Interleukin-6 Stimulates LDL Receptor Gene Expression via Activation of Sterol-Responsive and Sp1 Binding Elements

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Abstract—Inflammatory or malignant diseases are associated with elevated levels of cytokines and abnormal low density lipoprotein (LDL) cholesterol metabolism. In the acute-phase response to myocardial injury or other trauma or surgery, total and LDL cholesterol levels are markedly decreased. We investigated the effects of the proinflammatory cytokine interleukin (IL)-6 on LDL receptor (LDL-R) function and gene expression in HepG2 cells. IL-6 dose-dependently increased the binding, internalization, and degradation of 125I-LDL. IL-6–stimulated HepG2 cells revealed increased steady-state levels of LDL-R mRNA. In HepG2 cells transiently transfected with reporter gene constructs harboring the sequence of the LDL-R promoter extending from nucleotide −1563 (or from nucleotide −234) through −58 relative to the translation start site, IL-6 dose-dependently increased promoter activity. In the presence of LDL, a similar relative stimulatory effect of IL-6 was observed. Studies using a reporter plasmid with a functionally disrupted sterol-responsive element (SRE)-1 revealed a reduced stimulatory response to IL-6. In gel-shift assays, nuclear extracts of IL-6–treated HepG2 cells showed an induced binding of SRE binding protein (SREBP)-1a and SRE binding protein(SREBP)-2 to the SRE-1 that was independent of the cellular sterol content and an induced binding of Sp1 and Sp3 to repeat 3 of the LDL-R promoter. Our data indicate that IL-6 induces stimulation of the LDL-R gene, resulting in enhanced gene transcription and LDL-R activity. This effect is sterol independent and involves, on the molecular level, activation of nuclear factors binding to SRE-1 and the Sp1 binding site in repeat 2 and repeat 3 of the LDL-R promoter, respectively. (Arterioscler Thromb Vasc Biol. 2000;20:1777-1783.)

Key Words: transcription factors ■ cholesterol ■ lipoproteins ■ receptors ■ hypcholesterolemia

Elevated levels of interleukin (IL)-6 and other cytokines play a key role in the acute-phase reaction evoked by myocardial infarction, surgery, or infection. The acute-phase response induced by these disease states produces hypcholesterolemia in humans and nonhuman primates; this effect on lipoprotein metabolism appears to be mediated by cytokines. Systemic infusion of cytokines lowers serum cholesterol in animals and humans. Cytokines may affect plasma levels of cholesterol and lipoproteins by modulating the synthesis and secretion of apolipoproteins, lipolytic enzyme activities, or the expression of lipoprotein receptors.

One of the major determinants of plasma cholesterol levels is the activity of the LDL receptor (LDL-R). The LDL-R mediates the cellular uptake and degradation of plasma LDL. It is expressed in almost all organs and cell types. However, more than two thirds of the LDL-Rs in humans are present in the liver.

Expression of the LDL-R gene is predominantly controlled at the transcriptional level by feedback repression depending on intracellular sterol content. Transcription of the LDL-R gene is controlled by 3 imperfect direct repeats, designated repeats 1 to 3, within the LDL-R promoter. Repeats 1 and 3 represent binding elements for Sp1 or other members of the GC-box transcription factor family and appear to be constitutively positive elements. Repeat 2, sterol-responsive element (SRE)-1, is directly responsible for sterol regulation by acting as a conditionally positive element after binding of sterol regulatory element binding proteins (SREBPs). SREBP-1 and SREBP-2 are 2 members of the family of basic helix-loop-helix leucine zipper (bHLH-ZIP) nuclear proteins, which are highly homologous to each other. Both are synthesized as 125-kDa precursors that are embedded in the membranes of the endoplasmic reticulum and the nuclear envelope. In the absence of sterols, the amino-terminal part of the SREBPs harboring the DNA binding and transcription regulatory domains is proteolytically cleaved from its carboxy-terminal membrane-spanning domain. The truncated amino-terminal parts of the SREBPs translocate to the nucleus and bind to SRE-1 in target genes, resulting in enhanced gene expression. A number of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, IL-1β, and oncostatin M (OM), have

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been shown to increase LDL-R gene expression. Modulation of LDL-R transcription by these cytokines appears to occur through different mechanisms. TNF-α and IL-1β are capable of increasing LDL-R gene transcription only when cells are deprived of sterol, suggesting that both cytokines regulate LDL-R gene expression by a sterol-dependent mechanism.19 In contrast, OM, a cytokine predominantly produced by activated T cells and macrophages, stimulates LDL-R gene expression in HepG2 cells independent of the intracellular cholesterol level.20

IL-6 is a proinflammatory cytokine released by monocytes, endothelial cells, fibroblasts, and other cells during trauma, injury, and infection. It is a member of a family of cytokines including leukemia-inhibiting factor (LIF), OM, and IL-11. The IL-6 receptor consists of 2 types of subunits, the ligand-binding glycoprotein gp80 and the signal transducer gp130.21 On binding to its receptor, IL-6 induces the synthesis of acute-phase plasma proteins in liver cells.22 In the present study, we examine the action of IL-6 on LDL metabolism and LDL-R gene expression in HepG2 cells. We demonstrate that IL-6 induces LDL-R mRNA and activity in HepG2 cells. Furthermore, our findings indicate that the effect of IL-6 involves the synergistic activation of SRE-1 and Sp1 binding proteins, independent of the cellular sterol content.

Methods

Cell Culture and Reagents

HepG2 cells were grown as a monolayer culture in DMEM supplemented with 10% (vol/vol) FCS (Seromed) and 20 U/mL penicillin/streptomycin (Seromed) in a humidified 5% CO2 atmosphere.

The reporter plasmids pDLDR-CAT1563 and pDLDR-CAT234 were kindly provided by Dr D. Russell, University of Texas, Dallas. Recombinant human IL-6 was obtained from R&D Systems; antibodies for the supershift experiments SREBP-2(N19), anti–SREBP-1(IgG2A4), from Pharmingen; double-stranded poly(dI-dC) as sodium salt, from Amersham Pharmacia. The reporter gene construct plDLDR33, harboring 2 neighboring repeat 3 elements upstream from the native TATA-like element of the LDL-R promoter, was obtained by polymerase chain reaction amplification with the plasmid pDLDR234 as template and the sense primer 5'-CCCAACTTTAAAACTCTCTCCCTCGCAAACCTCTCTCCCCCTGCGTAG3'-containing the 2 repeat 3 elements (italic letters) and additional bases at the 5' end (underlined sequence) to generate a HindIII restriction site.

Promoter Assay

HepG2 cells were plated in 48-well polystyrene plates. Cells were transiently transfected with the reporter gene constructs (0.5 µg per well) by lipofection (Tfx50, Promega) for 4 hours. To normalize for transfection efficacy, a control plasmid harboring the Renilla luciferase gene driven by the viral SV40 promoter (pRL-SV40, Promega) was cotransfected. Transfected cells were treated with low-serum medium containing IL-6 at different concentrations in the presence or absence of sterols for 24 hours and lysed, and firefly and Renilla luciferase activities were determined by use of the Dual Luciferase Assay (Promega) on a luminometer (Lumat LB9501, EG&G Bertold).

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Analysis

HepG2 cells were seeded in 25-cm2 polystyrene flasks. After stimulation with IL-6 (25 ng/mL), nuclear extracts were prepared according to the method of Schreiber et al.,26 with slight modifications in buffer compositions (buffer A contained 10 mmol/L HEPES, 10 mmol/L KCl, 1.5 mmol/L MgCl2, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, and 1 mmol/L dithiothreitol; buffer C contained 20 mmol/L HEPES, 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, and 1.5 mmol/L MgCl2; both were supplemented with proteinase inhibitors [Complete, Roche Biochemicals]). To investigate the effect of sterol-suppressive conditions, cells were preincubated for 12 hours in the presence or absence of 10 µg/mL cholesterol and 1 µg/mL 25-OH cholesterol in low-serum medium. Protein concentrations were typically 3 to 5 µg/µL.

Oligonucleotides, containing repeat 2 or repeat 3 of the human LDL-R promoter and a 5'-TTGG extension (MWG Biotech), were annealed and labeled by random priming as described above. The oligonucleotide sequences were as follows, with the consensus binding sites underlined: repeat 2, 5'-TTGGAAAACTACCCACCTTGCA-3'; repeat 3, 5'-TTGGCTGCAAACCTCCTCCCTGCTTAG-3'.

Each binding reaction contained 4 to 8 µg nuclear protein and 0.5 to 1 ng 32P-labeled double-stranded oligonucleotide probe (5×105 to 10×105 cpm) in a final volume of 20 µL. Nuclear proteins were incubated for 30 minutes at room temperature in binding buffer: the buffer for repeat 2 contained 10 mmol/L HEPES, 0.5 mmol/L MgCl2, 80 mmol/L KCl, 1 mmol/L dithiothreitol, 10% glycerol (vol/vol), 0.3
mg/mL BSA, 50 ng/mL poly(dI-dC) · poly(dI-dC), and for repeat 3 contained 12.5 mmol/L HEPES, 6 mmol/L MgCl₂, 5.5 mmol/L EDTA, 50 mmol/L KCl, 0.5 mmol/L dithiothreitol, 10% glycerol (vol/vol), 0.25 mg/mL BSA, and 50 ng/mL poly(dI-dC) · poly(dI-

Results

IL-6 increased the binding of ¹²⁵I-labeled LDL at 4°C to HepG2 cells in a dose-dependent manner (Figure 1A). Maximum stimulation was 1.4-fold at 25 ng/mL IL-6. The increase in binding was accompanied by enhanced internalization (Figure 1B) and degradation of ¹²⁵I-LDL (Figure 1C). At 25 ng/mL IL-6, maximum stimulation of internalization and degradation was 1.4-fold and 1.3-fold, respectively. For comparison, lovastatin (10⁻⁶ mol/L), a potent 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitor, induced the binding of ¹²⁵I-labeled LDL 2-fold in our assay (data not shown).

Transcription of the LDL-R gene responds to stimuli initiating cell cycle traverse, which could be mediated by SRE-1. Growing cells create an additional need for cholesterol to support the synthesis of membranes and express maximum LDL-R activity, so that the IL-6–induced increase in LDL-R activity could result from a mitogenic effect of IL-6. In contrast to medium supplemented with 10% FCS, IL-6 did not induce cell proliferation under conditions used to study LDL-R expression in a nonradioactive cell proliferation assay (CellTiter96, Promega; see Figure I, which can be accessed online at http://atvb.ahajournals.org).

To assess whether the IL-6–induced increase in LDL-R activity was due to an increase in the steady-state level of LDL-R mRNA, HepG2 cells were stimulated with IL-6 (25 ng/mL) for up to 9 hours, and LDL-R mRNA was measured by Northern blot analysis. IL-6 treatment produced a rapid increase in LDL-R mRNA in HepG2 cells with a maximum of 5-fold at 2 hours (Figure 2).

The determination of the decay of LDL-R mRNA in IL-6–stimulated HepG2 cells by Northern blot analysis with an incubation of HepG2 cells with IL-6 (25 ng/mL) for 2 hours and subsequent incubation with actinomycin D (5 μg/mL) revealed that blockade of transcription with actinomycin D reduced the level of LDL-R mRNA over time in cells incubated in the presence or absence of IL-6. The half-lives of LDL-R mRNA did not significantly differ in the presence and absence of IL-6 and were ≈1 hour and 1.5 hours, respectively (Figure II, which can be accessed online at http://atvb.ahajournals.org). Hence, the induction of LDL-R mRNA in HepG2 by IL-6 was not due to increased mRNA stability, and the observed upregulation occurred at the transcriptional level.

We also examined the role of de novo synthesis of protein in the IL-6–mediated stimulation of LDL-R mRNA. Consistent with previous observations, treatment of HepG2 cells with cycloheximide alone increased LDL-R mRNA by ≈2-fold (Figure III, which can be accessed online at http://atvb.ahajournals.org). Cycloheximide pretreatment (5 μg/mL) did not prevent the upregulation of LDL-R mRNA by IL-6,
To identify the elements of the LDL-R promoter responsible for the upregulation of LDL-R mRNA, we generated reporter gene constructs consisting of parts of the 5′-flanking region of the LDL-R gene and the firefly luciferase gene. In the construct pLDLR1563, the luciferase gene was placed under control of a LDL-R promoter fragment extending from positions 58 to 1563 relative to the start site of translation. This fragment harbors repeats 1 through 3, known to be essential for the regulation of LDL-R gene transcription.13 The basal activity of this construct did not significantly differ from the activity of the wild-type construct pLDLR234. After transient transfection of the mutant LDL-R construct and stimulation for 24 hours, the stimulatory effect of IL-6 was decreased to 1.5-fold (Figure 3) compared with the constructs harboring the respective wild-type promoter region (2.4-fold, Figure 3). This indicates that SRE-1 participates in the IL-6–mediated stimulation but also suggests that other promoter elements may concomitantly be involved.

For this reason, we wished to examine the role of the Sp1 binding site in repeat 3 in the IL-6–induced stimulation of the LDL-R promoter. Mutations in either repeat 1 or 3, abolishing Sp1 binding, markedly reduce the transcription of LDL-R in sterol-depleted and in sterol-suppressed cells, indicating that intact Sp1 binding sites are required for basal and induced LDL-R promoter activity.13,14 We produced a reporter gene construct, pLDLRR33, harboring 2 tandem cop
ies of repeat 3. The basal activity of this construct was lower than the activities of the constructs pLDLR1563 and pLDLR234. The activity of this construct was increased to 1.9-fold of control at 25 ng/mL IL-6 (Figure 4). As expected, the activity of pLDLR33 was not regulated by cholesterol. These data provide evidence that the effect of IL-6 on the LDL-R promoter is mediated in part by repeat 3.

Electrophoretic mobility shift assays were performed to examine whether IL-6 stimulation leads to an enhanced binding of nuclear proteins to repeat 2 or 3 in the LDL-R promoter. Incubation of nuclear extracts of IL-6–treated HepG2 cells with 32P-labeled oligonucleotide containing repeat 2 showed that IL-6 increased the formation of a repeat 2/protein complex. By supershift analysis, these proteins were identified as SREBP-1a and SREBP-2 bound to repeat 2 (Figure 5, lanes 5 and 6). Complex formation started within 30 minutes of incubation, was maximal after 60 minutes, and declined thereafter (Figure 5, lanes 7 through 11). Binding of nuclear proteins to repeat 2 was also detected when cells were incubated with an excess of sterols (10 μg/mL cholesterol and 1 μg/mL 25-OH cholesterol), showing that this activation of repeat 2 was sterol independent (Figure 5, lanes 12 through 16). Binding was specific, because the formation was inhibited by competition with 40-fold molar excess of unlabeled repeat 2 32P-oligonucleotide (Figure 5, lane 3).

Three complexes of nuclear proteins with repeat 3 were detected on IL-6 stimulation. All of them were competed by the addition of 40-fold molar excess of unlabeled repeat 3 oligonucleotide (Figure 6, lane 3). The slowly migrating complex comigrated with recombinant human Sp1 bound to repeat 3 (Figure 6, lane 7). Antibody supershift analyses (Figure 6, lanes 4 through 6) identified these proteins as Sp1 (slowly migrating complex) and Sp3 (fast migrating complex) homodimers and Sp1/Sp3 heterodimer (intermediate complex). Binding of these 2 members of the Sp1 family of transcription factors to the Sp1 binding sequence in repeat 3 occurred as early as 90 minutes after the addition of IL-6, declining thereafter (Figure 6, lanes 9 through 13). These data are in line with the promoter assays and show that IL-6 stimulates the binding of nuclear proteins to the sterol-responsive element in repeat 2 and to the Sp1 binding site in repeat 3. Thus, the stimulatory effect of IL-6 on the LDL-R appears to involve at least 2 members of the Sp1 family of transcription factors (Sp1 and Sp3) and SREBP-1a and SREBP-2.

**Discussion**

Elevated plasma levels of proinflammatory cytokines, including IL-6, TNF-α, IL-1β, and interleukins, are found in inflammatory, infectious, and malignant diseases and have been implicated in the abnormal lipid metabolism in these disease states. Our data clearly show that one mechanism by which IL-6 affects lipid metabolism is the upregulation of the LDL-R in hepatic cells, which may eventually lead to a

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**Figure 4.** Promoter activity assay of a construct harboring 2 tandem copies of repeat 3 of LDL-R (pLDLRR33) in HepG2 cells stimulated with IL-6. After preincubation with low-serum medium for 24 hours, HepG2 cells were transiently cotransfected with pLDLRR33 and pRL-SV40 for 4 hours. Cells were subsequently stimulated with IL-6 in the presence or absence of sterols (10 μg/mL cholesterol and 1 μg/mL 25-OH cholesterol) for 24 hours or with lovastatin (10–6 mol/L), and luciferase activities were determined. The promoter activity, relative to transfection, of the control cells was set as 1. Data represent mean ± SEM of 4 independent experiments, each performed in quadruplicate. *P<0.05 vs control.

**Figure 5.** Electrophoretic mobility shift analysis with repeat 2 (Rep.2) of the LDL-R promoter and nuclear proteins derived from HepG2 cells stimulated with IL-6. Con indicates control. After preincubation with low-serum medium for 24 hours, HepG2 cells were stimulated with IL-6 (25 ng/mL) for 30, 60, 90, 120, and 180 minutes, followed by extraction of nuclear proteins. To investigate the influence of excess sterols, cells were incubated with sterols (10 μg/mL cholesterol and 1 μg/mL 25-OH cholesterol) 12 hours before stimulation. 32P-labeled oligonucleotides of Rep.2 of the LDL-R promoter were incubated with nuclear extracts, and free and bound probes were electrophoretically separated in a nondenaturing polyacrylamide gel.
LDL-R promoter activity, we wished to address the possibility that IL-6 might also act through Sp1 binding elements in repeats 1 and 3 of the LDL-R promoter. Dawson et al \(^1\)4 and others \(^2\)0 have shown that a nonfunctional repeat 3 leads to a loss of basal transcription. Consequently, we examined the effect of IL-6 on repeat 3 by using a promoter harboring a tandem copy of repeat 3 and the TATA-like sequence of the LDL-R promoter (pLDLR33) as described by others. \(^2\)0 The expression of this construct was inducible with IL-6 (although to a lesser extent than the wild-type construct) and, as expected, did not depend on cholesterol levels within the cell (Figure 4). These results indicate that the IL-6–dependent signal is partly mediated by sterol-independent activation of repeat 3 in the LDL-R promoter.

It has been shown that the transcription factors SREBP-1 and Sp1 contribute synergistically to the activation of the LDL-R promoter. Initially, SREBP stimulates the binding of Sp1 to its adjacent recognition site, and then both proteins activate transcription more efficiently than either alone. \(^2\)4 Thus, the diminished promoter activity of our repeat 2 mutant (pLDLR234/−152G) could be due to the loss of synergistic action of transcription factors binding to repeats 2 and 3 rather than to a stimulatory effect on repeat 3. Therefore, we separately investigated the binding of nuclear proteins to repeats 2 and 3 in gel-shift assays with nuclear extracts of IL-6–stimulated HepG2 cells. With repeat 2 used as a labeled probe, IL-6 induced the formation of one DNA/protein complex that reached a maximum concentration after 60 minutes of incubation (Figure 5). The increased binding of nuclear protein to repeat 2 was of the same magnitude even in the presence of sterols. These nuclear proteins were identified as the members of the SREBP family, SREBP-1a and SREBP-2. Gel-shift experiments using repeat 3 as a labeled probe revealed the formation of 3 DNA/protein complexes that were maximally induced 90 minutes after the addition of IL-6 (Figure 6). Through supershift analyses, these proteins were identified as Sp1 and the Sp1-related transcription factor Sp3, respectively. Hence, IL-6–dependent activation of the LDL-R involves the binding of Sp1 and Sp3 to repeat 3 and, at the same time, stimulates the binding of SREBP-1a and SREBP-2 to repeat 2. Interestingly, activation of the SRE-1 in repeat 2, the element predominantly regulated by sterols, by IL-6 appears to be unaffected by the cellular content of sterols (Figure 5). This finding is in contrast to the mechanism of LDL-R gene activation described for OM. Although IL-6 and OM share the same signal-transducing protein gp130 and both cytokines stimulate LDL-R in a sterol-independent manner, OM stimulation exclusively involves repeat 3 as a downstream target of mitogen-activated protein kinase kinase (MEK)/extracellular signal–regulated kinase (ERK) activation. \(^3\)0,\(^3\)5 A further difference between OM and IL-6 is that only IL-6 directly enhances the binding of Sp1 and Sp3 to repeat 3, whereas OM does not directly upregulate Sp1 binding activity. \(^3\)0 Thus, our data stand in contrast to the speculation by Li et al \(^3\)5 suggesting that IL-6 and OM upregulate LDL-R by a similar mechanism.

How IL-6 enhances the binding of nuclear proteins to repeats 2 and 3 is not clear at present. Because the effect of IL-6 on LDL-R gene expression does not require de novo synthesis of protein, a rather direct action of IL-6 on nuclear factors that bind to specific elements in the promoter is probable. It is conceivable that one or more kinases activated...
by IL-6 may alter the phosphorylation state of the nuclear proteins binding to the SRE-1 and Sp1 binding sites, thereby modulating their potency to stimulate LDL-R transcription. Alternatively, proteins that are involved in the maturation and degradation of these nuclear factors may be regulated by IL-6–activated kinases.

In summary, the present study has demonstrated that IL-6 activates LDL-R transcription by enhancing the binding of SREBP-1a and SREBP-2 as well as the binding of Sp1 and Sp3 to their cognate DNA sequence in repeat 2 and repeat 3 of the LDL-R promoter. Consequently, the LDL-R activity on the surface of liver cells is enhanced, leading to an increased uptake of LDL from the circulation. These data are consistent with the hypothesis that hypocholesterolemia after myocardial injury, surgery, or infection is partly due to an enhanced catabolism of LDL in the liver.

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