**Cytokine Regulation of Low Density Lipoprotein Receptor Gene Transcription in HepG2 Cells**

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Elevated plasma levels of cytokines have been demonstrated in inflammatory, malignant, and infectious diseases. These disease states are often associated with abnormal lipid metabolism and reductions in plasma cholesterol levels. To determine if inflammatory cytokines could influence hepatic lipid metabolism, we evaluated low density lipoprotein (LDL) receptor function and gene expression in cytokine-stimulated HepG2 cells, a hepatoblastoma-derived cell line which shares many functional similarities with normal hepatocytes. Tumor necrosis factor-α (TNF) and interleukin-1β (IL-1) increased LDL binding to HepG2 cells in a dose-responsive manner. Other cytokines including macrophage-colony stimulating factor, granulocyte macrophage-colony stimulating factor, and γ-interferon had no significant effects on LDL binding. Increased binding in response to TNF or IL-1 was paralleled by increased steady-state levels of LDL receptor mRNA. Evaluation of LDL receptor mRNA half-life revealed no significant change in mRNA stability between control and TNF- or IL-1-stimulated cells. A fusion gene construct consisting of 1563 base pairs of the 5'-flanking DNA of the human LDL receptor gene was coupled to a luciferase reporter gene, transfected into HepG2 cells, and promoter activity was assayed after TNF and IL-1 challenge to the cells. TNF and IL-1 increased promoter activity 200-400%, while treatment with LDL inhibited promoter activity by 70-85%. TNF or IL-1 co-incubation with LDL could not override transcriptional inhibition by LDL. Pretreatment with cycloheximide prevented induction of LDL receptor mRNA by TNF, but not by IL-1, suggesting stimulation of LDL receptor transcription by TNF requires protein synthesis. We propose that TNF and IL-1, acting via distinct signal transduction pathways, increase surface LDL receptors by increasing gene transcription. Our findings suggest that cytokine-induced hypocholesterolemia may be related to TNF and/or IL-1 stimulation of hepatic LDL receptor gene expression and function.

The LDL receptor is the primary receptor for binding and internalization of plasma-derived LDL-cholesterol and regulates plasma LDL homeostasis and LDL-cholesterol delivery to tissues and cells. Control of LDL receptor function occurs at both the transcriptional and post-transcriptional levels (1-6). Elevated intracellular levels of cholesterol or oxysterols regulate LDL receptor protein synthesis and surface expression by inhibiting LDL receptor gene transcription. Other mediators, including several growth factors, have been found to up-regulate LDL receptor activity, presumably by their mitogenic activity. By stimulating cell division, mitogens increase cellular demand for cholesterol, a major component in membrane biosynthesis. It has been shown that platelet-derived growth factor (7, 8), fibroblast growth factor (9, 10), insulin (11-13), transforming growth factor-β (14-16), oncostatin M (15), interleukin-1β (IL-1) (16), and tumor necrosis factor-α (TNF) (15, 16) promote LDL binding in various cell types.

Inflammatory or malignant diseases are often associated with elevated levels of cytokines and abnormal LDL-cholesterol metabolism (18-23). Cytokines may affect plasma levels of cholesterol and lipoproteins by modulating lipolytic enzyme activities (22, 24-27) and gene expression of apoproteins (22, 28). Systemic infusion of macrophage-colony stimulating factor (M-CSF), interleukin-2, granulocyte macrophage-colony stimulating factor (GM-CSF), and TNF lowers serum cholesterol levels in animals and humans (29-34). The liver regulates plasma LDL-cholesterol levels through the surface expression of LDL receptors which mediate plasma LDL and VLDL clearance (35-38). Thus, cytokine modulation of hepatic LDL receptor function may contribute to the hypocholesterolemia associated with several disease states.

To test this hypothesis, we examined the role of inflammatory cytokines in affecting LDL receptor function and gene transcription in HepG2 cells, a hepatoblastoma-derived cell line that mimics normal hepatocytes (39). We report for the first time cell-specific effects of the cytokines, TNF and IL-1, in increasing surface number of LDL receptors by stimulating transcription of the LDL receptor gene. Our results additionally suggest mechanisms by which TNF and IL-1 activate LDL receptor promoter activity.

**EXPERIMENTAL PROCEDURES**

**Cells**—HepG2 cells were purchased from ATCC (American Type Culture Collection). Cells were propagated in Minimal Essential Media supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% fungizone, and 25 mM HEPES.

**Cytokines and Growth Factors**—Recombinant TNF (specific activity = 4.75 × 10⁶ units/mg) was obtained from Genentech. GM-CSF and M-CSF (specific activity = 1.9 × 10⁶ units/mg) was a gift from Genetics Institute (Cambridge, MA). IL-1β (specific activity = 1 unit/100 pg) was purchased from Cistron Biotechnology (Pine Brook, NJ). γ-interferon (specific activity = >2 × 10⁶ units/mg) was purchased from Boehringer Mannheim.

Preparation of Lipoproteins—LDL (1.019-1.063 g/ml) was isolated...
by sequential ultracentrifugation (40), followed by dialysis against HEPES-buffered saline, sterilized by filtration through a 0.22-micron filter, and stored under nitrogen at 4°C. All LDL preparations were routinely screened for peroxides (41). Newly prepared LDL was radiolabeled by the method of Bilheimer et al. (42) as described by Goldstein et al. (43) yielding [32P]LDL with specific activity between 100-1000 cpm/ng. Greater than 98% of the [32P]LDL was trichloroacetic acid-precipitable. Radiolabeled LDL was used within 2 weeks of preparation.

To determine the LDL receptor promoter, cDNA fragments of the LDL receptor cDNA cloned into pSP64 was provided by Dr. David Russell (University of Texas-Southwestern Medical Center, Dallas, TX). The cDNA for glyceraldehyde phosphate dehydrogenase was provided by Dr. David DeWitt (Michigan State University, East Lansing, MI).

Mitogenesis Assay—Mitogenesis was assessed by the incorporation of [3H]thymidine into subconfluent HepG2 cells grown in 96-well plates. Cells were harvested in serum-free media and then exposed to media enriched with the various cytokines. Cells were harvested on glass fiber paper using an automated cell harvester (Mininorm) and quantitated by liquid scintillation counting.

Northern Blot Analysis—Total cellular RNA was extracted in guanidine thiocyanate as previously described (44). Isolated RNA was quantified by UV spectrophotometry. Twenty micrograms of RNA per lane was electrophoresed in 1% denaturing agarose gels containing formaldehyde and ethidium bromide, and the RNA was transferred to a nylon filter (Zetaprobe). cDNA probes were labeled with [32P]dCTP by the method of random hexamer primer extension and washed under high stringency conditions with a final wash in 0.1× SSC and 0.1% SDS at 65°C. The filters were exposed to Kodak XAR-5 film with intensifying screens at -70°C, and the amounts of RNA were quantitated by laser densitometry and normalized by comparison to glyceraldehyde phosphate dehydrogenase. Varying film exposures were utilized to ensure linearity of film response to hybridization signals.

[32P]LDL Binding—LDL binding was measured according to the methods of Goldstein and co-workers (43). Briefly, radiolabeled [32P]LDL (0-40 μg/ml) was added to cells in the presence or absence of 50-fold excess unlabeled LDL at 4°C. After 2 h, cells were washed extensively in PBS plus 0.2% bovine serum albumin, twice in PBS alone, and then incubated for 1 h in PBS plus dextran sulfate (4 mg/ml) to release surface-bound LDL (dextran-releasable counts) (42, 45). Specific binding was calculated as the difference between dextran-releasable counts with and without a 50-fold excess unlabeled LDL.

LDL Receptor Promoter-Reporter Gene Constructs—The LDL receptor promoter-CAT fusion gene (pLDLDR-CAT 1563) construct was generously provided by Dr. David Russell (University of Texas-Southwestern Medical Center). The LDL receptor promoter sequence, extending 1563 base pairs in the 5′ direction from the A (+1) of the translation initiation codon ATG, is excised with HindIII and ligated into the pBluescript plasmid (46) containing the coding sequence for the luciferase reporter gene to generate the new construct p1563LDLRLUC. Multiple transcription initiation sites have been identified in the LDL receptor gene between positions -93 and -79 (47, 48). Correct 5′ orientation of the LDL receptor promoter sequence in p1563LDLRLUC was verified by restriction endonuclease mapping.

Transient Expression Assays—HepG2 cells were transfected with plasmid DNA by the calcium phosphate co-precipitation technique. Twenty micrograms of plasmid DNA by the calcium phosphate co-precipitation technique. Twenty micrograms of plasmid DNA was co-precipitated with 100-1000 ng/ml of MG-CSF (10-1000 ng/ml), and γ-interferon (100-1000 units/ml) had no significant effect on LDL binding (Figs. 1 and 2). Measurement of specific LDL binding with increasing amounts of [32P]LDL generated concentration-dependent, saturable binding curves (Fig. 3). Scatchard analysis of binding isotherms revealed a 2-fold increase in the number of surface LDL binding sites (Bmax) without significant change in receptor affinity (Kd) in response to TNF or IL-1 stimulation. Co-stimulation of HepG2 cells with TNF and IL-1 increased LDL binding over stimulation with either cytokine alone (Table 1). Co-incubation with γ-interferon did not increase LDL binding significantly over TNF alone. Mitogenesis assays measuring [3H]thymidine incorporation revealed no significant change in the cytokine-treated HepG2 cells during the 24-h period in which LDL receptor studies were performed (data not shown).

Northern blot analysis of HepG2 cells treated for 24 h with TNF or IL-1 revealed increases in steady-state levels of LDL receptor mRNA consistent with the observed increases in specific binding (Fig. 4A). Increases in LDL receptor mRNA were observed as early as 1 h after TNF or IL-1 stimulation (Fig. 4B). M-CSF, GM-CSF, and γ-interferon treatment of HepG2 cells did not increase steady-state LDL receptor mRNA levels similar to that observed in the LDL binding studies (data not shown).

Changes in steady-state levels of mRNA may be secondary to increases in LDL receptor gene transcription or enhanced mRNA stability. We determined LDL receptor mRNA half-life in control and TNF- or IL-1-treated HepG2 cells by

![Fig. 1. Effect of cytokines on LDL binding.](image-url)
LDL Receptor Regulation

FIG. 2. Dose-response effects of TNF and IL-1. HepG2 cells were incubated for 24 h with increasing doses of TNF (A) or IL-1 (B), and specific LDL binding was calculated as per Fig. 1.

Fig. 3. TNF and IL-1 increase surface number of LDL receptors. HepG2 cells were treated for 24 h at 37°C with 1% ITS (control), 1% ITS + TNF (50 ng/ml), or 1% ITS + IL-1 (10 units/ml). LDL binding was assayed with increasing concentrations of 125I-LDL generating saturable binding curves. Scatchard analysis revealed a significant increase in $B_{\text{max}}$ without similar changes in $K_d$ (inset).

quantification of steady-state levels of mRNA during progressively longer incubations with actinomycin D (5 μg/ml). TNF or IL-1 treatment did not significantly lengthen LDL receptor mRNA half-life (stability) in comparison to untreated cells (Fig. 5). Our LDL receptor mRNA half-life estimation of 1–3 h is consistent with previous reported values (2, 49). We also examined the role of protein synthesis in TNF or IL-1 induction of LDL receptor mRNA. Cycloheximide pretreatment prevented induction of LDL receptor mRNA by TNF, but not by IL-1 (Fig. 6). Treatment of HepG2 cells with cycloheximide alone increased LDL receptor mRNA by approximately 2-fold as others have shown (50). Thus, taken together, these findings suggest that both cytokines are inducing LDL receptor gene transcription, but only TNF requires additional protein synthesis to stimulate transcription.

A fusion gene construct consisting of the 5'-flanking DNA of the human LDL receptor gene promoter coupled to the luciferase reporter gene was engineered in order to examine the effects of TNF and IL-1 on LDL receptor promoter activity. By utilizing a luciferase assay system, treatment with either TNF or IL-1 increased LDL receptor promoter activity by 200–400% at 24 h. Incubation of transfected cells with cholesterol or LDL markedly decreased the promoter's activity to 14–28% of control (Fig. 7). Transfected cells were co-incubated with LDL and TNF or IL-1 for 24 h, and luciferase activity was measured. Neither TNF nor IL-1 could override
whether TNF or IL-1 would similarly stimulate another binding and LDL receptor mRNA levels in smooth muscle cells. We additionally examined TNF and IL-1 effects on LDL physiologic states (18, 23), can significantly stimulate LDL tightly regulated gene involved in cholesterol biosynthesis, smooth muscle cells (data not shown). Furthermore, we tested TNF (100 ng/ml) without the addition of cytokines. Cells were incubated with 5 pg/ml CoA reductase mRNA levels (data not shown).

HepG2 cells resulted in up-regulation of steady-state HMG-CoA reductase mRNA or LDL binding in TNF- or IL-1-stimulated physiologic rather than pharmacologic doses of cytokines, as incorporation. Recently, IL-1 and transforming growth factor-β were found to induce a 2-fold increase in binding and internalization of [125I]LDL in HepG2 cells (15). Our data extend these results in several ways. Our effects on LDL receptor function and gene transcription were found utilizing constructs with mutations of the SP-1 binding sites of the LDL receptor promoter would be necessary to further test this hypothesis. Interestingly, neither TNF nor IL-1 treatment of HepG2 cells resulted in up-regulation of steady-state HMG-CoA reductase mRNA levels (data not shown).

**DISCUSSION**

We report that TNF and IL-1, at doses found in pathophysiologic states (18, 23), can significantly stimulate LDL receptor function and gene expression in HepG2 cells. Significantly, these effects were not part of a mitogenic response as neither cytokine increased DNA synthesis by [3H]thymidine incorporation. Recently, IL-1 and transforming growth factor-β were found to induce a 2-fold increase in binding and internalization of [125I]LDL in HepG2 cells (15). Our data extend these results in several ways. Our effects on LDL receptor function and gene transcription were found using physiologic rather than pharmacologic doses of cytokines, as low as 5 units/ml of IL-1 and 5 ng/ml of TNF. Additionally, we focused on the mechanism of LDL receptor up-regulation and LDL receptor gene transcription. TNF and IL-1 induce surface LDL receptors by increasing LDL receptor gene transcription.

Studies on LDL receptor gene transcription have focused on the 5'-flanking region where three imperfect 16-base pair repeats and a TATA box reside. The first and third of these repeats can bind the positive transcription factor SP-1. The second repeat is crucial in maintaining sterol-mediated repression of LDL receptor transcription and contains the octameric sequence, designated the sterol regulatory element 1 (SRE-1) that is also found in the 5' region of 2 other sterol-responsive genes, HMG-CoA reductase and HMG-CoA synthase (52). These three repeats are located in close proximity in a region approximately 150 base pairs upstream from the major transcription initiation sites between positions -93 and -79 and thus are included in our p1563LDLRLUC construct. Another publication has recently highlighted the simultaneous induction of the LDL receptor and SP-1 genes in human endothelial cells stimulated with TNF suggesting a possible link between SP-1 induction and LDL receptor gene transcription (2). Since we demonstrated that protein synthesis is necessary for induction of LDL receptor mRNA by TNF, it is conceivable that TNF induces synthesis of SP-1 which then activates LDL receptor gene transcription. Additional transfection studies utilizing constructs with mutations of the SP-1 binding sites of the LDL receptor promoter would be necessary to further test this hypothesis. Interestingly, neither TNF nor IL-1 could overcome the suppressive effects of LDL-cholesterol on the LDL receptor promoter. Thus, if TNF induces LDL receptor promoter activity by activating SP-1, steroids acting at repeat 2 can inhibit the neighboring SP-1 binding sites (repeats 1 and 3). This concurs with the observation of Dawson et al. (4), who found in their LDL receptor

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Specific binding ± S.E. (ng/mg protein)</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>21.37 ± 0.90</td>
<td>100</td>
</tr>
<tr>
<td>TNF (100 ng/ml)</td>
<td>55.93 ± 0.71</td>
<td>252</td>
</tr>
<tr>
<td>IL-1 (10 units/ml)</td>
<td>50.12 ± 0.89</td>
<td>235</td>
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<tr>
<td>TNF (100 ng/ml) + IL-1 (10 units/ml)</td>
<td>81.29 ± 2.93</td>
<td>380</td>
</tr>
<tr>
<td>TNF (100 ng/ml) + γ-IFN (100 units/ml)</td>
<td>50.38 ± 1.49</td>
<td>236</td>
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**TABLE I**

**Co-stimulation with TNF and IL-1 leads to additive effects in increasing LDL binding**

HepG2 cells were treated for 24 h in serum-free media with or without the addition of cytokines. Cells were incubated with 5 µg/ml 125I-LDL for 2 h at 4°C. Specific binding was calculated as the difference in dextran-releasable binding with and without a 50-fold excess unlabeled LDL. Binding is normalized to protein concentration.

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**FIG. 4. Increased steady-state levels of LDL receptor mRNA in TNF- and IL-1β-treated HepG2 cells**

Total cellular RNA was extracted from cytokine-treated HepG2 cells and hybridized with 32P-labeled LDL receptor and glyceraldehyde phosphate dehydrogenase cDNA probes. Histograms represent densitometric scanning of the LDL receptor mRNA band, normalized to glyceraldehyde phosphate dehydrogenase, and expressed as a percentage of control. *Insets* are respective autoradiograms with LDL receptor and glyceraldehyde phosphate dehydrogenase bands labeled. A, HepG2 cells were treated for 24 h with TNF (100 ng/ml) and IL-1 (10 units/ml). B, time course analysis with RNA harvested after 0, 1, 2, 4, 8, and 24 h of stimulation with TNF (200 ng/ml).
LDL Receptor Regulation

100
80
60
40
20
0

Act D (Hrs)

FIG. 5. TNF and IL-1 do not affect LDL receptor mRNA stability. HepG2 cells were treated for 24 h with 1% ITS ± TNF (50 ng/ml) or IL-1 (10 units/ml). After 24 h, the transcriptional inhibitor actinomycin D (5 μg/ml) was added for the indicated time periods and RNA was harvested for Northern analysis of LDL receptor mRNA. Autoradiograms were scanned and normalized to glyceraldehyde phosphate dehydrogenase. The data are expressed as the percent fall off in LDL receptor mRNA levels over time to allow for half-life estimation.

FIG. 6. Effect of cycloheximide on TNF and IL-1 induction of LDL receptor mRNA. HepG2 cells were incubated for 30 min with cycloheximide (5 μg/ml) prior to treatment with TNF (50 ng/ml) or IL-1 (10 units/ml) for 4 h. Total cellular RNA was harvested for Northern blot analysis. Histograms represent densitometric scanning of LDL receptor mRNA, normalized to glyceraldehyde phosphate dehydrogenase, and expressed as a percentage of control (1% ITS alone) cells.

gene promoter studies that sterol repression of the SRE-1 site (repeat 2) could inhibit transcriptional activation by repeat 3, a SP-1 binding site, when inserted into a herpes simplex virus thymidine kinase promoter. The addition of oxysterols also repressed platelet-derived growth factor-induced stimulation of LDL receptor protein or mRNA in human fibroblasts (53). Alternatively, as induction of LDL receptor gene transcription by IL-1 does not require protein synthesis, TNF may induce IL-1 synthesis in HepG2 cells. IL-1 could then stimulate LDL receptor promoter activity. Neither HepG2 cells nor hepatocytes are known to secrete IL-1, but we did not utilize IL-1 blocking antibodies or assay for IL-1 in our TNF-treated HepG2 cells.

TNF and IL-1 are often characterized as biologically redundant cytokines, i.e., two different cytokines activating two distinct receptors which produce an identical cellular response (54, 55). LDL receptor gene transcription appears to be another example of redundancy in the actions of these two cytokines. Our cycloheximide studies suggest TNF and IL-1 are acting via different signal transduction pathways. Furthermore, in our transfection experiments, the addition of IL-1 to maximally effective doses of TNF significantly increased LDL receptor promoter activity compared to the increase produced by either cytokine alone (data not shown). The utilization of distinct signal transduction pathways in inducing LDL receptor gene transcription would thus provide a mechanism for both the biological redundancy and additive responses exhibited by TNF and IL-1.

Interestingly, our initial transfection studies utilized three
CAT constructs differing in length in the 5’ direction of the LDL receptor promoter (47, 48). None of the CAT constructs was sensitive to the 2-4-fold increases in transcription found utilizing the identical LDL receptor promoter sequence in a luciferase construct. Others have previously failed to find transcriptional differences utilizing these CAT constructs and various agonists (56). Our results suggest that this failure may be the result of the reporter construct utilized and not necessarily a function of the particular agonist.

Elevated plasma levels of cytokines, including TNF, IL-1, and interferons have been found in various inflammatory, infectious, and malignant diseases and have been implicated as contributing factors in the wasting and cachexia characteristic of these disease states (18–21, 57–59). Plasma levels of insulin have been found to rise in response to TNF or IL-1 and/or IL-1 release (60, 61) and the well described toxicities of GM-CSF treatment of human mononuclear cells stimulate TNF production suggesting an avenue of GM-CSF administration including fever, chills, and fatigue (45, 53).

In summary, our experiments show that the inflammatory cytokines, TNF and IL-1, can affect LDL receptor function and gene expression in HepG2 cells. The effects of TNF and IL-1 are cell-specific as we and others have shown (2) and they do not necessarily affect other genes involved in cholesterol metabolism, i.e., HMG-CoA reductase. TNF and IL-1 appear to act via distinct signal transduction pathways. By demonstrating effects on LDL receptor function at doses of TNF and IL-1 found circulating during disease states, our studies provide a potential mechanism for reductions in serum lipid levels that contribute to changes in cholesterol homeostasis during inflammatory states.

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REFERENCES