Regulated Expression of the Apolipoprotein E/C-I/C-IV/C-II Gene Cluster in Murine and Human Macrophages

A CRITICAL ROLE FOR NUCLEAR LIVER X RECEPTORS α AND β

Puifying A. Mak‡, Bryan A. Laffitte§, Catherine Desrumaux¶, Sean B. Joseph§, Linda K. Curtiss¶, David J. Mangelsdorf¶, Peter Tontonoz‡**, and Peter A. Edwards‡***‡‡

From the ‡Department of Biological Chemistry and Medicine, the ¶Department of Pathology and Laboratory Medicine and the Howard Hughes Medical Institute, and the **Department of Molecular Biology, University of California, Los Angeles, California 90095, the ¶Howard Hughes Medical Institute, Department of Pharmacology, University of Texas Southwest Medical Center, Dallas, Texas 75390-9030, and the ***Departments of Immunology, The Scripps Research Institute, La Jolla, California 92037

Received for publication, March 27, 2002, and in revised form, May 22, 2002 Published, JBC Papers in Press, May 24, 2002 DOI 10.1074/jbc.M202993200

Lipid-loaded macrophage “foam cells” accumulate in the subendothelial space during the development of fatty streaks and atherosclerotic lesions. To better understand the consequences of such lipid loading, murine peritoneal macrophages were isolated and incubated with ligands for two nuclear receptors, liver X receptor (LXR) and retinoic acid receptor (RXR). Analysis of the expressed mRNAs using microarray technology led to the identification of four highly induced genes that encode apolipoproteins E, C-I, C-IV, and C-II. Northern blot analysis confirmed that the mRNA levels of these four genes were induced 2-14-fold in response to natural or synthetic ligands for LXR and/or RXR. The induction of all four mRNAs was greatly attenuated in peritoneal macrophages derived from LXR−/− null mice. The two LXR response elements located within the multihancer ME.1 and ME.2 were shown to be essential for the induction of apoC-II promoter-reporter genes by ligands for LXR and/or RXR. Finally, immunohistochemical studies demonstrate that apoC-II protein co-localizes with macrophages within murine arterial lesions. Taken together, these studies demonstrate that activated LXR induces the expression of the apoE/C-I/C-IV/C-II gene cluster in both human and murine macrophages. These results suggest an alternative mechanism by which lipids are removed from macrophage foam cells.

Apoe, apoC-I, apoC-IV, and apoC-II form a gene cluster that spans 45 kb on human chromosome 19 (1) and 30 kb on murine chromosome 7 (2). These four secreted proteins have important roles in lipoprotein/lipid homeostasis. ApoE is a component of chylomicrons, VLDL, and intermediate density lipoprotein (3), where it functions to mediate the clearance of these lipoproteins from the circulation by a process that is dependent on the interaction of apoE with specific cell surface receptors (3). The majority of plasma apoE is derived from the liver (4). However, other tissues, including brain glial cells and macrophages, synthesize and secrete apoE (5, 6). Data from studies that utilized either apoE null mice (7, 8) or bone marrow transplantation (9, 10) suggest that macrophage-derived apoE is important in preventing and/or reducing cholesteryl ester accumulation in macrophages in the artery wall. It has been proposed that this anti-atherosclerotic effect of apoE is a result of the apoE-dependent efflux of cholesterol from foam cells (11). However, the relative importance of this apoE-dependent cholesterol efflux, as compared with the ABCA1/apoAI-dependent lipid efflux (reviewed in Ref. 12) is currently unknown. Nonetheless, several studies suggest that the anti-atherosclerotic effect of macrophage-derived apoE is independent of its role in increasing the clearance of lipoproteins from the plasma (reviewed in Ref. 3).

ApoE mRNA has been identified in murine liver, intestine, and macrophages (13). ApoC-II is the obligate cofactor for lipoprotein lipase (LPL) and is required for the LPL-dependent hydrolysis of triglycerides present in chylomicrons, VLDL, and high density lipoprotein (14). Deficiency of either apoC-II or LPL results in hypertriglyceridemia (15). Paradoxically, transgenic mice expressing human apoC-II are also hypertriglyceridemic, suggesting that apoC-II may have other unknown functions in addition to acting as the obligate cofactor of LPL (16).

ApoC-I, like apoC-II, is expressed in the liver and is associated with triglyceride-rich chylomicrons and VLDL (17). ApoC-I has been reported to inhibit cholesteryl ester transfer protein, to activate the enzyme lecithin-cholesterol acyltransferase, and to inhibit lipoprotein binding to the LDL receptor-related protein (Ref. 18 and references therein). The physiological role of apoC-IV remains to be established. Compared with other members of this apolipoprotein gene cluster, apoC-IV hepatic mRNA and plasma protein levels are expressed at extremely low levels (1, 19). However, expression of the human apoC-IV transgene in mice led to hypertriglyceridemia, as a result of the accumulation of human apoC-IV-enriched VLDL

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Our studies support the hypothesis that induction of the apoE, apoC-II, and apoC-IV gene cluster in macrophages by LXR/RXR may be localizes with macrophages in murine atherosclerotic lesions. No histological studies demonstrated that apoC-II protein co-localizes with reporter genes suggest that the LXRE in the distal hepatic control regions (HCR.1 and HCR.2) that control the expression of apoE, apoC-I, and apoC-II (23). HCR.1 was identified independently (52). In addition, two multienhancer regions (ME.1 and ME.2, each 620 bp) control the expression of apoE in macrophages, adipose tissue (24), brain (25), and skin (26). The role of ME.1 and/or ME.2 in the regulated expression of apoC-I, apoC-IV, and apoC-II has not been reported. Functional response elements for the nuclear receptors FXR and LXR have been identified in HCRs (27) and MEs (28), respectively. These results are consistent with the emerging theme that LXR and FXR play key roles in regulating genes involved in lipoprotein metabolism. The FXR response elements in HCR.1 and HCR.2 were shown to be bound by the FXR/RXR heterodimer and to be required for bile acid-dependent activation of the apoC-II gene (27). In addition, the LXREs located in ME.1 and ME.2 were shown to be required for the induction of apoE in human macrophages, in response to ligands for LXR (28). There are two LXR genes, LXRα and LXRβ, that encode two forms of LXR and that share about 78% identity at the amino acid level in both the DNA- and ligand-binding domains (29). Each LXR isoform complexes with RXR to form a functional heterodimer that binds to the aforementioned LXREs (reviewed in Ref. 30). Among the 11 LXR-regulated genes identified to date, several are known to be expressed in macrophages; these include ABCA1 (31–33), ABCG1 (34), apoE (35), fatty acid synthase (36), and LPL (37). In the current study, we utilized mouse peritoneal macrophages treated with ligands for LXR and RXR and employed microarray technology to identify novel LXR target genes. These studies led to the identification of apoC-I, apoC-IV, apoC-II, and apoE, as target genes of LXR. All four genes were highly induced in both human and mouse primary macrophages following LXR activation. Induction was attenuated or abolished in macrophages derived from LXR- or RXR-null mice. Studies with reporter genes suggest that the LXRE in the distal enhancer, ME.2, has a critical role in regulating the expression of this gene cluster. Consistent with these observations, immunohistochemical studies demonstrated that apoC-II protein co-localizes with macrophages in murine atherosclerotic lesions. Our studies support the hypothesis that induction of the apoE/C-I/C-IV/C-II gene cluster in macrophages by LXR/RXR may be a critical event in the subsequent efflux of lipids to apolipoproteins in the artery wall.

Experimental Procedures

Reagents—Mouse apoC-2 antibody was a kind gift from Dr. Karl Weisgraber (Gladstone Institute, UCSF). pCMX expression plasmids for LXRα and RXRα were a gift from Ron Evans (Salk Institute, La Jolla, CA). The LXR- and FXR-specific agonists, T909317 (hereafter referred to as T) and GW4064, were generous gifts from Drs. Tim Willson and Patrick Maloney, respectively (GlaxoSmithKline, Research Triangle Park, NC). The RXR-specific agonist LG100153 (hereafter referred to as LG) was a gift from Dr. Richard Heyman (Ligand Pharmaceuticals, La Jolla, CA). The aforementioned agonists, the pregnane X receptor ligand pregnenolone 16α-carbonitrile, and oxysterols (Sigmas) were dissolved in ethanol or Me3SO prior to addition to cells (<1 μM/ml medium). DNA modification and restriction enzymes were obtained from New England Biolabs and Invitrogen. 1-3H]farnesylpyrophosphate (100 μM) was purchased from ICN Biomedicals. Lipoprotein-deficient fetal bovine serum (LPDS) was purchased from Intracel Corp. (Rockville, MD). All of the other reagents have been described previously (27, 38, 39).

Cell Culture—Human monocytes were isolated from peripheral blood by elutriation and plated on 100-mm dishes at a density of 1 × 10⁶ cells/ml in Iscove’s modified Dulbecco’s medium in the presence of 30% autologous serum, 2.2% HCS, 10% human AB serum, and 1% antibiotics. The medium was changed on the third and sixth days. On day 8, the medium was replaced with Iscove’s modified Dulbecco’s medium supplemented with either 10% fetal bovine serum (FBS), 10% LPDS, or 10% LPDS and mevalonic acid (100 μM) in the presence of either 5 μM compactin (unloaded) or ligands for LXR (1 μM T or 5 μM 2R)-hydroxysteroid dehydrogenase (HSD) inhibitors (HSD-3′). HepG2 cells were maintained in modified Eagle’s medium containing 10% FBS as described (27).

Isolation of Murine Peritoneal Macrophages—Eight-week-old female C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME), and injected intraperitoneally with 1 ml of 4% thioglycolate solution (Difco) 4 days prior to harvesting macrophages. Briefly, the mice were sacrificed, and ice-cold high glucose Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 20 μg penicillin/streptomycin was injected into the peritoneal cavity of each mouse. This fluid was carefully withdrawn and centrifuged, and the cell pellet was resuspended in high glucose Dulbecco’s modified Eagle’s medium containing 10% FBS and penicillin/streptomycin. The cells were pooled and plated at 1.2 million cells/ml, and the macrophages were allowed to adhere for 4–6 h. The medium was then replaced with Dulbecco’s modified Eagle’s medium supplemented with 10% LPDS, 100 μM mevalonic acid, and either 5 μM compactin, T, and/or 0.1 μM LG, and the cells were incubated for 8–36 h, as indicated in the text and legends.

RNA Isolation and Microarray Analysis—Total RNA was isolated using Trizol Reagent (Invitrogen) and further purified by using an RNaseasy kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. The GenChip murine genome MG-U74Av2 microarrays were purchased from Affymetrix Inc. (Santa Clara, CA). RNA was isolated from duplicate dishes of murine peritoneal macrophages incubated in 10% LPDS and 100 μM mevalonic acid and either 5 μM compactin or 5 μM T and 100 μM LG. Four complementary RNA samples were prepared, and each was hybridized to an individual microarray. Preliminary data analysis was performed by the Microarray Core Facility at University of California at Irvine. Further analysis and data mining were performed using Affymetrix microarray suite 4.0, and GeneSpring4.0 (Silicon Genetics, Redwood City, CA). These analyses provide a signal for each specific gene/EST that is subsequently normalized by comparing to the median signal (arbitrary value of 1.0) obtained from the whole array. Genes/ESTs were considered to be present when RXR target genes were hybridized with ≥5.0 Plots of DNA probes as described previously (27). Transcript abundance was determined using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) standardized against 18 S RNA and mathematically adjusted to establish a unit of 1.0 for the unloaded control condition.

Reporter Genes—The constructs of human apoC-II proximal promoter (27), human ME.1, and ME.2 reporter genes (28) have been described. The human ME.1 (620 bp) or ME.2 (620 bp) were cloned into the BamHI site upstream of the human apoC-II promoter in the previously described apoC-II-luciferase reporter gene (27) to give ME.1-CII and ME.2-CII-luc, respectively. ME.1 was also cloned into the SmaI-Xmal sites upstream of ME.2-CII-luc to give ME.1-CII-luc and ME.2-CII-luc, respectively. ME.1 was also cloned into the StmaI-Xmal sites upstream of ME.2-CII-luc to give ME.1-2C-II-luc. The LRX in ME.2 was mutated by using the QuickChange site-directed mutagenesis kit (Stratagene) according to manufacturer’s instructions using primers 5′-caccagctgccagAAcactggcgAAcaaaggcag-3′ and 5′-ctggctttgTCTcgacgcatTctggcagctg-3′.

Transient Transfections and Reporter Gene Assays—Transient transfections of HepG2 cells were performed in triplicate in a 48-well plate using an MBS mammalian transfection kit (Stratagene) with minor modifications. The cells were transfected with 100 ng of a reporter construct, pCMX-β-galactosidase (50 ng), and either the following plasmids pCMX-LRαX (50 ng) and pCMX-RXRα (5 ng) or the control pTKCI (55 ng), using a total of 205 ng of DNA/well. After transfection, the cells were incubated for 24 h in modified Eagle’s medium containing 10% LPDS supplemented with 100 μM mevalonic acid in the presence of either 5 μM compactin or ligands for LXRα (1 μM T) or RXRα (100 μM LG), before lysis. The luciferase activities were measured with the
Regulation of ApoE/C-I/C-IV/C-II Gene Cluster by LXR

**TABLE I**

Representative list of genes involved in lipid metabolism that are induced following activation of macrophage LXR and RXR

| Gene       | Normalized expression from Affymetrix arrays | GenBank accession number |
|------------|---------------------------------------------|-------------------------|
|            | Unloaded cells | T + LG-treated cells | Fold change |
| SREBP-1    | 2.2            | 8.8                | 4.0          | A1843895 |
| FAS        | 1.7            | 6.2                | 3.6          | X13135  |
| LPL        | 4.7            | 25.7               | 5.5          | M03535  |
| ABCA1      | 1.1            | 16.4               | 14.4         | X75926  |
| ABCG1      | 3.4            | 12.7               | 3.7          | Z48745  |
| ApoE       | 83.4           | 158.8              | ∞            | D00466  |
| ApoC-I     | ND             | 4.4                | ∞            | Z22661  |
| ApoC-II    | 4.8            | 40.9               | 9.3          | Z22216  |
| ApoC-IV    | ND             | 0.5                | ∞            | Z24722  |

**Experimental Procedures.**

Murine peritoneal macrophages were incubated for 36 h in medium supplemented with 10% LPDS and either vehicle (MeSO) and 5 μM compactin (unloaded) or 5 μM T and 100 nM LG. Total RNA was isolated from duplicate dishes of control and treated cells and processed for hybridization to Affymetrix U74Av2 microarrays. The expression data derived from four microarrays were analyzed as described under “Experimental Procedures.” The expression signal for each gene/EST was normalized to the median signal arbitrary value = 1.0 of all sequences on the array. The normalized expression level of the indicated genes under the control (unloaded) condition or after treatment with ligands for LXR and RXR are shown in the second and third columns. The calculated fold change is shown in the fourth column. Fold change for apoC-1 and apoC-IV are infinite because these genes have nondetectable (ND) signals when the mRNA is derived from unloaded cells. The GenBank™ accession number of each sequence is given in the last column. SREBP-1, sterol regulatory element-binding protein-1; FAS, fatty acid synthase.

**Immunohistochemical Studies**—Heart tissue cryosections were obtained from an LDL receptor-deficient mouse that had consumed a high fat diet (TD 94059, Harlan Teklad; 15.8% fat and 1.25% cholesterol) for 16 weeks. The cryosections were fixed in acetone at −20 °C for 2 min and immersed in PBS for 2 min to rehydrate the tissues. All further incubations were performed at room temperature in a humid chamber.

The sections were incubated for 30 min in 10% goat serum diluted in PBS. After blot drying, the sections were incubated with rabbit anti-mouse apoC-II (1:2000 dilution of antiserum) or biotinylated F4/80 antibody (Zymed Laboratories Inc., South San Francisco, CA). For apoC-II staining, the slides were incubated with biotinylated goat anti-rabbit IgG (1:20 dilution of antiserum in PBS/bovine serum albumin and 0.15% Triton X-100). After thorough washings, endogenous peroxidase was blocked for 2 min with a blocking agent (Zymed Laboratories Inc., South San Francisco, CA). For apoC-II staining, the slides were incubated with biotinylated goat anti-rabbit IgG (1:20 dilution of antiserum in PBS/bovine serum albumin/Triton X-100 for 1 h. All of the sections were then exposed to Vectastain ABC Elite solution (Vector Laboratories) for 30 min and developed with 9-aminon-3-ethylene carbazole (Vector Laboratories). The sections were counterstained with hematoxylin, mounted with an aqueous mounting medium (Shandon, Lipshaw, PA), and photographed.

**RESULTS**

**Identification of LXR Target Genes in Murine Peritoneal Macrophages**—To identify novel genes that are activated by the LXR/RXR heterodimer, peritoneal macrophages were isolated from 8-week-old female C57BL/6 mice 4 days after thio- glycollate injection. The cells were cultured overnight in medium containing 10% FBS and subsequently cultured for 36 h in a cholesterol-poor medium (10% LPDS) supplemented with 100 μM mevalonic acid and either compactin and vehicle (MeSO) or the ligands for LXR (T) and RXR (LG). RNA was isolated and subsequently processed for hybridization to Affymetrix microarrays (MG-U74Av2), which contain probes representing over 12,000 murine genes and ESTs sequences. Analysis of the data using Affymetrix standard protocols and GeneSpring software (see “Experimental Procedures”) indicated that 70 genes/EST sequences met the following criteria: (i) the signal derived from cells treated with ligands for LXR and RXR was greater than the median signal on the array and (ii) the ratio of the signal from induced:control cells was ≥2-fold. Using these criteria, the identified genes include sterol regulatory element-binding protein-1, fatty acid synthase, lipoprotein lipase (LPL), ABCA1 and ABCG1 (two members of the ATP-binding cassette family of transporter proteins), and apoE (Table I). These six genes served as positive controls, because they have all been previously identified as LXR target genes (28, 31–34, 36, 37, 42, 43). Analysis of the data indicated that apoC-II also met these criteria, suggesting that it might represent a gene that was induced by ligands for LXR and RXR (Table I). Northern blot analyses demonstrated that apoC-II and apoE mRNA levels were induced 10.55 ± 4.4 and 7.19 ± 3.9-fold (mean ± S.D., n = 4; p < 0.05 compared with unloaded cells), respectively, when cells were treated with LXR and RXR ligands. A representative Northern blot is shown in Fig. 1A. Apolipoproteins E, C-I, C-IV, and C-II form a gene cluster in both humans and mice (1, 2). Interestingly, analysis of the Affymetrix data indicated that the mRNAs for apoC-I and apoC-IV were expressed at low levels in unloaded cells but appeared to be induced in cells treated with ligands for LXR and RXR (Table I). The induction of these latter two mRNAs was confirmed by Northern blot analysis (Fig. 1A), consistent with the coordinate regulation of the whole gene cluster in macrophages by ligands for LXR and RXR.

**Induction of Murine ApoE, C-I, C-IV, and C-II mRNAs by Activated LXR/RXR—**RXR is a ubiquitously expressed nuclear receptor that heterodimerizes with several other nuclear receptors (44). To confirm that the induction of the apoE/C-I/C-IV/C-II gene cluster was a result of activation of LXR/RXR, murine peritoneal macrophages were treated for 36 h with specific ligands for RXR (LG) and/or LXR (T). Northern blot analysis shown in Fig. 1A demonstrates that treatment of the cells with ligands for both LXR and RXR (T and LG) resulted in the induction of mRNAs encoding apoE (9.1-fold), apoC-II (14.4-fold), apoC-IV (2.3-fold), ABCA1 (10.55-fold), ABCG1 (8.8-fold), FAS (3.6-fold), pregnenolone 16α-carbonitrile, which are ligands for FXR and pregnane X receptor, respectively (data not shown). Surprisingly, treatment of murine peritoneal macrophages with li-
gands for both LXR and RXR induced apoE and apoC-II mRNAs to levels that were greater than those observed in mouse liver (Fig. 1B).

The data of Fig. 2 demonstrate that, in the presence of an RXR agonist, maximal induction of all four apolipoprotein genes occurred when the cells were incubated for 36 h with 30–100 nM T (an LXR ligand). In the absence of an RXR ligand, maximal induction of the four genes occurred in the presence of 100 nM LG. Northern blot analysis was as described above, using 4 μg of RNA/lane.

Together, these observations demonstrated that treatment of murine peritoneal macrophages with ligands for LXR and/or RXR induce the expression of all four members of the apoE/C-I/C-IV/C-II gene cluster.

Induction of ApoE/C-I/C-IV/C-II Gene Cluster by LXR Is Attenuated in LXR Null Mice—To demonstrate that induction of murine peritoneal macrophages with ligands for LXR and/or RXR induce the expression of all four members of the apoE/C-I/C-IV/C-II gene cluster.

Induction of ApoE/C-I/C-IV/C-II Gene Cluster by LXR Ligand Is Attenuated in LXR Null Mice—To demonstrate that induction of the apoE/C-I/C-IV/C-II gene cluster is dependent on LXR, peritoneal macrophages were isolated from wild type, LXRα−/−, LXRβ−/−, and LXRαβ−/− mice and incubated for 48 h in the presence of ligands for LXR (T) and/or RXR (LG). Real time quantitative PCR (Taqman) assays were utilized to determine the relative expression levels of all four target genes (Fig. 4). Consistent with a previous report by Laffitte et al. (28), the data demonstrate that (i) apoE mRNA levels are induced
the LXR ligand (T) in cells derived from wild type, LXR<sup>-/-</sup>, or LXR<sup>β/-/-</sup> mice, (ii) the induction of apoE is further enhanced when cells from mice of these three genotypes were incubated with ligands for both LXR and RXR, and (iii) apoE mRNA levels are not induced when LXR<sup>-/-</sup> cells were incubated with ligands for LXR and/or RXR (Fig. 4).

Analysis of the data shown in Fig. 4 further demonstrates that the induction of apoC-I, apoC-II, and apoC-IV mRNA levels in response to T, the LXR ligand, was also attenuated in LXR<sup>-/-</sup> macrophages. However, there are a number of interesting differences in the response of the four genes to LXR and/or RXR ligands. For example, the induction patterns of apoC-II and apoC-IV mRNA were similar to each other; both transcripts were induced when macrophages, derived from either wild type, LXR<sup>-/-</sup>, or LXR<sup>β/-/-</sup> mice, were treated with the LXR ligand in the presence or absence of the RXR ligand (Fig. 4). In contrast, no induction of the apoC-II and apoC-IV mRNAs was observed when LXRβ<sup>-/-</sup> macrophages were incubated with the LXR ligand (Fig. 4). However, in contrast to the results with apoE, we noted that apoC-II and apoC-IV mRNAs were induced -2-fold when LXRβ<sup>-/-</sup> cells were incubated with LG (Fig. 4). Consequently, we conclude that apoC-II and apoC-IV genes can also be activated by an LXR-independent pathway that involves RXR.

ApoC-I mRNA levels were highly induced when wild type or LXR<sup>-/-</sup> macrophages were incubated with T and/or LG (Fig. 4). In contrast, the induction of apoC-I mRNA levels was significantly reduced or abolished when LXRβ<sup>-/-</sup> or LXR<sup>β/-/-</sup> macrophages, respectively, were treated with either LXR or RXR ligands (Fig. 4). These data suggest that regulation of apoC-I is more strictly dependent on the LXRβ/RXR heterodimer and that LXR<sup>-/-</sup> cannot fully substitute for LXRβ to activate the gene. Despite these minor differences, the studies of Fig. 4 clearly demonstrate that induction of each member of the apoE/C-I/C-IV/C-II gene cluster mRNA levels in response to an LXR ligand is greatly attenuated in macrophages derived from LXR<sup>-/-</sup> mice.

Human ApoE, C-I, C-IV, and C-II mRNAs Are Induced in Primary Macrophages Following Activation of LXR—The genomic organization of the apoE/C-I/C-IV/C-II gene cluster is largely conserved between mouse and human with the exception that in mouse there has been no duplication of the 10 kb genomic region containing ME.1, apoC-I, and HCR.1 (Fig. 5A) (2). In humans, this duplication gives rise to ME.2, the pseu-
dogene apoC-I', and HCR.2 (Fig. 5A). Fig. 5B shows the alignment of the potential LXREs that are contained within the human and mouse ME regions. The sequence of the mutated human ME.2 used in a reporter construct is also shown. The locations of these sequences relative to the 5' end of each ME are given (25). The mutant LXRE is also shown; mutated nucleotides are shown as underlined lowercase letters. C, human monocytes/macrophages were isolated and cultured as described under “Experimental Procedures.” Briefly, the cells were cultured in Iscove’s modified Dulbecco’s medium supplemented with either 10% FBS, 10% LPDS, or 10% LPDS and 100 μM mevalonic acid in the presence of either 5 μM compactin (unloaded) or ligands for LXR (either 1 μM T or 5 μM 22(R)-hydroxycholesterol) and/or RXR (100 nM LG). Northern blot analysis was carried out as described in the legend to Fig. 1. Similar results were obtained in two additional experiments. The results of quantitation of apoC-I mRNAs are not given because no signal was detectable in the control sample.

LXR Activates Transcription via LXREs in the Multienhancer Regions of the ApoE/C-I/C-IV/C-II Gene Cluster—The human apoE gene cluster contains two multienhancer regions, ME.1 and ME.2, which are 95% identical over the 620-bp sequence (24). Shih et al. used transgenic mice to demonstrate that ME.1 and ME.2 control the expression of apoE in a number of tissues, including macrophages (24). Recently, Laffitte et al. (28) reported that LXR/RXR binds to a newly identified LXRE that lies within both the human ME.1 and ME.2 (Fig. 5B). In addition, these authors reported that ligand activation of the LXR/RXR heterodimer activates reporter constructs under the control of human ME.1 or ME.2 fused to the apoE-proximal promoter (28).

We hypothesized that the same element may be important for regulated transcription of apoC-II, apoC-I, and apoC-IV in response to activated LXR. To test this hypothesis, we constructed luciferase reporter genes under the control of 600 bp of the human apoC-II proximal promoter (27) or both promoters containing sequences corresponding to wild type ME.1 and/or ME.2 or a mutant ME.2 containing a 4-bp mutation in the LXRE (Figs. 5B and 6). Each reporter construct was transiently transfected into HepG2 cells in the presence or absence of plasmids encoding RXR and LXR, and the cells were then incubated for 24 h in the presence of Me2SO or the nuclear receptor ligands T, LG, or T and LG. The data of Fig. 6 show...
that there was little or no induction of the reporter gene that was under the control of the apoC-II proximal promoter under any of the conditions tested. In contrast, the addition of either ME.1 or ME.2 to the apoC-II proximal promoter (ME.1-CII-Luc and ME.2-CII-Luc, respectively) resulted in the induction of the reporter genes in response to ligands for LXR and/or RXR (Fig. 6). In numerous experiments, we noted that the relative level of expression of ME.2-CII-Luc was greater than that of ME.1-CII-Luc (Fig. 6). Inclusion of both ME.1 and ME.2 in the reporter construct results in an additive effect on luciferase activity (Fig. 6; ME.1-ME.2-CII-Luc). In contrast, a reporter construct containing 4-bp mutations in the LXRE of ME.2 was completely unresponsive to ligands for LXR and/or RXR (Fig. 6; ME.2mut-apoC-luc). These data support the hypothesis that the LXREs contained within the two multienhancers are necessary for the LXR-dependent transcriptional activation of the genes encoding apoC-II (Fig. 6), apoE, apoC-I, and apoC-IV in response to ligands for LXR/RXR.

**Immunohistochemical Localization of apoC-II in Murine Arterial Lesions**

The data of Figs. 1–6 demonstrate that the mRNA of apoC-II is induced in isolated murine peritoneal macrophages and human monocyte macrophages by an LXR-dependent process. To investigate the possible physiological significance of these findings, we obtained mouse aortic root sections from LDL receptor-deficient mice that had been fed a high fat diet for 16 weeks. Sequential sections were immunostained using antibodies specific for either murine apoC-II (A) and murine macrophages (F4/80) (B). Primary antibody was omitted in C. Magnification for each panel is 40×. EC, endothelial cells; SMC, smooth muscle cells; Mφ, macrophages.

**FIG. 6.** The multienhancers ME.1 and ME.2 contain an LXRE that is sufficient to activate an apoC-II reporter gene in response to ligands for LXR/RXR. HepG2 cells were transiently transfected in triplicate with 100 ng of the indicated human apoC-II promoter-reporter gene construct in the presence or absence of plasmids encoding LXRα (50 ng) and RXRα (5 ng). The cells were incubated for 24 h in the presence of dimethyl sulfoxide (DMSO; unloaded), an RXR ligand (100 nM LG), and/or an LXR ligand (1 μM T), as indicated. The cell lysates were prepared and assayed for reporter gene activity and normalized for minor variations in transfection efficiency, as described under “Experimental Procedures.” The relative luciferase activities are given after normalization. Similar results were obtained in three additional experiments.

**FIG. 7.** ApoC-II co-localizes with macrophages in mouse aortic lesion sections. Atherosclerotic lesions obtained from the aortic root of LDL receptor-deficient mice were analyzed by immunohistochemistry. Cross-sections containing fatty streak lesions were stained for murine apoC-II (A) and murine macrophages (F4/80) (B). Primary antibody was omitted in C. Magnification for each panel is 40×. EC, endothelial cells; SMC, smooth muscle cells; Mφ, macrophages.
apoC-II mRNA levels are induced 15 reported. The current finding that apoC-I, apoC-IV, and expression of apoC-IV mRNA in macrophages has not been extremely low levels of apoC-I mRNA (48). To our knowledge, the antibody to apoC-II was highly specific and cross-reacted with only one protein (apoC-II) in murine plasma (data not shown).

**DISCUSSION**

Lipid-loaded macrophages, or foam cells, are found in both fatty streaks and more advanced atherosclerotic lesions. These cells are thought to have a critical but poorly understood role in the development of atherosclerosis (reviewed in Refs. 45 and 46). Recent studies with macrophages have shown that activation of endogenous LXR and RXR induces the expression of a number of genes involved in lipid metabolism; such genes include ABCA1, ABCG1, fatty acid synthase, apoE, sterol regulatory element-binding protein-1c, and LPL (28, 31, 34, 36, 37, 42, 43) (Table I).

In the current report we have used murine and human macrophages to demonstrate that all members of the apoE/C-I/C-IV/C-II gene cluster are highly induced following activation of LXR and/or RXR. In addition, we demonstrate that the LXREs within the multienhancer regions ME.1 and ME.2 are necessary for this activation. This result is consistent with an earlier publication demonstrating that the two LXREs located within the human ME.1 and ME.2 sequences are necessary for activation of the apoE gene in response to LXR ligands (28).

The apoE/C-I/C-IV/C-II gene cluster encodes four apolipoproteins with diverse functions. Through a series of elegant studies using transgenic mouse models, Taylor and coworkers (21–23, 47) demonstrated that the HCRs control the expression of all four genes in the liver, whereas the 620-bp MEs direct the expression of apoE in multiple tissues (24–26). However, the role of ME.1 and ME.2 in controlling the expression of apoC-I, apoC-IV and apoC-II in specific tissues has not been addressed. The current study identifies the LXREs within the two enhancers that are required for induction of apoC-II, and presumably all members of this gene cluster, in response to activated LXR. Utilization of macrophages derived from LXR null mice confirms the importance of LXREs and LXRβ in these inductive processes. Surprisingly, studies with the LXR null cells identify some important differences in the induction of different members of this gene cluster. For example, no induction of apoE or apoC-I was observed under any conditions tested with the LXRα/β null macrophages (4). In contrast, apoC-II and apoC-IV mRNAs were induced 2–3-fold by LQ, the RXR ligand (Fig. 4), implying that these two genes can be induced by activated RXR via a second process that is independent of LXR.

Macrophages have previously been reported to express extremely low levels of apoC-I mRNA (48). To our knowledge, the expression of apoC-IV mRNA in macrophages has not been reported. The current finding that apoC-I, apoC-IV, and apoC-II mRNA levels are induced 15–140-fold in LXR-activated cells implies that these apolipoproteins may have key functions in the artery wall. It was particularly surprising to note that the induced apoC-II and apoC mRNA levels in LXR-activated macrophages are greater than the levels measured in normal murine liver (Fig. 1B). The immunostaining studies confirm that macrophages within the fatty lesions of the artery wall express apoC-II (Fig. 7). Additional studies will be required to demonstrate that the secretion of both apoC-I and apoC-II proteins is induced when macrophages within the artery wall become lipid-loaded foam cells. Such studies are not possible until antibodies specific for murine apoC-I and apoC-II become available.

Why might this apolipoprotein gene cluster be induced when macrophages become lipid-loaded and accumulate LXR ligands, such as oxysterols? A number of studies have shown that activation of LXR by accumulating oxysterols within the cell induces a number of genes involved in lipid metabolism. One such gene encodes ABCA1 that is involved in the transport of phospholipids and cholesterol to lipid-poor protein acceptors, such as apoA-I (reviewed in Ref. 49). However, it has also been reported that other apolipoproteins, including apoC-I, apoC-II, and apoE, also can function as acceptors and promote cholesterol efflux from cultured cells by a process that likely involves ABCA1 (50). Thus, the LXR-dependent increase in the macrophage expression of apoE, apoC-I, apoC-II, and apoC-IV could result in increased levels of apolipoproteins in the subendothelial space that function to promote the efflux of cellular cholesterol and phospholipids.

Recent studies have also shown that treatment of macrophages with ligands for LXR leads to increased expression of LPL (37) (Fig. 1). Because apoC-II is an obligate co-factor for LPL, we hypothesize that the dual secretion of apoC-II and LPL from LXR-activated macrophages will result in high local lipase activity. Further studies will be necessary to determine whether this macrophage-derived LPL functions to modify small VLDL, intermediate density lipoprotein (51), or high density lipoprotein in the subendothelial space.

**Acknowledgments—We thank Drs. R. Evans, R. Heyman, P. Maloney, K. Weisgraber, and T. Willson for providing plasmids and reagents. We thank Dr. Heidi Kast-Woelbern for plasmids and for helpful discussions. We thank members of the Edwards and Tontonoz laboratories for critical comments during these studies.**

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