High-Density Lipoproteins Differentially Modulate Cytokine-Induced Expression of E-Selectin and Cyclooxygenase-2

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Abstract—Atherogenesis is a multifactorial chronic inflammatory disease in which low plasma levels of HDLs are a strong predictor of the condition. Although the mechanism of protection by HDLs is not precisely known, HDLs have been shown to influence many of the events involved in the development of atherosclerosis. Previously we have shown that HDLs inhibited the cytokine-induced expression of adhesion molecules (E-selectin, VCAM-1, and ICAM-1) by endothelial cells (ECs). As the complete transcriptional regulation of all 3 genes requires the NF-κB family of transcription factors, we examined the effect of HDLs on activation of NF-κB. We also investigated the effect of HDLs on 2 other cytokine-induced genes, granulocyte-macrophage colony-stimulating factor (GM-CSF) and cyclooxygenase (Cox-2; prostaglandin H2 synthase, EC 1.14.19.1). E-selectin expression in response to tumor necrosis factor-α (TNFα) was, as expected, inhibited in ECs that had been preincubated with HDLs. However, the level of secretion of GM-CSF in the same cultures was no different from control. In a similar manner, although HDLs had no effect on steady-state mRNA levels of GM-CSF, the levels of E-selectin were significantly inhibited by HDLs. In transient cotransfection experiments we found that HDLs inhibited the cytokine-induced expression of a reporter gene driven by the E-selectin proximal promoter (−383 to 80) but had no effect on the expression of a reporter gene driven under the control of the proximal promoter of GM-CSF (−627 to 28). As would be predicted from this differential response, HDLs did not influence the nuclear translocation or DNA binding of NF-κB, or alter the kinetics of degradation and resynthesis of the inhibitory protein IκBα. We found that HDLs synergized with cytokine to enhance the expression of Cox-2 and induce the synthesis of its main EC product, prostacyclin (PGI2), a potent inhibitor of platelet and leukocyte functions. In conclusion, HDL induces an antiinflammatory phenotype in cytokine-induced ECs, synergizing with cytokine to induce the synthesis of its main EC product, prostacyclin (PGI2), a potent inhibitor of platelet and leukocyte functions.

Key Words: inflammation • coronary artery disease • prostacyclin • granulocyte-macrophage colony-stimulating factor

Coronary artery disease remains one of the major causes of death in the Western world today. A great deal of epidemiological research shows a strong inverse correlation between the concentration of plasma HDL and the frequency of coronary artery disease. More direct evidence of the protective effects of HDL comes from studies in which elevation of HDL in animal models has led to a dramatic decrease in the frequency of coronary artery disease. It is not yet known whether the protective effects of HDL relate to their role in reverse cholesterol transport or to mechanisms unrelated to their lipid transport function.

Normal endothelium is one that maintains a nonadhesive, nonthrombogenic, and nonproliferative interface of cells that separate the blood from the underlying tissues. The phenotype of the endothelium changes in response to certain inflammatory mediators, such as tumor necrosis factor-α (TNFα) and interleukin-1β (IL-1β), to one that expresses and secretes several adhesion molecules and chemotactic proteins of the IκB family. Normally, NF-κB members are confined to the cytoplasm through association with inhibitory proteins of the IκB family. IL-1β or TNFα leads to the phosphorylation and degradation of the IκB proteins, resulting in the release, nuclear translocation, and DNA binding of NF-κB, with subsequent transcriptional activation of respon-
The complex effect on cytokine-induced gene expression than molecule expression and suggests that HDLs have a more to be the central mechanism by which HDL inhibits adhesion.(Sigma). In typical experiments, confluent HUVECs were incubated sion molecules.33 HDLs were found to significantly inhibit expression of cytokine-inducible endothelial cell (EC) adhe-

There is considerable evidence for the importance of leukocyte recruitment into the arterial intima, both for the development of atherosclerosis and for the maintenance of the mature plaque. The earliest observable cellular event in the genesis or development of atheroma is the binding of leukocytes to the endothelium.26,27 Furthermore, there are now several studies demonstrating the presence on endothelium of IL-1 or TNF-inducible adhesion molecules, such as P-selectin, E-selectin, VCAM-1, and ICAM-1, both in animal models of atherosclerosis and in human atherosclerotic tis-

In a previous study the possibility was explored that one of the actions of HDLs, contributing to protection from coronary artery disease, might be through inhibition of expression of cytokine-inducible endothelial cell (EC) adhesion molecules.33 HDLs were found to significantly inhibit the ability of TNFα or IL-1β to stimulate expression of E-selectin, VCAM-1, and ICAM-1 in human umbilical vein ECs (HUVECs), both at the level of cell surface expression and steady-state mRNA. As these adhesion molecules have all been demonstrated to require activation of the NF-κB family of transcription factors for complete expression, these experiments were initially designed to explore the possibility that the antiinflammatory effects of HDLs were mediated by inhibiting the activation of the NF-κB family of transcription factors. Our results show that inhibition of NF-κB is unlikely to be the central mechanism by which HDL inhibits adhesion molecule expression and suggests that HDLs have a more complex effect on cytokine-induced gene expression than was previously appreciated.

**Methods**

**Cell Culture**

HUVECs were isolated as described previously.33 All cells were plated onto 1% gelatin-coated culture plastic in medium 199 with Earle’s salts (ICN Biomedicals Inc) supplemented with 20% FCS (GIBCO), 2 mmol/L glutamine, penicillin and streptomycin, 50 μg/mL EC growth supplement (Sigma), and 50 mg/mL heparin (Sigma). In typical experiments, confluent HUVECs were incubated for 16 hours with native or reconstituted HDL (1 mg/mL apoAI), after which TNFα or IL-1α (1 mg/mL) was added to the culture medium for a further period before ending the experiment. From using this concentration of cytokines, no loss of cell viability was observed.

**Isolation of HDL**

Blood from normal healthy volunteers, <40 years of age, was collected in Vacutainers containing K-EDTA as the anticoagulant (Beckton Dickinson). Plasma was separated by spinning at 2000 rpm for 20 minutes at 4°C. HDLs were then purified by sequential ultracentrifugation in the 1.07 to 1.21 g/mL density range, according to the previously described method.34 Resulting HDLs were then dialysed against 4 changes of PBS and filter-sterilized by using a 0.2-mm Minisart microfilter (Sartorius). HDL isolated in the presence of EDTA loses paraoxonase,35 hence effects observed in these studies are independent of this enzyme.

**Analysis of HDL**

Concentrations of apoAI and apoB were determined by immunoturbidimetric methods, using a Cobas-Fara centrifugal analyzer (Roche Diagnostic) with commercially available antibodies (Boehringer Mannheim). HDL particle size was evaluated by electrophoresis through 3% to 30% nondenaturing gradient gels (Pharmacia). All preparations of HDL contained 2 main populations: 1 with a Stokes diameter of 10.4 nm (HDL 3a). HDL preparations contained no contaminating parti-

**Reconstituted HDL**

The reconstituted HDLs used in this study were kindly provided by the Swiss Red Cross, Blood Transfusion Service, Bern, Switzerland. The particles, containing apoAI as the sole protein, and soybean phosphatidylcholine as the sole phospholipid, were prepared by using cholate dialysis according to the reported method.39 Reconsti-

**Flow Cytometry**

HUVECs were plated at confluence in 24-well plates (2×10⁴ cells/400 μL per well), and incubated with HDL and TNFα (as above). Cells were then harvested with trypsin and washed in ice-cold PBS. Surface expression of E-selectin was then determined by resuspending the cells in 50 μL of anti-E-selectin monoclonal antibody38 for 45 minutes at 4°C. Cells were then washed in PBS.
containing 0.02% sodium azide and 5% newborn calf serum (Gibco) at 4°C and incubated for 45 minutes at 4°C in 50 µL of wash buffer containing FITC-labeled rabbit anti-mouse Ig (DAKO). After further washing, the cells were fixed with 2.5% formaldehyde. Expression of E-selectin was selected using a Coulter Epics Profile II flow cytometer, counting 10^4 cells per sample. Controls included an isotype-matched irrelevant antibody and no primary antibody.

**GM-CSF Enzyme-Linked Immunosorbent Assay**

GM-CSF protein concentrations in EC culture supernatants were determined by using a human GM-CSF ELISA kit (R & D Systems Ltd) according to the manufacturer’s recommendations.

**Northern Analysis**

Total RNA was prepared as described by Chomczynski and Sacchi. Equal aliquots of total RNA (10 µg/lane) were size-fractionated in a 1% formaldehyde gel and transferred to nylon membrane (Hybond N, Amersham). Blots were hybridized according to Church and Gilbert, using 10 ng/ml α-32P-labeled cDNA probes. After washing, the blots were exposed on Kodak BIOMAX film (Eastman Kodak). Relative amounts of RNA per lane were normalized to the ethidium bromide–stained ribosomal RNAs.

**Western Blotting**

After appropriate preincubation with HDL and TNFα, ECs were washed twice with ice-cold PBS and then harvested by scraping. ECs were then pelleted and lysed in 50 µL of lysis buffer (50 mM Tris, 1% NP-40, 0.25% deoxycholate, 5 mM EDTA, 0.5 mM aprotinin, and 1 mM PMSF) (Sigma) on ice for 15 minutes. Debris was then removed by centrifuging the lysate for 5 minutes at 4°C and the supernatants stored at −70°C until use. Protein concentration was estimated by using the Bio-Rad BCA kit (Bio-Rad). Protein was separated by running 10 µg of protein lysate in a 15% SDS-PAGE, using a Protean minigel apparatus. Proteins were then transferred to a nylon (Millipore) membrane, according to the manufacturer’s recommendations, using the Protean minigel wet transfer apparatus. Filters were then blocked overnight in 5% milk solid in PBS and washed once for 15 minutes in PBS with 0.05% Tween, the protein–antibody complex was identified by incubating the filter for 1 hour in a 1:10 000 dilution of an HRP-conjugated goat anti-rabbit immunoglobulin (Sigma) in hybridization buffer (50 mM NaCl, 5% milk, 0.5% Tween, and 80 g/mL leupeptin, and 8 µg/mL aprotinin) followed by 0.5 packed nuclei volume of high-salt buffer (as for low salt, with 1.2 mM/L KC1), added slowly while stirring the nuclei with a pipette tip. The extraction mixture was then agitated on a shaking platform for 30 minutes at 4°C and nuclear extract recovered by spinning down the nuclei at 13 000 rpm in an Eppendorf microfuge at 4°C. Supernatants containing the nuclear extracts were then recovered and stored at −70°C before use. Protein concentration of the nuclear extracts was measured by using the Bio-Rad BCA kit (Bio-Rad).

**Purification and Labeling of the Oligonucleotide Probe**

NF-κB–specific oligonucleotide duplexes had the following sequence with complimentary single-stranded regions at each end shown in lower case for the upper strand only: E-selectin NF-κB, aatCGTTTGTGATGCCATTGGATTTCCTCTTTACTGGATGTG; and Igk NF-κB, AACCGGGGATTCCTTGAG; and Igk NF-κB, AACAGGGAGCTTCGAGG;

**Plasmids**

The luciferase reporter gene plasmid pGM-Luc contains a 655-bp fragment of the human GM-CSF promoter (−627 to 28) upstream of the luciferase gene (Promega). The chloramphenicol acetyl transferase (CAT) reporter gene plasmid pCAT-ESL contains a 463-bp fragment of the human E-selectin promoter (−383 to 80) upstream from the CAT gene (pCAT-Basic, Promega). The β-galactosidase vector pCMVβ (Clontech) containing the constitutively active cyto-megalovirus (CMV) promoter was used as a cotransfent to correct for variations in transfection efficiency. All of the expression plasmids were, therefore, purified by CsCl equilibrium centrifugation. The luciferase reporter gene plasmid pNF-κB3 contains 3 concatenated sequences of the E-selectin NF-κB consensus sequence.

**Endothelial Cell Transfection**

HUVECs were cotransfected with reporter genes using electroporation, according to a method previously described. HUVECs were then harvested in HEPES-buffered saline. Cells were finally resuspended in normal growth medium and plated into 6×60-mm culture dishes precoated with 1% gelatin (Sigma). Growth medium was changed
Promoters have been shown to require NF-κB binding for complete activation. Although the proximal promoters of both E-selectin and GM-CSF have a major requirement for the NF-κB family of transcription factors, the GM-CSF gene is also regulated by a distal enhancer region, located at 716 to 625 bp upstream from the transcriptional start site of GM-CSF, controlled via a cyclosporin-sensitive nuclear factor of activated T cells binding site. Activation of nuclear factor of activated T cells requires both the elevation of Ca²⁺ and mobilization of protein kinase C. As HDLs have been shown to be capable of inducing both these responses, it was possible that the lack of effect on GM-CSF synthesis is by induction of the calcium-sensitive distal enhancer region, which could mask any inhibition of the proximal promoter region. To overcome this consideration, we compared the effect of HDLs on the TNF-inducible expression of reporter genes controlled by the E-selectin and GM-CSF proximal promoters in transient transfection assays in HUVECs. Whereas HDLs had no effect on the stimulation of luciferase activity under the control of the GM-CSF promoter (−627 to 28) bp, they suppressed, in the same cells, the ability of TNF to stimulate CAT activity under the control of the E-selectin promoter (−383 to 89) bp (Figure 3). As both these complex promoters have been shown to require NF-κB binding for regulation, these findings suggest that the differential effect afforded by HDL treatment is likely to be independent of NF-κB per se. However, as both these proximal

Figure 2. Northern blot analysis showing effect of HDLs on steady-state mRNA levels of E-selectin and GM-CSF. Confluent ECs were incubated for 20 hours without addition (NIL), or for 16 hours with no addition followed by 4 hours in the presence of TNFα (1 ng/mL) (TNF), or for 16 hours with HDL (1 mg/mL apoAI) followed by 4 hours with TNFα (1 ng/mL) and HDL (TNF,HDL), or for 20 hours in the presence of HDL (1 mg/mL apoAI) (HDL). In each case, 10 μg of total RNA was extracted and analyzed by northern analysis by using cDNA probes to E-selectin, GM-CSF, and GAPDH. Data are representative of 3 experiments, using HDL from 3 separate donors.

Figure 3. HDLs inhibit the TNFα-induced expression of the CAT reporter driven by the E-selectin promoter but have no effect on the expression of a luciferase reporter driven by the proximal promoter of GM-CSF. Forty-eight hours after cotransfection of confluent ECs with the E-selectin promoter (−627 to 28 bp) in a CAT reporter plasmid, the GM-CSF promoter (−388 to 80 bp) in a luciferase reporter plasmid and the β-galactosidase reporter gene, cells were incubated either with or without HDL (1 mg/mL apoAI) for 16 hours followed by a 9-hour incubation with TNFα (10 ng/mL) in the presence (HDL,TNF) or the absence (TNF) of HDL (1 mg/mL apoAI). Levels of gene activity were measured with respect to untreated cells (NIL). Levels of CAT (a) and luciferase activity (b) are expressed as relative values normalized to levels of β-galactosidase. Values represent the mean and SD and are representative of 6 similar experiments, using HDL from 6 separate donors. Differences between means were evaluated by using Student’s unpaired t test. *P, not significant; **P<0.005.

Results

The data shown are representative experiments where the HDL preparations used are native heterogeneous samples isolated from different donors as described in Methods. In parallel experiments, the use of reconstituted discoidal HDL particles, in which apoAI is the sole apolipoprotein and phosphatidylcholine is the sole phospholipid, gave similar results (data not shown).

If HDLs were able to inhibit cytokine-induced adhesion molecule expression by inhibiting some element of NF-κB activation, then it is likely that most genes known to require the NF-κB family of transcription factors should be inhibited by treatment with HDLs. We therefore examined the effect of HDL treatment on a range of cytokine-inducible genes. Preincubation of HUVECs with HDLs resulted in an inhibition of cytokine-induced adhesion molecule expression, as has been shown previously, although clearly having no effect on GM-CSF secretion (Figure 1) or steady-state mRNA level (Figure 2).

Although the proximal promoters of both E-selectin and GM-CSF have a major requirement for the NF-κB family of transcription factors, the GM-CSF gene is also regulated by a distal enhancer region, located at 716 to 625 bp upstream from the transcriptional start site of GM-CSF, controlled via a cyclosporin-sensitive nuclear factor of activated T cells binding site. Activation of nuclear...
The isoforms of this enzyme, Cox-1, which is present in several cell types and is constitutively expressed and relatively stable,56–58 and Cox-2, which is the inducible form.56–58 As shown in Figure 7, not only could HDL alone stimulate Cox-2, but HDL synergized with TNFα and IL-1β to augment this increase. Consistent with the findings of others (Dr...
J. Mitchell, personal communication, (1998), IL-1α was a far more potent activator of the enzyme than TNFα. We confirmed that this elevation in the level of Cox-2 was concomitant with an increase in the accumulation of prostacyclin in the culture medium, by measuring the accumulation of the stable breakdown product of PGI₂, 6-oxo-PGF₁α (Figure 7).

Discussion

In this study, we have shown that in addition to inhibiting the expression of adhesion molecules for leukocytes, HDL can synergize with TNFα and IL-1β to induce levels of Cox-2 and the secretion of prostacyclin. The in vitro EC phenotype induced by HDL is therefore one of reduced surface adhesiveness for leukocytes, together with increased secretion of a factor that can inhibit platelet and leukocyte activation and induce vasodilation. This phenotype might be expected to contribute to the in vivo mechanisms by which this multipotent lipoprotein reduces the risk of atherosclerosis.

Our findings that HDL have differential effects on expression of E-selectin, GM-CSF, and Cox-2 in response to IL-1β and TNFα stimulation challenge that HDL has a general inhibitory effect on the NF-κB pathway. All 3 genes have been shown to have a requirement for the NF-κB family of transcription factors for full cytokine-mediated expression.97–99 The mechanism by which HDLs modulate cytokine-mediated E-selectin expression is at least mediated at the transcriptional level, as we also observed inhibition of steady-state mRNA level and repression of a reporter gene driven by a full E-selectin proximal promoter. Furthermore, by using a reporter construct containing 3 tandem E-selectin NF-κB binding domains, we were unable to show inhibition of expression by HDL treatment. Consistent with this, we were unable to detect any inhibition by HDL of TNF-mediated NF-κB nuclear translocation or DNA binding, or a difference in the kinetics of degradation and resynthesis of IκBα, a process known to be NF-κB dependent.23–25 These data therefore suggest that HDL primarily influences transactivating factors other than those of the NF-κB pathway itself. Similar conclusions may be drawn from the recent work of Wolle et al,62 who have shown that flavone (PD 09806) inhibits VCAM-1 expression by human aortic ECs but does not affect the binding or translocation of NF-κB or inhibit the transactivation of a reporter construct containing 2 tandem VCAM-1 NF-κB sites. Further deletion analysis of the E-selectin promoter is now required to establish the specific domains involved in HDL-mediated inhibition.

The original observation that HDL could induce EC synthesis of prostacyclin was attributed to the ability of the lipoprotein to provide the substrate, arachidonic acid.53–55,62,63 Our findings that HDL synergizes with IL-1β and, to a lesser extent TNFα, to increase Cox-2 protein levels and prostacyclin production is novel, and pertinent to atherogenesis, in which both cytokines are likely to play a role.64 Our studies offer an additional mechanism whereby prostacyclin is elevated by HDL, but do not preclude the possibility that HDL may also supply exogenous substrate for the synthesis of the eicosanoid. However, as reconstituted discoidal HDL particles, containing only apoAI as the sole protein and phosphatidylcholine as the sole phospholipid, had effects on 6-oxo-PGF₁α accumulation similar to native HDL, it is likely that HDL-induced Cox-2 elevation in confluent ECs is sufficient to stimulate the eicosanoid in the absence of exogenous arachidonic acid. Furthermore, although studies by Stuhlmeier et al65 have shown that arachidonic acid can inhibit the TNFα-induced expression of E-selectin by bovine aortic ECs, provision of this component cannot explain the broad spectrum of antiinflammatory effects of HDL, where both VCAM-1 and ICAM-1 are also inhibited.34,48 As recent work by Vinals et al66 has shown that HDL also induce Cox-2 in vascular smooth muscle cells, it will be interesting to see whether the effect is also enhanced by IL-1β and TNFα.

The mechanism whereby HDL synergizes with cytokines to induce Cox-2 remains to be clarified. It has been shown that EC incubation with HDL can result in the production of lysophosphatidylcholine (LPC), and as exogenous addition of LPC to EC has been demonstrated to elevate not only Cox-2, but also ET-1, another vasoactive agent known to be elevated

![Figure 7](image-url) HDLs synergize with the cytokines TNFα and IL-1β to elevate the level of Cox-2 expression in ECs. Western blot analysis of whole cell lysates (10 μg/lane) prepared from confluent ECs preincubated with HDL (1 mg/mL apoAI) alone for 8 hours or preincubated with HDL for 4 hours and then treated with either TNFα (1 ng/mL) or IL-1β (1 ng/mL) and HDL for a further 4 hours. The lanes have been probed with antibody against Cox-2 (Biogenics Ltd). Results are representative of 3 experiments, using HDL from 3 separate donors.

![Figure 8](image-url) HDL stimulates the accumulation of 6-oxo-PGF₁α. After preincubation of confluent ECs with HDL (1 mg/mL apoAI) for 16 hours, IL-1β (1 ng/mL) or TNFα (1 ng/mL) was added for a period of 16 hours after which the supernatants were assayed for levels of 6-oxo-PGF₁α. Levels in supernatants from cells treated with cytokine in the presence of HDL (HDL,IL-1; or HDL,TNF) and absence of HDL (IL-1 or TNF) were compared with cells incubated with HDL alone (HDL) and cells left untreated (NIL). The data are representative of 3 separate experiments, using HDL from 3 separate donors, and show mean and SD values. Differences between means were evaluated by using Student’s unpaired t test. *P, not significant; **P<0.001.
HDLs Modulate Expression of E-Selectin and Cox-2

by HDL,\textsuperscript{67} it is tempting to consider that the synergy we have seen is secondary to the elevation in LPC. However, elevation of LPC cannot explain the full actions of HDL, as LPC has been shown to elevate, rather than inhibit, the expression of adhesion molecules that we and others have shown to be inhibited by HDL.\textsuperscript{33,48} In addition, it is possible that HDL may be able to prolong messenger RNA stability of Cox-2, as has been suggested for IL-1 induction of the enzyme.\textsuperscript{68} It is also possible that HDL may mediate its effects transcriptionally. Analyses of the transcriptional requirements of the Cox-2 promoter have been conducted in bovine aortic EC with lipopolysaccharide or 12-O-tetradecanoylphorbol 13-acetate (TPA),\textsuperscript{69} in murine osteoblasts with TNFα,\textsuperscript{70} in rheumatoid synovial fibroblasts by using IL1β,\textsuperscript{71} and in A549 by using IL-1β,\textsuperscript{72} and all provide evidence that NF-κB is required for induced expression of the enzyme. Although our data suggest that HDL does not affect NF-κB translocation or transactivation in ECs, mutational analysis of the effect of HDL on cytokine-induced activation of the Cox-2 promoter may provide important clues as to the mechanism of the synergy between HDL and cytokine.

Although at face value it might seem paradoxical that HDLs inhibit expression of 1 cytokine-induced response while synergistically increasing another, it may be that the differential effects are functionally complementary. Thus, prostacyclin (PGI\textsubscript{2}) has been shown to be a potent inhibitor of leukocyte activation and adhesion, platelet aggregation, and vascular smooth muscle cell contraction, migration, and growth, and it is also shown to inhibit cholesterol ester accumulation in vascular cells.\textsuperscript{73,74} The ability of HDL to enhance TNFα- or IL-1β-mediated prostacyclin production would therefore be expected to act in conjunction with the inhibition of E-selectin, VCAM-1, and ICAM-1 expression to suppress leukocyte–EC interactions and thereby to protect against the progression of atherosclerosis and its thrombotic complications.

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