HDL Remodeling During the Acute Phase Response

Anisa Jahangiri, Maria C. de Beer, Victoria Noffsinger, Lisa R. Tannock, Chandrashekar Ramaiah, Nancy R. Webb, Deneyes R. van der Westhuyzen, Frederick C. de Beer

Objective—The purpose of this study was to examine the interactive action of serum amyloid A (SAA), group IIA secretory phospholipase A2 (sPLA2-IIA), and cholesteryl ester transfer protein (CETP) on HDL remodeling and cholesterol efflux during the acute phase (AP) response elicited in humans after cardiac surgery.

Methods and Results—Plasma was collected from patients before (pre-AP), 24 hours after (AP-1 d), and 5 days after cardiac surgery (AP-5 d). SAA levels were increased 16-fold in AP-1 d samples. The activity of sPLA2-IIA was increased from 77.7±38.3 U/mL (pre-AP) to 281.4±57.1 U/mL (AP-1 d; P<0.001). CETP mass and activity reduction was commensurate to the reduction of HDL cholesterol levels. The combined action of SAA, sPLA2-IIA, and CETP in vitro markedly remodeled HDL with the generation of lipid-poor apoA-I from both pre-AP and AP-1 d HDL. The net result of this remodeling was a relative preservation of ABCA1- and ABCG1-dependent cholesterol efflux during the acute phase response.

Conclusions—Our results show that the many and complex changes in plasma proteins during the acute phase response markedly remold HDL with functional implications, particularly the relative retention of cholesterol efflux capacity. (Arterioscler Thromb Vasc Biol. 2009;29:261-267.)

Key Words: SAA ■ HDL ■ CETP ■ apoA-I ■ inflammation

Inflammation induces major changes in HDL levels and composition. Mediators of inflammation such as tumor necrosis factor (TNF)-α and interleukin (IL)-6 induce expression of serum amyloid A and group IIA secretory phospholipase A2 (sPLA2-IIA), which dramatically alter HDL apolipoprotein content and levels, respectively. Acute phase SAA in the plasma is associated with HDL, where it can comprise the major apolipoprotein. The increase in sPLA2-IIA activity results in hydrolysis of HDL surface phospholipids and a decrease in HDL particle size. The plasma cholesteryl ester transfer protein (CETP) is an integral component of reverse cholesterol transport and regulates HDL cholesterol concentrations. By promoting the transfer of cholesteryl esters (CE) from HDL to apoB-containing lipoprotein particles, HDL-derived CE is taken up via the LDL receptor and cleared by the liver. An additional result of CETP action is the generation of lipid-poor apoA-I, a key acceptor in ATP-binding cassette transporter A1 (ABCA1)-mediated lipid efflux. The presence of SAA on HDL holds the potential to impact both the CE transfer and the apoA-I liberating ability of CETP. sPLA2-IIA could also impact the latter action of CETP as apoA-I was shown to dissociate more readily from CETP-remodeled reconstituted HDL after hydrolysis by bee venom phospholipase A2. Given the centrality of inflammation in atherogenesis, there is a paucity of information regarding CETP function when acute phase HDL is the “substrate.” In the present study, we used plasma from patients undergoing cardiac surgery with cardiopulmonary bypass as a “standardized” insult where the oxygenator membrane activates macrophages to produce cytokines. We characterized the SAA-containing acute phase (AP) HDL during the acute phase to define the polydisperse HDL “substrate” that CETP would encounter. We further investigated CETP function in the acute phase, particularly as it relates to the presence of SAA and SAA2 on AP HDL, with respect to its CE transfer and apoA-I liberating functions. Teleologically, the dramatic changes in HDL composition and metabolism during inflammation must serve a short-term purpose to allow the organism to survive a noxious assault. Acute tissue injury results in cell death with large quantities of cell membranes rich in phospholipids and cholesterol generated. Macrophages are mobilized to such sites, ingest these fragments, and acquire considerable lipid load. We thus examined the influence of the AP response on the ability of serum to promote cholesterol efflux as a removal mechanism to mobilize this cholesterol in an ABCA1- and ABCG1-dependent manner.
Methods

Human Subjects

Patients undergoing cardiac surgery donated plasma before (preacute phase, pre-AP), 24 hours postoperatively (acute phase, AP-1 d), and at discharge, 5 days after surgery (AP-5 d) as outlined in the supplemental materials (available online at http://atvb.ahajournals.org). This study was approved by the University of Kentucky Medical Institutional Review Board (IRB). For the full descriptions of the methods used, please see the supplemental materials.

Statistical Analyses

Data are presented as mean±SEM. Differences between pre-AP and AP parameters were tested by paired t test (SigmaStat 3.5). Statistical analyses between pre-AP, AP-1 d, and AP-5 d were performed using repeated measures 1-way ANOVA with the Holm-Sidak multiple comparisons test. Significance was set at P<0.05. A Wilcoxon signed rank test was used for post test of CETP mass. The power in all tests was >0.9.

Results

SAA, sPLA2, CETP, and HDL in AP Plasma

As expected, plasma SAA levels increased from pre-AP levels of 47.2±19.9 µg/mL to 785.6±66.4 µg/mL in AP-1 d samples (P<0.05) and were still elevated at discharge (AP-5 d; 567.2±50.2 µg/mL; P<0.05; Figure 1A). The activity of sPLA2-IIA followed a similar pattern: 77.7±38.3 U/mL (pre-AP), 281.4±57.1 U/mL (AP-1 d), and 250.5±43 U/mL (AP-5 d; P<0.05; Figure 1B). Quantitative immunoblot analysis showed a 2.9-fold reduction in CETP mass from 2.6±0.6 µg/mL in pre-AP plasma to 0.9±0.2 µg/mL in AP-1 d plasma, (Figure 2A P<0.01). Consistent with this, CETP activity was 2.2-fold lower in AP compared to pre-AP plasma (16.9±3.0 nmol/mL/h and 37.9±4.1 nmol/mL/h respectively, P<0.001; Figure 2B). HDL-C levels were reduced 1.9-fold, from 372.2±44.6 µg/mL to 195.0±22.2 µg/mL (P<0.001; Figure 2C), and apoA-I dropped from 1590.0±84.2 µg/mL to 771.4±138.7 µg/mL (P<0.001; Figure 2D). The decrease in

Figure 1. SAA (A) and sPLA2 (B) concentrations in pre-AP, AP-1 d, and AP-5 d plasma. Data are presented as mean±SEM; n=12 (SAA), n=6 (sPLA2); *P<0.05 vs pre-AP by 1-way repeated measures ANOVA.

Figure 2. CETP, HDL cholesterol, and apoA-I are reduced in AP-1 d plasma. A, Plasma CETP concentrations were quantified by densitometric analysis of Western blots. CETP activity (B), HDL-C concentrations (C), and apoA-I (D) concentrations in pre-AP and AP-1 d plasma. **P<0.01; ***P<0.001 by paired t test.
CETP activity was commensurate with the reduction in HDL-C and apoA-I, hence the activity of CETP normalized to HDL-C or apoA-I levels was not different between pre-AP and AP plasma (not shown).

Characterization of Pre-AP and AP HDL by Immunoaffinity Chromatography

It was reported that the presence of apoA-II on reconstituted HDL particles inhibits the CETP-mediated dissociation of apoA-I.\textsuperscript{11} To assess the influence of SAA, we studied pre-AP and AP-1 d HDL as well as HDL\textsubscript{2} enriched in vitro with SAA (SAA-HDL). We subjected \textsuperscript{125}I-HDL to immunoaffinity chromatography to determine the proportion of the LpAI fraction (ie, HDL particles lacking both apoA-II and SAA, and containing only apoA-I) in AP HDL. Figure 3A is a Coomassie-stained SDS-PAGE gel showing the major apolipoproteins present in these HDL. Whereas SAA is virtually undetectable in pre-AP HDL, it is a major component of AP-1 d HDL (27.2\% of total protein by mass). In SAA-HDL, SAA comprised a major apolipoprotein. Figure 3B shows the autoradiograph (ARG) of the SDS-PAGE analysis of the fractions when pre-AP HDL was passed through an anti–apoA-II column so that the FT comprises only LpAI particles. Figure 3C shows the autoradiograph of the SDS-PAGE analysis of the fractions when pre-AP HDL was passed through an anti–apoA-II column so that the FT comprises only LpAI particles. Figure 3D shows the autoradiograph of the SDS-PAGE analysis of the fractions when pre-AP HDL was passed through an anti–apoA-II column so that the FT comprises only LpAI particles. Figure 3E shows the autoradiograph of the SDS-PAGE analysis of the fractions when pre-AP HDL was passed through an anti–apoA-II column so that the FT comprises only LpAI particles. Figure 3F shows the autoradiograph of the SDS-PAGE analysis of the fractions when pre-AP HDL was passed through an anti–apoA-II column so that the FT comprises only LpAI particles. Figure 3G shows the autoradiograph of the SDS-PAGE analysis of the fractions when pre-AP HDL was passed through an anti–apoA-II column so that the FT comprises only LpAI particles. Figure 3H shows the autoradiograph of the SDS-PAGE analysis of the fractions when pre-AP HDL was passed through an anti–apoA-II column so that the FT comprises only LpAI particles. Figure 3I shows the autoradiograph of the SDS-PAGE analysis of the fractions when pre-AP HDL was passed through an anti–apoA-II column so that the FT comprises only LpAI particles. Figure 3J shows the autoradiograph of the SDS-PAGE analysis of the fractions when pre-AP HDL was passed through an anti–apoA-II column so that the FT comprises only LpAI particles. Figure 3K shows the autoradiograph of the SDS-PAGE analysis of the fractions when pre-AP HDL was passed through an anti–apoA-II column so that the FT comprises only LpAI particles. Figure 3L shows the autoradiograph of the SDS-PAGE analysis of the fractions when pre-AP HDL was passed through an anti–apoA-II column so that the FT comprises only LpAI particles. Figure 3M shows the autoradiograph of the SDS-PAGE analysis of the fractions when pre-AP HDL was passed through an anti–apoA-II column so that the FT comprises only LpAI particles. Figure 3N shows the autoradiograph of the SDS-PAGE analysis of the fractions when pre-AP HDL was passed through an anti–apoA-II column so that the FT comprises only LpAI particles. Figure 3O shows the autoradiograph of the SDS-PAGE analysis of the fractions when pre-AP HDL was passed through an anti–apoA-II column so that the FT comprises only LpAI particles. Figure 3P shows the autoradiograph of the SDS-PAGE analysis of the fractions when pre-AP HDL was passed through an anti–apoA-II column so that the FT comprises only LpAI particles. Figure 3Q shows the autoradiograph of the SDS-PAGE analysis of the fractions when pre-AP HDL was passed through an anti–apoA-II column so that the FT comprises only LpAI particles. Figure 3R shows the autoradiograph of the SDS-PAGE analysis of the fractions when pre-AP HDL was passed through an anti–apoA-II column so that the FT comprises only LpAI particles. Figure 3S shows the autoradiograph of the SDS-PAGE analysis of the fractions when pre-AP HDL was passed through an anti–apoA-II column so that the FT comprises only LpAI particles. Figure 3T shows the autoradiograph of the SDS-PAGE analysis of the fractions when pre-AP HDL was passed through an anti–apoA-II column so that the FT comprises only LpAI particles. Figure 3U shows the autoradiograph of the SDS-PAGE analysis of the fractions when pre-AP HDL was passed through an anti–apoA-II column so that the FT comprises only LpAI particles. Figure 3V shows the autoradiograph of the SDS-PAGE analysis of the fractions when pre-AP HDL was passed through an anti–apoA-II column so that the FT comprises only LpAI particles. Figure 3W shows the autoradiograph of the SDS-PAGE analysis of the fractions when pre-AP HDL was passed through an anti–apoA-II column so that the FT comprises only LpAI particles. Figure 3X shows the autoradiograph of the SDS-PAGE analysis of the fractions when pre-AP HDL was passed through an anti–apoA-II column so that the FT comprises only LpAI particles. Figure 3Y shows the autoradiograph of the SDS-PAGE analysis of the fractions when pre-AP HDL was passed through an anti–apoA-II column so that the FT comprises only LpAI particles. Figure 3Z shows the autoradiograph of the SDS-PAGE analysis of the fractions when pre-AP HDL was passed through an anti–apoA-II column so that the FT comprises only LpAI particles.

Comparative Displacement of ApoA-I in Pre-AP and AP HDL by CETP

Because ultracentrifugation displaces CETP from HDL,\textsuperscript{12} recombinant CETP was exogenously added in the remodeling experiments. The CETP activity in the incubations corresponded to the activity of CETP in normal human plasma. When the total pre-AP and AP HDL were incubated with CETP in vitro for 24 hours, a dose-dependent dissociation of apoA-I was observed (Figure 4A).\textsuperscript{13} The dissociation of apoA-I from pre-AP and AP HDL was comparable. Although it appears that 50\% or more of the apoA-I is in the lipid-poor state, the enhanced immunoreactivity of dissociated apoA-I precludes exact quantification. In addition to generating lipid-poor apoA-I, CETP action also resulted in the formation of larger HDL particles which are likely TG-enriched because...
of CE/TG exchange. CETP action on AP HDL liberated a very limited amount of “lipid-poor” SAA (Figure 4B). Recombinant SAA exhibited slower mobility than apoA-I on gradient gels, likely because of increased aggregation under the nondenaturing conditions (Figure 4B). SAA is dispersed on a broader spectrum of particle sizes, some likely containing little apoA-I (compare Figure 4A lane 8 and Figure 4B lane 4).

**Comparative Displacement of ApoA-I in Pre-AP and AP HDL by Concomitant Action of CETP and sPLA2-IIA**

During the acute phase, dissociation of apoA-I from HDL could be influenced by acute phase secretory phospholipase (ie, sPLA2-IIA), which is present in plasma and associates with HDL.13 We thus used SAA-HDL (Figure 1A) to study the remodeling of HDL by CETP and sPLA2-IIA. Treatment of SAA-HDL with CETP alone resulted in the generation of comparable amounts of lipid-poor apoA-I (Figure 5B, lane 3) as observed with control HDL2 (Figure 5A, lane 3). CETP treatment did not result in the displacement of significant amounts of lipid-poor SAA from SAA-HDL2 (Figure 5C, lane 3). Surface hydrolysis of HDL phospholipids by sPLA2 action alone resulted in a reduction in HDL particle size not accompanied by the dissociation of apoA-I or SAA (Figure 5A, 5B, 5C, lane 2). However the combined action of sPLA2 and CETP converted the majority of the apoA-I from the HDL-bound to the lipid-poor form in both HDL2 and SAA-HDL (Figure 5A and 5B, lane 4). This combined action may also result in the dissociation of limited amounts of lipid-poor SAA from SAA-HDL (Figure 5C, lane 4). We conclude that CETP action on the core, and sPLA2-IIA hydrolysis on the HDL surface, synergize to liberate lipid-poor apoA-I.

**ABCA1 and ABCG1-Dependent Cholesterol Efflux**

To test the effects of the acute phase response on HDL function, efflux assays were carried out using pre-AP, AP-1 d, and AP-5 d serum diluted to 2.5%. When compared to pre-AP serum, ABCA1-dependent efflux was not significantly decreased at AP-1 d despite a highly significant 53% fall in plasma apoA-I concentrations (Table). At AP-5 d, ABCA1 efflux was still maintained despite apoA-I remaining 1.5-fold lower than pre-AP levels. Similarly, ABCG1 efflux was modestly reduced at AP-1 d, whereas HDL-C decreased...
because the decrease in plasma HDL during inflammation decreased apoA-I and HDL levels. However, this is unlikely to increase HDL. A number of mechanisms could operate to reduce HDL despite the normal tendency of CETP to liberate apoA-I. (3) sPLA2-IIA enhances the surface disequilibrium that is alleviated by the dissociation of lipid-poor apoA-I. Increased sPLA2-IIA activity during the acute-phase response may further potentiate the generation of lipid-poor apoA-I.

It was reported that apoA-II abrogates the CETP-mediated liberation of apoA-I from reconstituted HDL containing both apoA-I and apoA-II.11 In pre-AP HDL, we demonstrated that approximately 80% of apoA-I is present in LpAI particles (Figure 3B). Lipid-poor apoA-I dissociating from pre-AP HDL in our study is thus likely derived from these LpAI particles. In contrast, in AP HDL, less than 20% of the apoA-I was present as LpAI, the remainder being associated with SAA and/or apoA-II (Figure 3C). However, CETP action resulted in the liberation of the bulk of apoA-I from AP HDL. This establishes that SAA, in contrast to apoA-II, does not interfere with the liberation of apoA-I induced by CETP remodeling.

Our data indicate that SAA is present on most AP HDL particles (Figure 3C). Unlike SAA, CETP is present as a dynamic exchange protein rather than a structural protein and undergoes rapid bidirectional transfer between HDL particles and acceptors. At any given time, only ~1 in 1000 HDL particles carry a CETP molecule. The fact that the activity of CETP when normalized to HDL-C is unaltered during the acute phase suggests that CETP dynamics are also unchanged during the APR. Our results strongly suggest that any decrease in total cholesteryl ester transfer from HDL during the acute phase is the result of the concomitant reduction of plasma CETP and HDL-C rather than the reduced functionality of the HDL/CETP interaction.

It is notable that CETP action results in an apparent increase in size of the HDL particles (Figures 4 and 5). In addition to liberating apoA-I, CETP-mediated remodeling has been reported to result in particle fusion.22 The increased particle size of CETP-remodeled HDL may also relate to the exchange of CE for TG by CETP action. As TG molecules are larger than CE molecules, HDL size would increase as TG content increases.23 CETP action on HDL results in core/surface disequilibrium that is alleviated by the dissociation of lipid-poor apoAI.24 Notably, in our study, CETP-remodeling of HDL resulted in both an increase in size of the particles, as well as the dissociation of lipid-poor apoA-I. Increased sPLA2-IIA activity during the acute-phase response may further potentiate the generation of lipid-poor apoA-I.

The increased sPLA2 activity in acute phase plasma (Figure 1B) is likely attributable to an increase in sPLA2-IIA.25 sPLA2-IIA was shown to be present in atherosclerotic plaques bound to heparan-sulfate proteoglycans of the subendothelial extracellular matrix.26 Proteoglycan binding of sPLA2-IIA particularly sPLA2-IIA, which increases HDL catabolism.18,19 SAA itself can also enhance the activity of sPLA2.20 The proinflammatory cytokines that induce SAA and sPLA2-IIA, simultaneously decrease apoA-I expression.21 Thus the decreased levels of apoA-I and increased levels of SAA on HDL during inflammation are attributable at least in part to reciprocal coordinated regulation. Finally, the combined remodeling action of sPLA2-IIA and CETP during the acute phase response (Figure 5) may also result in the increased catabolism of HDL. Given the numerous factors that operate during inflammation it is notable that the ratio of CETP to HDL is maintained. CETP reduction during inflammation could be viewed as a defensive adaptation to prevent “excessive” HDL reduction mediated by the mechanisms discussed.

Discussion

Data presented here indicate the following: (1) The reduction in CETP concentration and activity during the acute phase response is commensurate with the reduction in HDL levels. (2) Despite the striking alterations in HDL composition during the acute phase, CETP activity was maintained both with respect to its cholesteryl ester transfer function and its capacity to liberate apoA-I. (3) sPLA2-IIA enhances the ability of CETP to liberate apoA-I, and this is not impaired by the presence of SAA on the HDL. (4) Although SAA has been shown to be an effective acceptor in lipid efflux, CETP action liberates it to a limited extent in a lipid-poor form. (5) The interplay between the numerous acute phase proteins impacting HDL remodeling result in the relative preservation of ABCA1- and ABCG1-dependent cholesterol efflux.

There is an inverse relationship between CETP activity and HDL concentrations in the normal state.14 This was the basis for developing CETP inhibitors to increase HDL levels for potential therapeutic benefit. During inflammation this inverse relationship does not hold as both CETP and HDL levels are reduced. Studies have shown that inflammatory cytokines reduce CETP transcription and levels.15,16 This could result in increased HDL levels. However, our data shows a commensurate decrease in HDL and CETP in the acute phase. This suggests that during inflammation factors operate to reduce HDL despite the normal tendency of CETP to increase HDL. A number of mechanisms could operate to decrease plasma HDL during the acute phase. It was originally assumed that SAA enrichment of HDL was the basis for decreased apoA-I and HDL levels. However, this is unlikely because the decrease in plasma HDL during inflammation occurs rapidly, before SAA accumulation.17 An alternative explanation for decreased HDL levels during inflammation could be remodeling by acute phase group II phospholipases.

Table. Cellular Cholesterol Efflux During the Acute Phase Response

<table>
<thead>
<tr>
<th></th>
<th>Pre-AP</th>
<th>AP-1 Day</th>
<th>AP-5 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCA1- efflux (%)</td>
<td>100</td>
<td>82.5 ± 3.5</td>
<td>99.2 ± 2.9</td>
</tr>
<tr>
<td>ABCG1- efflux (%)</td>
<td>100</td>
<td>70.0 ± 14.3</td>
<td>108.5 ± 16.7</td>
</tr>
<tr>
<td>Plasma apoA-I (μg/ml)</td>
<td>1161.7 ± 117.6</td>
<td>545.0 ± 62.4</td>
<td>785.0 ± 36.6</td>
</tr>
<tr>
<td>Plasma HDL-C (μg/ml)</td>
<td>389.7 ± 60.5</td>
<td>196.6 ± 39.1</td>
<td>238.7 ± 27.9</td>
</tr>
</tbody>
</table>

ABCA1- and ABCG1-dependent cholesterol efflux was determined as outlined in the methods. Efflux experiments were performed at 37°C by incubating cells with serum (diluted to 2.5%) from patients pre-AP, AP-1 d, and AP-5 d for 16 hours in DMEM containing 0.2% BSA. Results are presented as a percentage of pre-AP serum efflux. ApoA-I was measured using an automated turbidimetric immunoassay (Mayo Medical Laboratories). HDL was quantitated using a commercial kit. *P<0.05 vs pre-AP and AP-5 d; † P<0.001 vs AP-1 d and AP-5 d; ‡ P<0.05 vs pre-AP.
serves to “concentrate” the enzyme, and it is more active in the bound form.26 Thus the effect of sPLA2-IIA on HDL remodeling may be more pronounced in the intima of a vessel. Furthermore, sPLA2 is more active when SAA is present on HDL.20 It may also be relevant that like sPLA2, SAA is also bound by proteoglycans in lesions.27 Although efflux of cholesterol from macrophages at this site represents only a small fraction of overall cellular cholesterol efflux, it is critically protective in the context of atherosclerosis.7 The interaction of sPLA2 and SAA might constitute a defensive mechanism against lipid accumulation. SAA-HDL is particularly enriched in SAA compared to AP HDL, with SAA present on 80% of particles.3 In our study, CETP action on SAA-HDL liberated significant amounts of lipid-poor apoA-I. This confirms that the presence of SAA does not impair the dissociation of apoA-I. In our study, there was no clear evidence that SAA was significantly displaced in a lipid-poor form by either CETP or sPLA2-IIA (Figures 4 and 5), though one has to recognize the limitations of analyzing SAA on non-denaturing gels as its tendency to self-aggregate and associate with the acrylamide matrix is well established.

Given the extensive remodeling of HDL during the acute phase that affects both the polydisperse particles themselves as well as the equilibrium between bound and free apolipoproteins, we evaluated the integral of all these actions on ABCA1- and ABCG1-dependent cholesterol efflux. We show an overall preservation of cholesterol efflux capacity of serum during the acute phase response. A large body of evidence suggested that HDL is part of the innate immune system,28 and that acute phase HDL remodeling could impact the unique cargo of proteins on HDL,29 reducing its antiinflammatory properties. We showed the presence of ABCA1- and ABCG1-dependent cholesterol efflux. This leads to the conclusion that the presence of antiatherogenic functions in SAA-HDL is not diminished by CETP action. However, during chronic inflammatory conditions, the latter might constitute a much more important risk factor for atherogenesis.

Inhibition of CETP results in elevated HDL levels,14 but this could abrogate its two antiatherogenic functions. The recent cessation of a human clinical trial testing such an inhibitor31 illustrates the need to better understand CETP function, not only on circulating lipoproteins, but also on apoA-I liberation at the level of the atherosclerotic lesion in the vessel wall. One can imagine a scenario where CETP inhibition could increase plasma HDL but also alter the equilibrium between HDL and lipid-poor apoA-I and consequently efflux potential. Our results indicate that AP HDL is not impaired in its ability to liberate apoA-I after CETP remodeling. This combined with the potentiation of apoA-I release by the combined action of CETP and the acute phase sPLA2-IIA, supports our results showing that during the acute phase, cholesterol effluxing capacity may be preserved despite a reduction of plasma HDL. However, prolonged inflammation and continual HDL remodeling may eventually lead to proatherogenic conditions by limiting the levels of HDL and apolipoprotein cholesterol acceptors and the proinflammatory nature of AP HDL having an impact.

Acknowledgments
The authors acknowledge the technical assistance of John Cranfill, Nathan Whitaker, Nick Whitaker, Matt Mains, and Connie Dampier.

Sources of Funding
This study was supported by an NIH Program Project Grant (PO1HL086670; to D.R.v.d.W.) and a VA Merit Review Funded by Veterans Affairs (VACO to F.C.D.).

Disclosures
None.

References


HDL Remodeling During the Acute Phase Response
Anisa Jahangiri, Maria C. de Beer, Victoria Noffsinger, Lisa R. Tannock, Chandrashekar Ramaiah, Nancy R. Webb, Denesys R. van der Westhuyzen and Frederick C. de Beer

Arterioscler Thromb Vasc Biol. 2009;29:261-267; originally published online November 13, 2008;
doi: 10.1161/ATVBAHA.108.178681
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/29/2/261

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2008/11/14/ATVBAHA.108.178681.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Methods

Human subjects

Patients undergoing cardiac surgery (coronary artery bypass, valve replacement) using a membrane oxygenator, donated blood prior to (pre-acute phase, pre-AP), 24 hr post-operatively (acute phase, AP- 1d), and at discharge, 5 days after surgery (AP- 5d). This study was approved by the University of Kentucky Medical Institutional Review Board (IRB) and blood was only collected from patients who underwent successful uncomplicated surgery and who gave informed consent.

Blood sampling

Blood was collected into EDTA-tubes. Plasma was isolated after a 10 min centrifugation at 530 x g and stored at minus 80°C after addition of protease inhibitor (aprotinin (2 µg/ml)). Measurements of SAA, sPLA₂, CETP, HDL-C and apoA-I were performed on individual patient plasma samples. Due to IRB restrictions, only small volumes of blood could be obtained from each patient. This necessitated the pooling of plasma from 4 – 7 patients for HDL isolation. The HDL remodeling studies are representative of two such HDL preparations. For efflux studies, plasma was clotted in a glass tube using 25 mM CaCl₂.

Measurement of plasma proteins and lipids

Plasma SAA levels were determined by ELISA (Biosource International, Camarillo, CA). CETP activity was determined using the Roar Biomedical kit (NY, New York). sPLA₂ activity was measured according to the protocol of Wooton-Kee using 1-palmitoyl-2-oleoyl-
phosphatidylglycerol (POPG) as a substrate. Plasma CETP and apoA-I was determined by densitometric scanning of Western blots (Kodak 1D, New Haven, CT). Briefly, 1 µl of plasma from patients was separated by 4 – 20% SDS-PAGE, transferred onto a PVDF membrane and immunoblotted using either anti-human CETP TP2 (University of Ottawa, Canada) or anti-human apoA-I (Calbiochem, San Diego, CA). A standard curve was obtained from the same gel by quantifying the densitometric signal produced by four different amounts of recombinant human CETP (Cardiovascular Targets, Inc, NY) or apoA-I (Biodesign International, Saco, Maine). CETP or apoA-I mass in plasma samples was determined by densitometric analysis against this standard. HDL-cholesterol levels were determined using a commercial kit (Wako Diagnostics, Richmond, VA). For the efflux experiments, apoA-I was measured using an automated turbidimetric immunoassay (Mayo Medical Laboratories, Rochester, MN). Total protein was measured by the Lowry method.

**HDL isolation**

Pre-AP and AP-1d HDL (1.063 < ρ < 1.21 g/ml) were isolated from pooled patient plasma by sequential ultracentrifugation as described, dialyzed against 150 mM NaCl, 0.01% (w/v) EDTA (pH 7.4), sterile filtered and stored at 4°C under argon gas. VLDL was isolated from normal volunteers by ultracentrifugation (ρ < 1.019 g/ml), dialyzed and stored as above. SAA-HDL was prepared by incubating 1.4 mg HDL₂ with 1 mg recombinant human SAA (Biovision, Mountain View, CA) for 4 hr at room temperature. The SAA-HDL was recovered by ultracentrifugation (ρ < 1.25 g/ml), dialyzed and stored as above.
In vitro HDL remodeling

Pre-AP and AP- 1d HDL (0.7-0.9 mg/ml total HDL protein, 30 µg apoA-I) were incubated in a final reaction volume of 50 µl in Tris-buffered saline, pH 7.4 at 37ºC for 24 hr with human recombinant CETP (0, 1, 2 or 4 µg per mg HDL protein, Cardiovascular Targets, Inc, NY), human VLDL (4 mM final triglyceride concentration), fatty-acid free bovine serum albumin (BSA) (10 mg/ml) and CaCl$_2$ (2 mM). We attempted to make our incubations as close as possible to physiological conditions, by using physiological concentrations of HDL, CETP, VLDL, calcium and albumin. In order to allow the HDL remodeling to proceed to completion, reactions were incubated for 24 hr. These methods are comparable to those employed in the literature$^5$, $^6$, $^7$. Reactions were terminated by the addition of EDTA (final concentration 20 mM). Human VLDL was used as a cholesteryl ester acceptor and triglyceride donor for CETP action. SAA-HDL (1 mg/ml) was incubated with CETP (2 µg/mg HDL) and human recombinant sPLA$_2$-IIA (2 µg/mg HDL) under the same conditions described above. The purity of the recombinant CETP was assessed by SDS-gel electrophoresis which showed only one band corresponding to the size of CETP at ~ 70kDa. The specific activity was reported by the manufacturer at 25 fold over normal human plasma. In the in vitro studies, we used CETP at physiological concentrations (approx 1 in 25 dilution of the stock CETP) which is in the activity range of CETP in vivo.

Gradient gel electrophoresis and Western blots

Aliquots of incubation mixtures (1 µg apoA-I) were electrophoresed on 4-20% non-denaturing polyacrylamide gels for 3.5 hr at 200 V, 4ºC. They were then transferred to PVDF membranes (100 min at 100 V at 4ºC) for subsequent Western blotting with anti-human apoA-I (Calbiochem) or anti-human SAA (Behring, Germany) antibodies. Bound antibodies were detected by enhanced chemiluminescence (GE Healthcare, NJ).
Immunoaffinity chromatography

HDL was radiolabeled by the iodine monochloride method\textsuperscript{8} and characterized using the Seize\textsuperscript{®} Primary Mammalian Immunoprecipitation kit (Pierce, Rockford, IL). Briefly, immunoaffinity spin columns were made containing anti-SAA or anti-apoA-II antibodies (200 µg antibody/ 200 µl coupling gel) according to the manufacturer’s instructions. \textsuperscript{125}I-labeled pre-AP and AP HDL (10 µg total protein) was allowed to bind to the column (with rotation) overnight at 4ºC. Unbound HDL particles (flow through, FT) were spun down (1 min at 1180 x g). After 5 washes with 400 µl Tris-buffered saline (pH 7.4), the bound HDL was eluted using 200 µl elution buffer containing a primary amide (pH 2.8) into 10 µl of 1M Tris (pH 9.5) to give a final pH of 7.4. Equal counts (2000 dpm) of the FT and eluted fractions (E1-E5) were subjected to SDS-PAGE using 4 – 20% acrylamide gels and analyzed by subsequent autoradiography (ARG).

Cholesterol Efflux

ABCA1-dependent cholesterol efflux was determined in skin fibroblasts isolated from normal and Tangier disease patients as described previously\textsuperscript{9}. Cells were labeled with 0.2 µCi/ml \[^{3}H\]cholesterol in medium for 48 h. Cellular ABCA1 expression was stimulated with free cholesterol loading by incubating fibroblasts with 30 µg/ml cholesterol in medium containing 0.2% fatty acid-free BSA for 48 h. Total cellular efflux was calculated following overnight incubation in 300 µl serum-free DMEM containing 0.2% fatty acid-free BSA and 2.5% pre-AP, AP- 1d or AP- 5d AP- 5d serum. ApoA-I (10 µg/ml) was used as a positive control. ABCA1-specific efflux was calculated by subtracting the value obtained in Tangier disease fibroblasts from the value in normal fibroblasts. ABCG1-dependent cholesterol efflux was determined in mock- and ABCG1 transfected cells as described previously\textsuperscript{10}. Cells were labeled as described above and treated with 10 nM mifepristone for 18 hr prior to efflux experiments which were
carried out as outlined above. ABCG1-dependent cholesterol was calculated as the difference in cholesterol efflux between mock- and ABCG1 transfected cells. HDL (25 µg/ml) was used as a positive control). All the cell lines were kindly provided by Dr. John Oram (University of Washington).
References


