellular apolipoprotein A1. The ability of oxysterol ligands of LXR to stimulate efflux is dramatically reduced in Tangier fibroblasts, which carry a loss of function mutation in the ABCA1 gene. Taken together, these results indicate that cellular cholesterol efflux is controlled, at least in part, at the level of transcription by a nuclear receptor–signaling pathway. They suggest a model in which activation of LXR by oxysterols in response to cellular sterol loading leads to induction of the ABCA1 transporter and the stimulation of lipid efflux to extracellular acceptors. These findings have important implications for our understanding of mammalian cholesterol homeostasis and suggest new opportunities for pharmacological regulation of cellular lipid metabolism.

Human and murine macrophages express cell surface receptors that bind and then rapidly internalize oxidized or modified (e.g., acetylated) low density lipoprotein (LDL) (1). This rapid and unregulated uptake of the cholesterol-rich LDL results in the formation of intracellular cholesteryl ester inclusions that give the macrophages a foamy appearance (2). Such cholesteryl ester–rich foam cells have been identified in both early and advanced atherosclerotic lesions of humans and animals and are thought to play a critical role in the development of the disease (3, 4). Thus, the differential expression of specific genes in lipid loaded macrophages is likely to play an important role in the pathology of atherosclerosis.

Recent studies have identified two genes, ATP-binding cassette (ABC)A1 (5) and ABCG1 (6, 7), whose mRNAs are induced 7- to >100-fold in lipid-loaded macrophages. In earlier publications, ABCA1 was referred to as ABC1, whereas ABCG1 was referred to as either ABC8 (murine) or white (human). ABCA1 and ABCG1 are members of the ATP binding cassette (ABC) superfamily of transporter proteins that bind and hydrolyze ATP to provide energy for transmembrane transport (8–10). The ABCA1 gene encodes a full transporter protein containing 12 putative transmembrane domains and two ABCs (11), whereas the ABCG1 gene encodes a half transporter protein containing six putative transmembrane domains and one ABC (12, 13).

Studies from a number of laboratories have provided compelling evidence that the ABCA1 protein facilitates the efflux of cellular cholesterol to extracellular apolipoprotein A1 (apoAI) or high density lipoprotein (HDL). Defective efflux of cellular lipids to serum apolipoproteins is thought to account for the abnormally low plasma HDL levels noted in ABCA1 null mice (14) and in patients with Tangier disease or familial HDL deficiency (15), in which the ABCA1 gene is mutated (16–19). Defective lipid efflux is also likely to underlie the accumulation of lipid-loaded reticuloendothelial cells and cholesteryl ester–loaded macrophages in the atherosclerotic lesions of patients with Tangier disease (16), as well as the presence of excess foam type II pneumocytes and lipid-loaded macrophages in the lungs of ABCA1 null mice (14). In support of this hypothesis, it has been shown that treatment of cells with antisense oligonucleotides to ABCA1 (16, 17) leads to a decrease in the efflux of cellular cholesterol and phospholipids to HDL or apoA1 in the medium. Moreover, fibroblasts derived either from patients with Tangier disease (16–19) or from ABCA1 null mice (20) exhibit a >60% reduction in the rate of efflux of cholesterol and/or phospholipids to HDL or apoA1. Studies have also shown that absorption of dietary cholesterol is increased in ABCA1 null mice, consistent with an important role for this transporter protein in cholesterol absorption (14). Taken together, these observations imply a central role for ABCA1 in regulation of cholesterol and/or phospholipid efflux to extracellular acceptors. Although the physiological function of ABCG1 is less well understood, antisense oligonucleotides to ABCG1 have also been reported to inhibit lipid efflux (6).

The ability of ABCA1 to facilitate cholesterol efflux suggests that it may be a useful target for intervention in human disease. However, the molecular mechanism that links cellular lipid loading to the induction of ABCA1 expression is not known. Here we demonstrate that the nuclear receptor LXRα and its oxysterol ligands regulate expression of ABCA1 in macrophages and fibroblasts. Moreover, we show that ligand activation of LXRα is sufficient to promote cholesterol efflux to extracellular acceptors through ABCA1. These observations suggest that cellular cholesterol efflux is controlled, at least in part, by a nuclear receptor signaling pathway.

Materials and Methods

Reagents. Acetylated LDL (Ac-LDL) and highly oxidized LDL (Ox-LDL) were prepared from human LDL as described (7). Cholesterol (Steraloids, Wilton, NH) and oxysterols (Sigma) were dissolved in ethanol before addition to cells (<1 μl/ml).

Abbreviations: ABC, ATP-binding cassette; LDL, low density lipoprotein; HDL, high density lipoprotein; apoAI, apolipoprotein AI; Ac-LDL, acetylated LDL; Ox-LDL, highly oxidized LDL; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OHC, hydroxycholesterol; RXR, retinoid X receptor.

1A.V. and B.A.L. contributed equally to this work.

2To whom reprint requests should be addressed. E-mail: pedwards@mednet.ucla.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Article published online before print: Proc. Natl. Acad. Sci. USA, 10.1073/pnas.0306769.

Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.030676977
ApoAI (Intracel) was resuspended in PBS, quick frozen and stored in aliquots at −80°C. LG268 was a gift from R. Heyman (Ligand Pharmaceuticals). [3H]Cholesterol (45 Ci/mmol) was from NEN.

**Cell Culture and RNA Analysis.** Human monocytes were isolated from peripheral blood, allowed to adhere to plastic dishes, and cultured in autologous serum for 8 days as described (7). RAW264.7 macrophages and NIH 3T3 fibroblasts (ATCC) were grown in DMEM plus 10% FBS. Cells were washed with PBS and cultured for 16–24 h in medium A (DMEM containing 10% lipoprotein-deficient FBS). The cells were rewashed before addition of 1.0 ml medium B, which was free of lipoprotein but containing the indicated oxysterol or LG268. The results presented in Fig. 1 strongly suggest that specific oxidosters activate transcription of the ABCA1 gene by a process that is independent of continued protein synthesis. Moreover, the stereochemical requirements for 22-OHC induction of ABCA1 are similar to those of 25-OHC (Fig. 1C) and some other 24-hydroxycholesterol derivatives (27–29). In contrast, ABCA1 mRNA levels were not increased in response to 20(S)-OHC (Fig. 1D), an oxysterol that is unable to activate LXR. The induction of ABCA1 mRNA in THP-1 cells was attenuated when the cells were coincubated with actinomycin D, an inhibitor of transcription (Fig. 1D).

The results presented in Fig. 1 strongly suggest that specific oxysterols activate transcription of the ABCA1 gene by a process that is independent of continued protein synthesis. Moreover, the stereochemical requirements for 22-OHC induction of ABCA1 are similar to those of 25-OHC (Fig. 1C) and some other 24-hydroxycholesterol derivatives (27–29). In contrast, ABCA1 mRNA levels were not increased in response to 20(S)-OHC (Fig. 1D), an oxysterol that is unable to activate LXR. The induction of ABCA1 mRNA in THP-1 cells was attenuated when the cells were coincubated with actinomycin D, an inhibitor of transcription (Fig. 1D).
ligand 20(S)-OHC, the retinoid X receptor (RXR)-specific ligand LG268, or both. As shown in Fig. 2A, mRNAs encoding ABCA1 and ABCG1 were very low in RAW-vector cells in the absence of oxysterol. By contrast, RAW-LXRα cells showed significant expression of both transcripts even in the absence of exogenously added oxysterol. Addition of 20(S)-OHC resulted in induction of ABCA1 and ABCG1 mRNAs in both cell types. However, maximal induction was observed at an oxysterol concentration of \( \leq 1.25 \mu g/ml \) in the RAW-LXRα cells as compared with a concentration of 2.5 \( \mu g/ml \) in RAW-vector cells (Fig. 2B). Thus, the dose-response was shifted significantly to the left in the cells that overexpressed LXRα. Addition of LG268 also resulted in a small but significant induction of both mRNAs (Fig. 2A). This latter result is consistent with the finding that LXR/RXR is a permissive heterodimer and activates transcription in response to ligand for either partner (23, 30). Taken together, these observations are consistent with an important role for both LXRα and oxysterols in transcriptional regulation of macrophage ABCA1 and ABCG1 expression. In control cells, oxysterols presumably induce expression of these mRNAs through LXRβ or and/or low levels of endogenous LXRα (see below). The oxysterol response is significantly enhanced through ectopic overexpression of LXRα.

Next, we asked whether the LXR-dependent induction of ABCA1 was restricted to macrophages or whether it might be functional in other cell types. We examined the ability of retroviral LXRα expression to influence ABCA1 expression in NIH 3T3 fibroblasts. Northern blot analysis revealed high-level expression of LXRα mRNA in cells transduced with the LXRα expression vector (NIH-LXRα) but not in those transduced with the retroviral vector alone (NIH-vector; Fig. 3A). Expression of LXRβ mRNA was similar in both cell lines. ABCA1 mRNA levels were very low in control cells but increased significantly upon incubation of the cells with either 20(S)- or 22(R)-OHC. By contrast, stable expression of LXRα in NIH 3T3 cells resulted in significant expression of ABCA1 mRNA, even in the absence of exogenously added oxysterol (Fig. 3A). Addition of oxysterol ligands to these cells further induced ABCA1 expression. Comparison of the results obtained with NIH-vector and NIH-LXRα cells indicates that overexpression of LXRs shifts the dose-response to oxysterols to the left (Fig. 3B). Thus, in both RAW264.7 macrophages and NIH 3T3 cell fibroblasts, induction of ABCA1 mRNA depends on specific oxysterols and the level of expression of LXRs.

We hypothesized that the basal ability of oxysterols to induce ABCA1 expression in control RAW-vector and NIH-vector cells (as shown in Figs. 2 and 3) was attributable to endogenous LXRα and/or LXRβ. To verify this hypothesis, we used retroviral vectors to overexpress dominant negative forms of either LXRα or LXRβ (LXRαΔAF2, LXRβΔAF2; see Materials and Methods) in NIH 3T3 cells. These mutant receptors form heterodimers with RXR that bind to LXR response elements but fail to activate transcription in response to oxysterol ligands (data not shown). NIH-vector, NIH-LXRαΔAF2, and NIH-LXRβΔAF2 cells were treated for 24 h with increasing concentrations of 20(S)-OHC. As shown in Fig. 4, expression of either dominant-negative receptor significantly blunted the induction of ABCA1 by 20(S)-OHC. Expression of ABCA1 mRNA was lower in NIH-LXRαΔAF2 (Fig. 4A) and NIH-LXRβΔAF2 (Fig. 4B) cells compared with control cells at each dose of oxysterol tested. These data strongly suggest that oxysterol induction of
ABC1 expression in RAW and NIH 3T3 cells is mediated by endogenous LXRα and/or LXRβ.

ABC1 has recently been reported to play a role in regulating the efflux of cellular cholesterol and/or phospholipids to specific lipid acceptors (HDL or apoAI) in the medium (16, 17, 20). Our demonstration that expression of ABCA1 is controlled by LXR led us to hypothesize that the LXR pathway may be a central mechanism by which cellular lipid efflux is regulated. This idea was tested using the NIH-LXRs and NIH-vector cells described above. As shown in Fig. 5A, cholesterol efflux from murine NIH 3T3 cells to apoAI was significantly enhanced when the cells overexpressed LXRα from a retroviral vector (compare lane 4 vs. 1). Preincubation of the cells with 20(S)- or 22(R)-OHC for 28 h resulted in an increase in the rate of cholesterol efflux to apoAI (Fig. 5A, lanes 2 and 3 vs. 1, lanes 5 and 6 vs. 4). Under each condition, cholesterol efflux was greater from the LXRx-overexpressing cells than from the control cells (Fig. 5A). In data not shown, we have observed that LG268 also promotes efflux in these cells, whereas 22(S)-OHC (which is unable to activate LXRx) does not. Thus, ectopic expression of LXRx in fibroblasts is sufficient to alter the rate of cholesterol efflux to extracellular acceptors. The stereospecificity of the effect of 22-OHC on efflux strongly suggests that the activity of oxysterols in these assays is the result of ligand activation of LXR.

To demonstrate that the ability of the LXR signaling pathway to regulate cholesterol efflux was mediated at least in part by ABCA1, we used primary skin fibroblasts from a patient with Tangier disease that carry a loss of function mutation in the ABCA1 gene (see Materials and Methods). Induction of ABCA1 mRNA by oxysterols was similar in both cells (Fig. 5B Inset). As shown in Fig. 5B, cholesterol efflux to extracellular apoAI from wild-type human fibroblasts was strongly stimulated by treatment with 22(R)-OHC but not 22(S)-OHC. The combination of 22(R)-OHC and the RXR ligand LG268 had a greater than additive effect. By contrast, the ability of 22(R)-OHC to stimulate efflux was dramatically reduced in Tangier fibroblasts. These results provide genetic evidence that stimulation of cellular cholesterol efflux by LXR and its oxysterol ligands is mediated predominantly by the ABCA1 pathway.

Discussion

The LXRs were originally cloned as orphan receptors based on their homology to RXR (23). LXRx is expressed primarily in liver, kidney, intestine, and certain macrophages, whereas LXRxβ is widely expressed (31). The physiologic ligands for both receptors are now recognized to be specific oxysterols (27–29, 32). Previous studies have delineated an important role for LXRx in the regulation of cholesterol homeostasis. LXRx null mice fail to induce transcription of the gene encoding cholesterol 7α-hydroxylase (Cyp7a1), the rate-limiting enzyme in bile acid synthesis, in response to dietary cholesterol (31, 33). In addition, evidence suggests that the gene encoding cholesteryl ester transfer protein (CETP) is also regulated by LXR (34). We have recently shown that both LXRx and LXRxβ are expressed in macrophages (ref. 7; P.T. and R. M. Evans, unpublished data) and that the induction of ABCG1 in response to macrophage lipid loading is mediated by LXR (7). These observations led us...
to explore a functional role for the LXRα in macrophage lipid metabolism.

Previous work established that expression of ABCA1 is dramatically induced in lipid-loaded macrophages and that this protein plays an important role in facilitating cellular cholesterol efflux (6, 14, 15). However, the nature of the molecular sensor that links lipid loading to the transcriptional induction of ABCA1 expression and the stimulation of cholesterol efflux has remained obscure. We have shown here that activation of the nuclear receptor LXRα by oxysterol ligands leads to the transcriptional induction of ABCA1 in macrophages and fibroblasts. Moreover, retroviral expression and activation of LXRα was shown to be sufficient to promote cellular cholesterol efflux to apoA1. The ability of oxysterols of LXRα to stimulate cholesterol efflux was dramatically reduced in cells that lack functional ABCA1. This study represents the first demonstration that expression of ABCA1 and the process of cellular cholesterol efflux are controlled, at least in part, by a nuclear receptor–signaling pathway.

Additional studies will be required to better define the specific roles of ABCA1 and ABCG1 in regulating lipid efflux from cells. Consistent with its proposed role in regulating lipid efflux, indirect evidence suggests that the ABCA1 protein is expressed at the cell surface (16). However, it has yet to be established whether ABCA1 functions directly to transport phospholipid and/or cholesterol across the plasma membrane to exogenous lipid acceptors. Alternatively, ABCA1 might act as a phospholipid flipase and thus control lipid asymmetry. Such asymmetry could drastically affect lipid desorption/efflux to exogenous lipid acceptor proteins such as apoA1.

The role of ABCG1 in such lipid movement is even less well understood. ABCG1 encodes a half transporter protein that has been reported to be localized to both the plasma membrane and to perinuclear membranes (6). However, we have observed that an ABCG1-green fluorescent protein fusion localized to perinuclear membranes and was absent from the plasma membrane (A.V. and P.A.E., unpublished data). These latter results are consistent with many studies that demonstrate that mammalian half transporter proteins are localized to intracellular membranes, including peroxisomes, mitochondria, and endoplasmic reticulum and are excluded from the plasma membrane (35, 36). Interestingly, the small residual induction of cholesterol efflux by oxysterols observed in Tangier cells (Fig. 5B) leaves open the possibility that additional proteins or pathways may be involved, perhaps including ABCG1. It is also possible that ABCG1 acts upstream of ABCA1 in the efflux pathway. Additional studies are underway to define both the cellular location of ABCG1 and its potential role in modulating cholesterol efflux.

The demonstration that ABCA1 and ABCG1 expression is highly induced in macrophages in response to oxysterol-activated LXR is consistent with an important role for these proteins in macrophage biology, especially during lipid-loading. Such lipid-loaded macrophages are thought to influence the etiology of atherosclerosis (4, 37). Our results suggest a novel mechanism whereby cholesterol, through its conversion to oxysterol ligands of LXR, can regulate sterol efflux from cells (Fig. 6). This model suggests that the LXR pathway may be a useful target for the pharmacological regulation of cellular lipid metabolism. It is possible that increased expression of ABCA1 and/or ABCG1 in macrophages may limit the accumulation of lipid and thus protect against the development of fatty lesions and atherosclerosis. Clearly, in vivo studies and the development of high affinity synthetic ligands for LXR will be required to test this possibility.

We thank Drs. S. E. Antonarakis for human ABCG1 cDNA, R. Lawn for human ABCA1 cDNA, R. Davis for murine ABCA1 cDNA, D. Mangelsdorf for LXRx and β cDNAs, and R. Heyman for LG268. Normal and Tangier fibroblasts were a gift of Dr. John F. Oram. We thank Drs. Oram, Rothblat, and Fielding for useful discussions, Ms. Rosa Chen for typing, and Drs. J. Berlinger and A. Lusis for a critical reading of the manuscript. This work was supported by grants from the National Institutes of Health (HL 30568 to P.A.E.), the Laubisch Fund (to P.A.E.), and the Johnson Comprehensive Cancer Center (to P.T.). P.T. is an Assistant Investigator of the Howard Hughes Medical Institute at the University of California, Los Angeles.