Mechanisms of Dysregulation of Low-Density Lipoprotein Receptor Expression in Vascular Smooth Muscle Cells by Inflammatory Cytokines

Xiong Z. Ruan, John F. Moorhead, Jian L. Tao, Kun L. Ma, David C. Wheeler, Stephen H. Powis, Zac Varghese

**Objective**—Although inflammation is a recognized feature of atherosclerosis, the impact of inflammation on cellular cholesterol homeostasis is unclear. This study focuses on the molecular mechanisms by which inflammatory cytokines disrupt low-density lipoprotein (LDL) receptor regulation.

**Methods and Results**—IL-1β enhanced transformation of vascular smooth muscle cells into foam cells by increasing uptake of unmodified LDL via LDL receptors and by enhancing cholesterol esterification as demonstrated by Oil Red O staining and direct assay of intracellular cholesterol concentrations. In the absence of IL-1β, a high concentration of LDL decreased LDL receptor promoter activity, mRNA synthesis and protein expression. However, IL-1β enhanced LDL receptor expression, overriding the suppression usually induced by a high concentration of LDL and inappropriately increasing LDL uptake. Exposure to IL-1β also caused overexpression of the sterol regulatory element binding protein (SREBP) cleavage-activating protein (SCAP), and enhanced its translocation from the endoplasmic reticulum to the Golgi, where it is known to cleave SREBP, thereby enhancing LDL receptor gene expression.

**Conclusions**—These observations demonstrate that IL-1β disrupts cholesterol-mediated LDL receptor feedback regulation, permitting intracellular accumulation of unmodified LDL and causing foam cell formation. The implication of these findings is that inflammatory cytokines may contribute to intracellular LDL accumulation without previous modification of the lipoprotein. (Arterioscler Thromb Vasc Biol. 2006;26:1150-1155.)

**Key Words:** atherosclerosis ■ cytokine ■ LDL receptor ■ SREBP cleavage-activating protein ■ vascular smooth muscle cells

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Hyperlipidemia is a recognized risk factor for cardiovascular morbidity and mortality. Among plasma lipid components, increased serum levels of low-density lipoprotein (LDL) cholesterol have been most closely correlated with the incidence of cardiovascular disease, whereas high-density lipoprotein cholesterol (HDL) is considered protective.1,2 A series of large-scale clinical trials have shown that lipid-lowering therapy reduces morbidity and mortality from coronary heart disease (CHD). Statin therapy, which lowers plasma LDL cholesterol levels by 30% to 50%, reduced major coronary events by 27%.3 Recent experimental evidence suggests that inflammation is an aggravating factor in atherogenesis,4 as might be predicted from the fact that all the main cell types in an atherosclerotic lesion produce a variety of inflammatory cytokines. Elevated plasma levels of inflammatory cytokines have also been found in patients with established atherosclerotic disease,5,6 whereas patients with elevated levels of acute-phase reactants (reflecting enhanced hepatic production in response to circulating inflammatory cytokines) have a less favorable clinical course than those with normal levels.7 Furthermore, observational studies in patients with chronic inflammatory diseases have also shown an increased risk of premature cardiovascular disease morbidity and mortality.8

The mechanisms by which activation of the inflammatory response may contribute to atherosclerosis are not fully understood but it is possible that inflammation interacts with other modifiable risk factors such as hypercholesterolemia in the initiation and progression of atherosclerotic lesions. Such interaction may help to explain why in patients with chronic activation of the inflammatory response, such as those receiving hemodialysis, low rather than high plasma total cholesterol levels are associated with a higher risk of death caused by cardiovascular disease (termed “reverse epidemiology”).8,9 It is possible that inflammation modifies cholesterol homeostasis in this condition and promotes the progression of atherosclerosis. However, research into the role of inflammation in modulation of cholesterol homeostasis has been limited.

The LDL receptor is of primary importance in binding and internalization of plasma-derived LDL cholesterol and in
regulating plasma LDL concentrations. Brown and Goldstein observed that LDL receptor activity is under tight metabolic control via a feedback system that depends on intracellular cholesterol concentration.10 This mechanism maintains a constant level of cholesterol in hepatocytes and other cells by controlling both the rates of cholesterol uptake from LDL, cholesterol synthesis, and cholesterol export through bile salt synthesis. Regulation of LDL receptor transcription in mammalian cells is complex. It is mediated by interaction between the cholesterol-sensitive sterol regulatory element-binding proteins (SREBPs) and promoter-specific but generic coregulatory transcription factors such as Sp1 YY1 and NF-Y/CBF.11 SREBP1 and 2 are critical molecules in the cholesterol feedback system.12 with SREBP2 being more selective than SREBP1 in controlling LDL receptor gene expression. SREBP cleavage-activating protein (SCAP) has been identified as a cholesterol sensor and chaperone of SREBP.13 When the cellular demand for cholesterol increases, SCAP shuttles SREBPs from the endoplasmic reticulum (ER) to the Golgi, where they are cleaved by 2 proteases (site 1 protease and site 2 protease). The cleaved N-terminal fragment enters the nucleus, binds to the sterol regulatory element in the LDL receptor promoter, and increases LDL receptor gene transcription. A high intracellular concentration of cholesterol prevents transport of the SCAP-SREBP complex from ER to the Golgi and downregulates LDL receptor expression.14 It follows that incubation with native LDL should not lead to the generation of lipid-rich foam cells. In contrast, modified unmodified LDL in VSMCs by disrupting SCAP-SREBP promoter, and increases LDL receptor gene transcription. A high intracellular concentration of cholesterol prevents transport of the SCAP-SREBP complex from ER to the Golgi and downregulates LDL receptor expression.14 It follows that incubation with native LDL should not lead to the generation of lipid-rich foam cells. In contrast, modified LDL (oxidized or glycosylated) is thought to contribute to atherosclerosis because of its uncontrolled uptake via scavenger receptors.15 However, recent evidence has challenged this paradigm by showing that loss of receptor-mediated lipid uptake via scavenger receptor A or CD36 pathways does not ameliorate atherosclerosis caused by oxidized LDL in hyperlipidemic mice16 and that native LDL can, in some circumstances, transform human mesangial cells and macrophages into foam cells.17,18

LDL is the major cholesterol carrier in the circulation and the LDL receptor is one of the main lipoprotein receptors in vascular smooth muscle cells (VSMCs), particularly during proliferative responses to various endogenous mitogens.19 The present experiments set out to demonstrate that inflammatory cytokines increased intracellular accumulation of unmodified LDL in VSMCs by disrupting SCAP-SREBP mediated feedback regulation of LDL receptor.

Methods

Cell Culture

Human VSMCs from coronary artery were cultured in human VSMC growth medium (TCS cell works, Buckinghamshire, UK). Experiments were performed in serum-free experimental medium containing DMEM/F-12 (1:1), 0.2% bovine serum albumin (BSA), 2 mmol/L calcium with the anti-oxidants EDTA, and butylated hydroxytoluene (BHT) at final concentrations of 100 μmol/L and 20 μmol/L, respectively (Sigma, Poole, Dorset, UK). All reagents for cell culture were obtained from TCS cell works. Recombinant IL-1 (IL-1β, 1.0 to 3.3×10^4 Unit/ml) was obtained from R&D Systems (Abingdon, UK). The anti-apoB100 receptor monoclonal antibody MB47 was kindly supplied by Professor LK Kurttis (The Scripps Research Institute, USA).

Preparation of Lipoprotein

Plasma was collected from healthy human volunteers and LDL was isolated by sequential ultracentrifugation as described in our previous publication.20

Morphological Examination

VSMCs were plated in chamber slides (Nunc Inc, Naperville, Ill). After 24 hours of treatments by LDL and IL-1β, the cells were washed 3 times with PBS, fixed for 30 minutes with 5% formalin solution in PBS, stained with Oil Red O for 30 minutes, and counter-stained with hematoxylin for another 5 minutes. Finally, the cells were examined by light microscopy.

Quantitative Measurement of Intracellular Free Cholesterol/Cholesterol Ester

VSMCs were plated in 12-well plates (Nunc Inc, Naperville, Ill). The total and free cholesterol within pretreated VSMCs were analyzed using the fluorometric method described by Gamble.21 The concentration of total and free cholesterol per well was analyzed using a standard curve and normalized by measuring the concentration of total cell protein using the Lowry protein assay. The concentration of cholesterol ester was calculated using total cholesterol minus free cholesterol.

ACAT1 Activity Assay

The method described by Gillies was modified and used for in vitro assay of ACAT1 activity.22 [1-14C]oleoyl-coenzyme A (CoA) (Amersham, Buckinghamshire, UK) was reacted with the homogenerate from VSMCs at 37°C for 2 hours. The lipids were extracted from the cells by Chloroform/Methanol and separated by TLC in hexane/ethyl acetate. The radioactive counts of cholesterol ester, which reflects ACAT1 activity, were read by Bioscan System-200 imaging scanner. The specific activity of ACAT1 was expressed as picomoles of oleoyl-CoA as was converted to cholesterol ester per minute per milligram protein.

Cell Labeling and Flow Cytometric Analysis

VSMCs were incubated in serum-free medium alone or with 5 ng/mL of IL-1β in the absence or presence of a high concentration of native LDL (200 μg/mL) for 24 hours. The medium was then replaced by fresh serum-free medium containing 10 μg/ml DiI labeled LDL for 5 hours at 37°C. Mean fluorescence intensity (MI) from the treated cells were analyzed by fluorescence-activated-cell sorter (FACS) analysis using a flow cytometer (Coulter, EPICS XL-MCL) as described previously.18

Western Blot Analysis

Identical amounts of total protein from cultured VSMCs extracts were denatured and then subjected to Western blotting analysis using a chicken anti-human LDL receptor polyclonal antibody and a goat anti-cholesterol HRP labeled secondary antibody (Abcam, Cambridge, UK). Actin was also examined using a rabbit anti-actin antibody (Sigma, Poole, Dorset, UK) and a goat anti-rabbit HRP linked IgG (New England Biolabs, Herts, UK). These procedures were performed as described in the literature.18

LDL Receptor Promoter Activity Assay

An LDL receptor promoter-luciferase fusion gene pGL3-LDLR6500 was constructed and cotransfected into VSMCs with a control plasmid of psv-β-galactosidase by electroporation as described in our previous publication.21 The transfected cells were treated with LDL and IL-1β for 8 hours. The luciferase activity in the cells was measured and normalized by comparison with β-galactosidase activity using Promega luciferase and β-galactosidase assay systems (Promega, Southampton, UK).
Real-Time Reverse-Transcription Polymerase Chain Reaction

Total RNA (500 ng) was used as a template for reverse-transcription polymerase chain reaction (RT-PCR). The RT reaction was set up using a kit from Applied Biosystems (Warrington, Cheshire, UK). After cDNA synthesis by RT, cDNA was split for the separate amplification of the LDL receptor, SCAP, SREBP2, ACAT1, and β-actin using specific primers designed using Taqman Primer Express as shown in the Table. Real-time PCR was performed in an ABI 7000 using SYBR Green dye according to the manufacturer’s protocol.

Confocal Microscopy

A polyclonal antibody specific for human SCAP was produced by immunizing rabbits with the synthetic peptide PVDS-DRKQGEPTEQC (amino acids 66 to 69 of human SCAP). Other antibodies were purchased from Molecular Probes Europe (Leiden, The Netherlands). VSMCs cultured in chamber slides (Nunc Inc) were washed, fixed, and permeabilized. The cells were then incubated with rabbit anti-human SCAP antibody (1:100 dilution) and an anti-human Golgi antibody (mouse anti-human Golgi-97, 1:100 dilution), followed by a secondary fluorescent antibodies (goat anti-rabbit Fluor 488 for SCAP and goat anti-mouse Fluor 594 for Golgi). After washing, the cells were examined by confocal microscopy (Bio-Rad, UK).

Data Analysis

Groups of data were evaluated for significance by 1-way analysis of variance using Minitab software. Data were considered significant at P<0.05.

Results

Staining of VSMCs with Oil Red O after incubation with a high concentration of native LDL (200 μg/mL) in the presence of anti-oxidants (100 μmol/L of EDTA and 20 μmol/L BHT) showed no intracellular Oil Red O stained lipid droplets (supplemental Figure IA, available at http://atvb.ahajournals.org). However, lipid accumulation was observed if 5 ng/mL of IL-1β was also added to the medium (supplemental Figure IB). This process was not inhibited by polyniosinic acid (Poly I) (supplemental Figure IC), which blocks scavenger receptors, but was prevented by heparin (supplemental Figure ID), which displaces LDL bound to the cell surface. The electrophoretic mobility of LDL isolated from the culture medium after a 24-hour incubation period was the same as that of fresh LDL, which excluded the presence of oxidized LDL and the involvement of scavenger receptors accounting for the observed lipid accumulation (supplemental Figure IE). Quantitative intracellular cholesterol analysis demonstrated that IL-1β increased cholesterol ester but not free cholesterol accumulation in VSMCs, suggesting that IL-1β may enhance cholesterol esterification (Figure 1). To prove the specificity of involvement of LDL receptor, we used the apolipoprotein (apo) B100 antibody MB47, which specifically recognizes an epitope at the LDL receptor-binding domain of apoB100. Like heparin, MB47
(50 μg/mL) reduced cholesterol ester accumulation induced by IL-1β by ≈84% (Figure 1). To determine whether the increased cholesterol ester content was dependent on an ACAT1 based mechanism, we examined ACAT1 activity by analyzing cholesterol esterification using C14 labeled oleoyl-CoA, which reflects ACAT1 activity. The result demonstrated that IL-1β increased ACAT1 protein activity (Figure 2). IL-1β (5 ng/mL) also increased ACAT1 mRNA expression (Figure 3B).

We examined the effect of IL-1β on LDL receptor promoter activity using a reporter gene pGL3LDLR6500. A high concentration of native LDL (200 μg/mL) markedly decreased LDL receptor promoter activity. However, IL-1β (5 ng/mL) increased promoter activity even in the presence of a high concentration of native LDL (Figure 3A). This result suggests that the inflammatory cytokine IL-1β increases LDL receptor expression at the transcription level, even in the presence of high concentrations of cholesterol.

Next, we examined how mRNA expression of SCAP, SREBP2, ACAT1, and the LDL receptor was influenced by IL-1β (5 ng/mL) in the absence or presence of a high concentration of native LDL (200 μg/mL). IL-1β increased the expression of SCAP, SREBP2, ACAT1, and LDL receptor mRNAs (Figure 3B) and overrode the suppression of SCAP, SREBP2, and LDL receptor genes induced by a high concentration of LDL (Figure 3C).

The influence of IL-1β on the regulation of LDL receptor protein expression was examined by flow cytometry. At a concentration of 5 ng/mL, the cytokine increased LDL uptake by VSMCs in the presence of a high concentration of native LDL and anti-oxidants (supplemental Figure IIA). This result was confirmed using western blotting (supplemental Figure IIB) and suggests that IL-1β overrides LDL receptor protein suppression induced by a high concentration of native LDL.

Finally, we investigated the effect of IL-1β on SCAP cycling between the ER and the Golgi in the presence of cholesterol using confocal microscopy. By dual staining with anti-human SCAP and anti-human Golgi antibodies, we demonstrated that cholesterol depletion achieved by incubation of cells with 1% hydroxypropyl-β-cyclodextrin (HPCD) increased SCAP accumulation in the Golgi, whereas cholesterol loading reduced it. Importantly, exposure to IL-1β (5 ng/mL) led to the localization of SCAP to the Golgi, even in the presence of high cholesterol concentration (Figure 4).

**Discussion**

Foam cells have traditionally been regarded as being derived from macrophages because they express macrophage markers. However, it is now known that VSMCs can also be converted into foam cells. In advanced lesions in both WHHL and FF rabbits, simultaneous thymidine autoradiography and immunostaining for cell type-specific markers has revealed that foam cells are derived from VSMCs.
Figure 4. IL-1β increases SCAP accumulation in the Golgi. VSMCs were incubated for 5 hours in experimental medium containing 1% HPCD to deplete lipid, or containing sterols (10 μg/mL of 25-hydroxycholesterol and 10 μg/mL of cholesterol), or sterols plus 5 ng/mL of IL-1β. The cells were then fixed, stained with anti-human SCAP and human Golgi antibodies as described in the Methods section, and imaged by confocal microscopy. The results are typical of those observed in 3 separate experiments (×400).

revealed that only ≈30% of the labeled cells are macrophages whereas 45% have a smooth muscle cell phenotype.

Many pathways may be involved in lipid accumulation and foam cell formation. Initially, uptake of modified LDL (oxidized or glycosylated) via the scavenger receptor was thought to be the major pathway for atherogenesis. However, scavenger receptor expression in VSMCs is very limited. Instead, VSMCs may express LDL receptor, VLDL receptor, and LRP. It has been demonstrated that exposure to macrophage conditioned medium resulted in an approximate doubling of LDL degradation by human arterial smooth muscle cells, endothelial cells, and skin fibroblasts.26,27 Our experiments demonstrated that IL-1β significantly increased native LDL accumulation in VSMCs specifically via the LDL receptor pathway. These data suggest that chronic inflammation may fundamentally modify cholesterol homeostasis by disrupting LDL receptor regulation.

All experimental medium contained the anti-oxidants ethylenediaminetetraacetic (EDTA) and BHT, which prevented oxidation of LDL by VSMCs, as confirmed by the lack of any change in the electrophoretic mobility of lipoprotein isolated from the culture medium at the end of the experiments. Because no oxidation of LDL occurred during the experiments, it is highly unlikely that a suitable ligand for the scavenger receptor was present. LDL-related protein (LRP) is a large, multifunctional, endocytic receptor that is a member of the LDL receptor superfamily. LRP may also be expressed by VSMCs, but its expression is much lower than levels of LDL receptor. Because no oxidation of LDL occurred during the experiments, it is highly unlikely that a suitable ligand for the scavenger receptor was present. LDL-related protein (LRP) is a large, multifunctional, endocytic receptor that is a member of the LDL receptor superfamily. LRP may also be expressed by VSMCs, but its expression is much lower than levels of LDL receptor.

Expression under influence of IL-1β, possibly as a result of posttranslational modification of the protein and before increases in SCAP mRNA expression.

Recently, Insig-1 has been identified as a sterol-regulated ER retention factor that interacts with SCAP-SREBP complexes in the ER. When intracellular cholesterol concentration increases, Insig-1 binds to the sterol sensing domain in the SCAP, preventing the exit of SCAP-SREBP complexes from the ER.29 We demonstrated that inflammatory stress increased the expression of SCAP, which may in turn increase the ratio of the SCAP/Insig-1 and result in escape of the SCAP-SREBP2 complex from the ER to the Golgi. This seems a likely hypothesis, because Insig-1 is quantitatively limited in the ER and may be present in insufficient concentrations to retain the increased SCAP-SREBP2 complex under inflammatory stress. This result is consistent with the observation that over expression of SCAP abolishes sterol suppression of SREBP cleavage.30

ACAT-1 protein is ubiquitously expressed in tissues. In contrast, ACAT-2 is expressed mainly in the intestine in humans.31 To determine whether the increased cholesterol ester content occurred via an ACAT1 based mechanism, we examined ACAT1 activity by analyzing cholesterol esterification using C14-labeled oleoyl-CoA and ACAT1 mRNA expression under influence of IL-1β and LDL. The results demonstrated that IL-1β increased both ACAT1 protein activity and mRNA expression.

Taken together, these results imply that the normally tight sterol-dependent feedback regulation of the LDL receptor in VSMCs is disrupted by IL-1β. Thus, in the presence of this inflammatory cytokine, native LDL is taken up in excess via the LDL receptor and results in massive cholesterol ester accumulation by activating the ACAT1 pathway. These processes convert VSMCs into foam cells. The implications of these findings are that native LDL can be atherogenic without prior modification by oxidation or glycosylation under inflammatory stress. Therefore, anti-inflammatory...
drugs may be useful adjunctive therapeutic agents to antioxidants and cholesterol-lowering agents in the management of atherosclerotic vascular disease.

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Fig.I. Visualisation of lipid droplets in VSMCs after IL-1β treatment and electrophoretic mobility of LDL recovered from the culture medium. VSMCs were incubated for 24 hours in experimental medium containing 200 µg/ml of native LDL in the absence (A) or presence of 5 ng/ml of IL-1β (B), IL-1β plus Poly I (C), and IL-1β plus Heparin (D). The cells were examined for lipid inclusions by Oil Red O staining (x 400). The supernatants were collected and subjected to electrophoresis in a BECKMAN Paragon LIPO Gel (E). Lane 1: fresh native LDL (negative control), lane 2: Ox-LDL (positive control), lane 3: LDL recovered after 0 hours incubation with VSMCs, lane 4: LDL recovered after 24 hours incubation with VSMCs. The results are typical of those observed in 4 separate experiments.

Fig.II. The effect of IL-1β on LDL receptor protein expression. VSMCs were incubated in serum-free medium alone (control) or with 5 ng/ml of IL-1β in the absence or presence of a high concentration of native LDL (200 µg/ml) for 24 hours. A: The medium was then replaced by fresh serum-free medium containing 10 µg/ml DiI labelled LDL for 5 hours at 37°C, and the cells were analysed by FACS. Mean fluorescence intensity (MFI) was calculated by subtracting the autofluorescence intensity from the observed MFI of labelled cells. Results represent means±SD of duplicate wells from four experiments. B: The cell extracts were prepared and subjected to SDS-PAGE, followed by immunoblotting analysis using anti-human LDL receptor and anti-actin antibodies as described in the Methods section. One of three representative experiments is shown. * p<0.001 vs. control, ** p<0.05 vs. LDL group.
Supplementary Fig. I

A B

C D

E

1 2 3 4
Supplementary Fig. II

A:

![Graph showing MFI (% of control) with conditions: LDL (200 µg/ml) - , +; IL-1β (5 ng/ml) - , +; with statistical significance symbols.]

LDL (200 µg/ml)        -                   +                   -                  +
IL-1β (5 ng/ml)         -                   -                    +                 +

B:

![Images of Western blots with annotations: LDL receptor (160kDa) and Actin (42kDa).]

LDL (200 µg/ml)        -                   +                   -                  +
IL-1β (5 ng/ml)         -                   -                    +                 +