High-Density Lipoproteins Neutralize C-Reactive Protein Proinflammatory Activity

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Background—C-reactive protein (CRP), a well-recognized marker of atherosclerosis, has recently been suggested to have a direct proinflammatory effect. The constitutive expression of low levels of CRP in normal plasma suggests the likelihood that a natural factor exists to neutralize the effect of CRP. This factor(s) has not yet been identified.

Method and Results—The proinflammatory effect of CRP was measured by the induction of inflammatory adhesion molecules in human umbilical vein endothelial cells (HUVECs). We show that CRP significantly induced upregulation of adhesion molecules in both protein and mRNA levels. The CRP-induced expression of these inflammatory adhesion molecules was completely suppressed when the cells were preincubated with a physiological concentration (1 mg/mL apolipoprotein A-I) of HDLs derived from human plasma (native HDL) or reconstituted HDL (rHDL) at a very low concentration (0.01 mg/mL apolipoprotein A-I). A novel mechanism of HDL inhibition is likely to operate, because (1) rHDL was 100 times more potent than native HDL, (2) preincubation with HDL and its sustained presence were obligatory, and (3) oxidized 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine was the fundamental active component.

Conclusions—The CRP-induced upregulation of inflammatory adhesion molecules in HUVECs was completely prevented by HDL via their oxidized phospholipid components. (Circulation. 2004;109:2116-2122.)

Key Words: lipoproteins | protein | inflammation | atherosclerosis

C-reactive protein (CRP), an acute-phase reactant, has long been considered a nonspecific but sensitive marker of inflammatory diseases, including atherosclerosis and associated cardiovascular diseases. More recently, emerging evidence suggests that CRP may have direct proinflammatory effects involved in the pathogenesis of these inflammatory diseases. Elevated CRP has been shown to exert proatherogenic effects on vascular cells exemplified by increasing the secretion of monocyte chemoattractant protein (MCP-1), reducing nitric oxide bioactivity, and inducing adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin. However, constitutive expression of low levels of CRP is detected in normal plasma, and the actual in vivo effect of CRP observed from the experimental animals is controversial. It is possible that the proinflammatory activity of CRP is masked in vivo by endogenous neutralizing factors and/or antiinflammatory agents.

Much recent work reveals that vascular inflammation can be limited by antiinflammatory counterregulatory mechanisms that maintain the integrity and homeostasis of the vasculature (reviewed by Tedgui and Mallat). Our previous work shows that HDLs inhibit the cytokine-induced expression of inflammatory adhesion molecules in endothelial cells (ECs), which has been confirmed by many laboratories both in vitro and in vivo. These findings demonstrate a potent antiinflammatory capacity of HDL, which could account for the protective effect of HDL against atherogenesis. Indeed, epidemiological and clinical studies showed that HDL concentration is often inversely correlated with the plasma levels of proinflammatory agents, such as cytokines and CRP, in atherosclerotic cardiovascular diseases, revealing the importance of the balance between antiinflammatory and proinflammatory potentials in the pathogenesis of these diseases. The aim of the present study was to examine whether HDL could inhibit the proinflammatory effect of CRP.

Methods

Cell Culture and Flow Cytometry Analysis
Human umbilical vein ECs (HUVECs) and bovine aortic ECs (BAECs) were isolated and cultured as described previously. Cells were used at passages 2 to 3 for HUVECs and passages 3 to 10 for
BACEs. For detection of adhesion molecules, HUVECs were incubated overnight in Opti-MEM serum-free medium (Gibco, Invitrogen Corp) in the presence or absence of HDL and then treated with recombinant human CRP (Calbiochem) for 5 hours unless indicated otherwise. After the treatment, cells were washed with medium M199 and incubated with primary monoclonal antibodies against E-selectin, VCAM-1, ICAM-1, or isotype-matched nonrelevant control antibodies for 30 minutes as described previously. Cells were then incubated with fluorescein isothiocyanate–conjugated secondary antibody for 30 minutes. The cells were then harvested by trypsinization and fixed in 2.5% formaldehyde (antibody binding control antibodies for 30 minutes as described previously. Cells were then incubated with fluorescein isothiocyanate–conjugated secondary antibody for 30 minutes. The cells were then harvested by trypsinization and fixed in 2.5% formaldehyde (antibody binding control antibodies for 30 minutes as described previously. Cells were then incubated with fluorescein isothiocyanate–conjugated secondary antibody for 30 minutes. The cells were then harvested by trypsinization and fixed in 2.5% formaldehyde (antibody binding control antibodies for 30 minutes as described previously. Cells were then incubated with fluorescein isothiocyanate–conjugated secondary antibody for 30 minutes. The cells were then harvested by trypsinization and fixed in 2.5% formaldehyde (antibody binding control antibodies for 30 minutes as described previously. Cells were then incubated with fluorescein isothiocyanate–conjugated secondary antibody for 30 minutes. The cells were then harvested by trypsinization and fixed in 2.5% formaldehyde (antibody binding control antibodies for 30 minutes as described previously. Cells were then incubated with fluorescein isothiocyanate–conjugated secondary antibody for 30 minutes. The cells were then harvested by trypsinization and fixed in 2.5% formaldehyde (antibody binding control antibodies for 30 minutes as described previously. Cells were then incubated with fluorescein isothiocyanate–conjugated secondary antibody for 30 minutes. The cells were then harvested by trypsinization and fixed in 2.5% formaldehyde (antibody binding control antibodies for 30 minutes as described previously. Cells were then incubated with fluorescein isothiocyanate–conjugated secondary antibody for 30 minutes. The cells were then harvested by trypsinization and fixed in 2.5% formaldehyde (antibody binding control antibodies for 30 minutes as described previously. Cells were then incubated with fluorescein isothiocyanate–conjugated secondary antibody for 30 minutes. The cells were then harvested by trypsinization and fixed in 2.5% formic acid. Samples (20 μL) were then added into the BAEC cultures and incubated for 1.5 hours. The MTT-labeled U937 cell suspension (200 μL/well) was then added into the BAEC cultures and incubated for 30 minutes at 37°C. Nonadherent cells were removed by rinsing the plates 3 times with PBS, and the number of adherent cells was counted under microscopy in at least 4 fields per well.

Adherence of U937 Cell to ECs
U937 cells (CRL 1593.2; ATCC) were colorimetrically labeled with 0.2 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Promega) in normal culture medium for 30 minutes at 37°C. The cells were collected by low-speed centrifugation and resuspended at a density of 2×10⁶ cells/mL in medium without FCS. BAECs were seeded into 24-well plates and treated as desired in triplicate. After the treatment, BAECs were washed twice with RPMI-1640 medium. The MTT-labeled U937 cell suspension (200 μL/well) was then added into the BAEC cultures and incubated for 30 minutes at 37°C. Nonadherent cells were removed by rinsing the plates 3 times with PBS, and the number of adherent cells was counted under microscopy in at least 4 fields per well.

Isolation and Preparation of Lipoproteins and Small Unilamellar Vesicles
As described previously, the lipoproteins were isolated from normal healthy adult donors by sequential ultracentrifugation in their appropriate density range: total HDL 1.07<d<1.21 and LDL 1.019<d<1.055 g/mL. The resulting preparations of lipoproteins were dialyzed against endotoxin-free PBS (pH 7.4) before use. Discoidal reconstituted HDL containing apolipoprotein (apo) A-I and 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine (PLPC) were prepared by the cholate dialysis method described by Matz and Jonas. Small unilamellar vesicles (SUVs) containing either PLPC or 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) in butylated hydroxytoluene (BHT) (molar ratio of PC to BHT, 10:1) were prepared in PBS precisely as described by Jonas. Oxidation of SUVs was produced by incubation with 5 μmol/L CuSO₄ in the absence of BHT for 48 hours at 37°C.

Mass Spectrometry
Samples of phospholipid were extracted with CHCl₃:CH₃OH (2:1), diluted 10-fold in acetonitrile±0.05% formic acid. Samples (20 μL) were infused at 10 μL/min into a PE/Sciex API-100 electrospray-ionization mass spectrometer (PE Biosystems) with ionization at 4000 V, the orifice was set at 65 V, and the spectra were acquired (range, 200 to 1000 Th at 0.1-Th resolution).

Reverse Transcription–Polymerase Chain Reaction
The primers used to amplify E-selectin, VCAM-1, and ICAM-1 were as described by Meagher et al and were designed to span intron–exon boundaries. Total RNA was extracted from HUVECs using TRIzol (Gibco BRL) according to the manufacturer’s instructions. First-strand cDNA was synthesized from 1 μg total RNA using Omniscript reverse transcriptase (Qiagen) and Adaptor primer (GeneWorks). E-selectin, VCAM-1, and ICAM-1 were amplified over 27 cycles with an internal GAPDH control. Amplified products were visualized by electrophoresis on 1.5% agarose gel stained with ethidium bromide.

Results
CRP Stimulates Adhesion Molecule Expression in Conditioned Serum-Free Medium
Because the induction of adhesion molecules by ECs is critical for proinflammatory reactions in the vasculature, we examined the effect of CRP on expression of VCAM-1, ICAM-1, and E-selectin in HUVECs. Treatment of HUVECs with CRP resulted in a significant increase in the cell-surface expression of E-selectin, VCAM-1, and ICAM-1 (Figure 1A). The time course of CRP-induced adhesion protein expression was similar to the effect of cytokines such as tumor necrosis factor (TNF) (Figure 1B). The activity of CRP was dose-dependent, reaching a maximum at 10 to 20 μg/mL (Figure 1C), and ranged in pathophysiological concentrations that are often seen in chronic inflammatory diseases including atherosclerosis.

Pasceri et al previously reported that the CRP-induced expression of adhesion molecules was serum dependent. We found that CRP was capable of inducing adhesion molecule expression in HUVECs cultured in either medium containing
20% FCS or serum-free Opti-MEM medium that had been conditioned by HUVECs for 16 hours (Figure 2A). However, there was no inflammatory effect when CRP was added to cells in fresh serum-free medium (Figure 2B), which was consistent with Pasceri’s report. CRP-induced adhesion molecule expression was restored when HUVEC-conditioned medium was added back to the cells in a concentration-dependent manner (Figure 2B). These data indicate that the effect of CRP is dependent on a factor that is secreted by ECs or present in serum.

Lipopolysaccharide (LPS) is known to induce adhesion molecule expression; therefore, it was necessary to exclude LPS contamination as a factor in the CRP-induced effect. LPS at concentrations of up to 1 ng/mL was insufficient to induce adhesion molecule expression (Figure 2C), whereas the contamination by LPS detected in the purified CRP was <0.1 ng/mL. In addition, heating of CRP at 65°C significantly decreased the inflammatory effect in a time-dependent manner, and heating for 1 hour completely eliminated the activity of CRP, whereas heating at 65°C for 1 hour did not alter the effect of LPS (Figure 2C). These data strongly indicate that LPS is not responsible for the observed inflammatory effect of CRP.

### HDL Inhibits CRP-Induced Expression of Adhesion Molecules

Remarkably, the CRP-induced expression of adhesion molecules was profoundly inhibited by native HDL in serum-free medium in a concentration-dependent manner (Figure 3A). Native HDL at a physiological level (1 mg/mL of apoA-I) completely blocked the effect of CRP on expression of E-selectin, ICAM-1, and VCAM-1. In addition, the mRNA levels of these adhesion molecules induced by CRP were also significantly reduced by HDL (Figure 3B). To minimize possible confounding effects of the heterogeneity of native HDL particles and of any coisolated contaminants, we investigated the effects of reconstituted HDL (rHDL). Preincubation of HUVECs with rHDL containing PLPC and apoA-I (molar ratio, 100:1) resulted in a marked reduction in the CRP-induced expression of E-selectin, VCAM-1, and ICAM-1 (Figure 3C). Complete inhibition of the expression by rHDL was attained at a 100-fold lower concentration of HDL particles (10 μg/mL apoA-I) compared with native HDL (Figure 3A). However, treatment with lipid-free apoA-I had no effect (Figure 3A). In contrast, PLPC presented to the cells as SUVs had an inhibitory effect similar to that of rHDL (Figure 4, A and B), suggesting a major role of the unsaturated phospholipids in the inhibitory activity of HDL. As controls, preincubation of HUVECs with LDL or POPC SUVs had no inhibitory effect on the activity of CRP (data not shown).

### HDL Inhibits Adherence of U937 Cells to BAECs

To verify the biological consequences of adhesion molecule expression influenced by CRP and HDL, leukocyte adherence to aortic ECs was determined. Figure 5 shows that adherence of U937 cells increased 6-fold after incubation of BAECs with CRP for 24 hours, comparable to the level of binding after TNF stimulation. The induction of adhesion molecules measured by their mRNA levels was similar to that in HUVECs (data not shown). Significantly, in the presence of rHDL, the number of U937 cells binding to the CRP- or TNF-activated BAECs was markedly reduced (Figure 5).
Mechanism of HDL Inhibition on CRP Differ From That on Cytokine-Induced Adhesion Molecule Expression

The ability of HDL to inhibit adhesion protein expression induced by cytokines (such as TNF-α or interleukin-1) has been well documented.\textsuperscript{17} We thus investigated whether the inhibitory effect of HDL on CRP is mediated by a common mechanism of inhibition on the adhesion molecule expression induced by cytokines. In previous reports, we showed that a short-term preincubation (\(1\) hour) with HDL was sufficient for reduction in the TNF-induced expression of VCAM-1\textsuperscript{17} and that the inhibition did not require HDL to be physically present during the activation of adhesion molecule expression by TNF.\textsuperscript{17,18} However, no inhibitory effect on CRP-induced adhesion molecule expression was discernible after a 1-hour preincubation with either rHDL or PLPC (Figure 6A) or when these reagents were added simultaneously with CRP (data not shown). Furthermore, when HDL or PLPC was removed from the medium after a 16-hour preincubation before activation of the cells with CRP, the inhibitory effect did not persist (Figure 6A). In addition, in contrast to our previous findings of the inability of phospholipids alone to suppress the TNF-induced adhesion protein expression,\textsuperscript{7,19} PLPC had an inhibitory effect similar to that of whole HDL particles on CRP (Figures 4 and 6A). Thus, these data suggest different mechanisms underlying HDL inhibition of the CRP- and cytokine-induced proinflammatory actions.
Oxidation of PLPC Is Necessary to Inhibit the CRP Proinflammatory Effect

The inhibitory activity of rHDL or PLPC depends on a prolonged preincubation or preconditioning by cultured HUVECs (Figure 6A), suggesting that an interaction with ECs is required for the inhibitory effect of rHDL or PLPC on CRP. CRP has recently been shown to bind to the phosphorylcholine (PC) head group of oxidized LDL and oxidized phospholipids, although the biological consequences of the binding are yet unknown. We therefore investigated whether oxidation of PLPC is involved in the PLPC-dependent inhibition the proinflammatory effect of CRP. Mass spectrometric analysis of unoxidized PLPC revealed a single predominant ion peak at m/z 758.7 (Figure 6B), which was lost after incubation of PLPC with cultured HUVECs for 16 hours, presumably because of oxidation (Figure 6A, center). Interestingly, the PLPC-dependent inhibition of CRP-induced E-selectin expression was reversed in a dose-dependent manner by the presence of the antioxidants 

Figure 5. HDL inhibits adherence of U937 cells to BAECs. BAECs were treated with TNF (0.5 ng/mL) or CRP (10 μg/mL) for 24 hours in presence or absence of rHDL (10 μg/mL). Prelabeled U937 cells were incubated with treated BAECs for 30 minutes. A, Adherence of U937 cells was microscopically photographed (×20) and (B) determined by visually counting 4 microscopic fields per well in triplicate. *P<0.001.

E-selectin expression was abrogated (Figure 7B). No period of preincubation was required for the effect of oxidized PLPC. In contrast, oxidized PLPC did not inhibit TNF activity (Figure 7B), suggesting a specific effect on CRP. As a control, POPC that had been exposed to CuSO₄ did not affect CRP-induced adhesion molecule expression (data not shown), presumably because POPC is less readily oxidized. Taken together, these data strongly indicate that oxidized PLPC is a key molecule accounting for the inhibitory effect of HDL on CRP proinflammatory activity.

Discussion

It has been shown previously that CRP induces the cell-surface expression of adhesion molecules in HUVECs in the presence of serum, suggesting a proinflammatory action of CRP. We were able to confirm and extend the previous findings and show that E-selectin, VCAM-1, and ICAM-1 are all induced in HUVECs in the absence of serum after stimulation with CRP in HUVEC-conditioned medium. Thus, the CRP-induced adhesion molecule expression provides us with a reliable model for investigation of CRP proinflammatory activity in vitro. We demonstrate that the proinflammatory activity of CRP can be completely abolished by native HDL at physiological levels. In addition, reconstituted HDL, composed of lipoprotein apoA-I with PLPC as the sole phospholipid, also profoundly inhibited the CRP-induced expression of E-selectin, VCAM-1, and ICAM-1. Consequently, the physiological significance of these findings was
confirmed by the inhibition of CRP-induced adherence of U937 cells to aortic ECs in the presence of HDL (Figure 5). These findings thus reveal a novel function of HDL to neutralize CRP-mediated proinflammatory activity on the vasculature.

One important finding in the present report is that the inhibitory effect of HDL on CRP differed in several respects from the effect of HDL on cytokine-induced adhesion molecule expression, suggesting at least 2 mechanisms of protection against endothelial activation and vascular inflammation by HDL. Interestingly, PLPC alone in the form of SUVs had an inhibitory activity equivalent to that of rHDL, whereas neither lipid-free apoA-I nor POPC had any inhibitory effect. These data differ from previous findings that phospholipids alone were unable to mimic the inhibitory effect of whole HDL particles on the cytokine-induced adhesion molecule expression,17 implying a specific role of phospholipids in the protective capacity of HDL against the proinflammatory action of CRP.

The finding that preconditioning by incubation with HUVECs was required for the inhibitory effect of PLPC or HDL on CRP-induced adhesion molecule expression (Figure 6A) suggested that preconditioning converts the HDL or PLPC from an inactive form to a form that has inhibitory activity. To investigate whether endothelial lipases are involved in this process, we used tetrahydrolipstatin, a specific inhibitor of lipases. We found that the antiinflammatory activity of PLPC was not affected by treatment of cells with the lipase inhibitor (data not shown). Furthermore, the addition of up to 100 \( \mu \text{mol/L} \) phosphorylcholine, the product of lipase, to the medium had no effect on the activity of CRP (data not shown). Thus, hydrolysis of PLPC is unlikely to account for the antiinflammatory activity of PLPC or HDL. A recent report that CRP binds to the phosphorylcholine moiety of oxidized phospholipids but not to unoxidized phospholipids20 indicated that oxidation may be the mechanism converting PLPC to an active form. In support of this proposition, the antioxidants \( \alpha \)-tocopherol and NDGA were able to completely abolish the inhibitory effect of PLPC. In addition, oxidized PLPC inhibited the proinflammatory effect of CRP without requiring preincubation with the cells, suggesting an antiinflammatory potential of this phospholipid. Although oxidized phospholipids, especially within oxidized LDL, are generally considered proinflammatory agonists, recent reports have shown that some oxidized phospholipids indeed inhibit LPS-induced upregulation of inflammatory genes.21,22
tory activity of CRP. Oxidation of phospholipids could result in a conformational change that reveals “cryptic” binding sites to CRP.20 Thus, the interaction of HDL with ECs may expose CRP binding sites by oxidation of the phospholipids within the HDL particles, leading to a competitive inhibition of the interaction between CRP and ECs.

The mechanism of interaction between CRP and ECs is unknown. FcγRI and FcγRII, the high- and low-affinity IgG receptors, respectively, have been found to bind to CRP, and the latter receptor has been proposed to be the major CRP receptor in phagocytic cells.23 FcγRII expression is very low on the surface of ECs but has been reported to be upregulated on cytokine-stimulated ECs.24 However, the increased expression was time-dependent, requiring 2 to 3 days of exposure to cytokine, and thus was unlikely to have occurred during the short exposure of HUVECs to either TNF or CRP shown here. Further studies are needed to identify whether a specific receptor for CRP exists on ECs.

In summary, we have shown a novel function of HDL that, via oxidation of its principal phospholipid, neutralizes the proinflammatory potential of CRP in ECs, revealing a balance between antiinflammatory and proinflammatory actions within the vascular wall. Factors that decrease the quantity and/or quality of HDL, such as obesity, diabetes, and age, are often associated with increased CRP concentration,8,9,25–27 which could ultimately disturb the antiinflammatory and proinflammatory balance to contribute to the development of inflammatory cardiovascular diseases. Therefore, the data presented here have important implications for the development of new strategies to prevent atherosclerosis and associated cardiovascular diseases.

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References

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