Nascent HDL formation by hepatocytes is reduced by the concerted action of serum amyloid A and endothelial lipase

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Abstract  Inflammation is associated with significant decreases in plasma HDL-cholesterol (HDL-C) and apoA-I levels. Endothelial lipase (EL) is known to be an important determinant of HDL-C in mice and in humans and is upregulated during inflammation. In this study, we investigated whether serum amyloid A (SAA), an HDL apolipoprotein highly induced during inflammation, alters the ability of EL to metabolize HDL. We determined that EL hydrolyzes SAA-enriched HDL in vitro without liberating lipid-free apoA-I. Coexpression of SAA and EL in mice by adenoviral vector produced a significantly greater reduction in HDL-C and apoA-I than a corresponding level of expression of either SAA or EL alone. The loss of HDL occurred without any evidence of HDL remodeling to smaller particles that would be expected to have more rapid turnover. Studies with primary hepatocytes demonstrated that coexpression of SAA and EL markedly impeded ABCA1-mediated lipidation of apoA-I to form nascent HDL. Our findings suggest that a reduction in nascent HDL formation may be partly responsible for reduced HDL-C during inflammation when both EL and SAA are known to be upregulated.—Wroblewski, J. M., A. Jahangiri, A. Ji, F. C. de Beer, D. R. van der Westhuyzen, and N. R. Webb. Nascent HDL formation by hepatocytes is reduced by the concerted action of serum amyloid A and endothelial lipase. J. Lipid Res. 2011. 52: 2255–2261.

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It has been recognized for decades that plasma concentrations of HDL cholesterol (HDL-C) are inversely related to the risk of atherosclerotic cardiovascular disease (1). Accordingly, substantial interest is directed toward understanding the genetic and metabolic factors that influence HDL-C levels. It is well documented that endothelial lipase (EL) is an important determinant of HDL metabolism in both humans and rodents. In mice, overexpression of EL leads to significantly reduced plasma HDL-C (2–4) whereas decreased EL activity through either gene deletion (3, 5) or antibody inhibition (6) leads to increased HDL-C. In humans, plasma EL concentrations are inversely correlated with HDL-C levels (7) and rare missense mutations in EL are more common in humans with high HDL-C (8).

EL is a member of the triglyceride lipase gene family that also includes pancreatic lipase, lipoprotein lipase, and hepatic lipase (2). Unlike other members of this lipase family, EL exhibits relatively high phospholipase activity and low triglyceride lipase activity, and its favored substrate is HDL (9). Turnover studies in mice with adenoviral vector-mediated overexpression indicate that EL hydrolyzes HDL phospholipids in vivo and increases the fractional catabolic rate of HDL apolipoproteins in a dose-dependent manner (4). Based on studies carried out in vitro, EL phospholipase activity remodels HDL to smaller particles without dissociating lipid-poor apoA-I (10). This is in contrast to what occurs with hepatic lipase, where triglyceride hydrolysis of core lipids leads to shedding of lipid-poor apoA-I from destabilized HDL particles (11).

Evidence suggests that EL is increased in humans during inflammation, a condition associated with reduced HDL-C (12, 13). In cross-sectional studies, plasma markers of inflammation are strongly associated with plasma levels of EL (14, 15). EL concentrations are also associated with

Abbreviations:  EL, endothelial lipase; GGE, gradient gel electrophoresis; HDL-C, HDL cholesterol; LXR, liver X receptor; LPS, lipopolysaccharide; rEL, recombinant EL; rHDL, reconstituted HDL; SAA, serum amyloid A; SR-BI, scavenger receptor class B type I.

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measures of the metabolic syndrome, which is considered a chronic low-grade inflammatory condition (7). Furthermore, low-dose endotoxin injection in healthy volunteers produces significantly increased levels of EL in the plasma 12 h after administration (15). Thus, an interesting question to address is how EL influences HDL metabolism during inflammation, when HDL undergoes substantial changes in lipid and apolipoprotein composition (16). Most notably, HDL becomes enriched in serum amyloid A (SAA), a major acute phase reactant whose secretion from the liver may be increased more than 1,000-fold during inflammation. The vast majority of SAA in the plasma is found associated with HDL, where it can comprise the major apolipoprotein (17). Studies by Caiazza et al. (18) indicate that apolipoproteins on HDL may regulate EL-mediated remodeling, such that reconstituted HDLs (rHDL) containing both apoA-I and apoA-II are hydrolyzed more readily by EL compared with particles containing apoA-I only. Whether the presence of SAA on HDL can modulate HDL remodeling by EL has not been investigated. Previous studies from our laboratory showed that in the presence of SAA, perturbation of HDL core and surface lipid by scavenger receptor class B type I (SR-BI) leads to generation of small, lipid-depleted apoA-I that is susceptible to catabolism (19). In this study, we carried out in vitro and in vivo studies to investigate whether the presence of SAA influences EL-mediated metabolism of HDL.

METHODS

Mice

C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA) and used at 8-12 weeks of age. Mice were maintained on a 10 h light/14-h dark cycle and received standard mouse chow and water ad libidum. To induce an acute phase inflammatory response, mice were injected with 1 µg/g body weight lipopolysaccharide (LPS; Sigma, St. Louis, MO, Cat # L2630). Animal procedures were performed in accordance with the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals and with the approval of the Lexington Veterans Affairs Medical Center Institutional Animal Care and Use Committee.

Adenoviral vectors

SAA was expressed alone or in combination with EL or SR-BI in livers of mice using adenoviral vector-mediated gene transfer. The indicated dose of AdSAA, a vector encoding mouse CE/J SAA iso-type (20), AdSR-BI, a vector encoding mouse SR-BI (21) or AdEL, a vector encoding human endothelial lipase [generously provided by Dr. Daniel Rader (9)] was administered via tail vein injection in 100 µl PBS. For estimates of EL expression 72 h after adenoviral vector infusion, mice were injected i.p. with 2.5 U heparin in 100 µl PBS containing 10 mM glucose, 10 mM HEPES, and 0.3 mM EDTA and then with HBSS containing 0.05% collagenase type IV (Sigma), 1.3 mM CaCl₂, 0.5 mM MgCl₂, 10 mM glucose, and 10 mM HEPES. Hepatocytes were washed by repeated low speed centrifugation (50 g for 2 min), and viability was assessed by trypan blue exclusion. Cells were plated onto 12-well collagen-coated plates at a density of 2 × 10⁶ cells/per well and incubated overnight at 37°C in 5% CO₂ in Williams’ Medium E (GIBCO) containing 10 mM glucose, 10 mM HEPES, and 0.3 mM EDTA and then with HBSS containing 0.05% collagenase type IV (Sigma), 1.3 mM CaCl₂, 0.5 mM MgCl₂, 10 mM glucose, and 10 mM HEPES. Hepatocytes were incubated with 4% FBS (GIBCO), 2% penicillin-streptomycin, 1% sodium pyruvate, 1% L-glutamine, and 1% insulin-transferrin-selenium (GIBCO). Cells were treated with the Liver X Receptor (LXR) agonist T0901317 (5 µM; Cayman Chemical) in serum-free medium containing 0.2% fatty acid-free BSA for 8 h to induce the expression of ABCA1 and then incubated with 15 µg/ml lipiddfree human apoA-I (Meridian Life Science, Inc.) in medium containing 0.2% fatty acid-free BSA. Cell lysates and culture supernatants were harvested after 18 h incubation for analysis.

Plasma HDL-C quantifications

Plasma HDL-C was measured using a commercially available kit (Wako) according to the manufacturer’s protocol.

Gel electrophoresis and immunoblot analysis

Plasma samples from mice were separated by 4-20% SDS-PAGE and stained with Coomassie Blue or transferred to PVDF membranes (100 min at 100 V, 4°C) and immunoblotted with anti-mouse apoA-I (Biodesign International) or rabbit anti-human EL (Cayman Chemical). Protein bands were visualized using enhanced chemiluminescence reagents (GE Healthcare) and quantified by densitometry. For other studies, plasma or cell culture supernatants were subjected to nondenaturing gradient gel electrophoresis (GGE). Electrophoresis was carried out in 4-20% polycrylamide gels for 3.5 h at 200 V, 4°C and the samples were then transferred to PVDF membranes and immunoblotted using anti-mouse apoA-I or anti-human apoA-I (Calbiochem) as indicated in legends to figures. For assessment of ABCA1 expression in primary hepatocytes, total cell lysates (10 µg) were separated on a 4-20% polycrylamide gradient gel, transferred to polyvinylidene fluoride (PVDF) membranes and immunoblotted with anti-ABCA1 (ABCAM ab7360).

Statistical analysis

Data are presented as mean ± SEM. Statistical analyses to compare differences between plasma HDL levels at selected intervals after viral vector administration were carried out using two-way ANOVA with a Bonferroni posttest.
EL remodeling of normal and SAA-containing mouse HDL in vitro

Previous studies demonstrated that EL hydrolyzes rHDL or HDL isolated from human plasma without generating lipid-poor apoA-I (10). In this study, we assessed whether EL remodels mouse HDL in a similar manner. Incubations with EL resulted in dose-dependent hydrolysis of normal mouse HDL as indicated by an increase in the release of FFAs (Fig. 1A). At maximal hydrolysis, ~55% of the HDL phospholipids was hydrolyzed. Analysis by non-denaturing GGE indicated that lipid-poor apoA-I (which migrates faster than the 7.1 nm standard) is not released from mouse HDL, even when >50% of phospholipids are hydrolyzed (Fig. 1B).

Studies by Caiazza et al. (18) demonstrated that rHDLs containing both apoA-I and apoA-II are better substrates for EL hydrolysis compared with rHDLs containing apoA-I only, suggesting that apolipoproteins modulate EL-mediated HDL remodeling. Thus, it was of interest to determine whether the presence of SAA on HDL alters the ability of EL to remodel the particle to liberate apoA-I. To prepare HDLs for these studies, mice were injected with either LPS to induce an acute phase response, or an adenoviral vector (1.5 × 10^11 particles) that expresses high levels of SAA in the absence of inflammation (AdSAA). Analysis by SDS-PAGE indicated that HDL isolated from LPS or AdSAA-injected mice were highly enriched in SAA compared with mice infused with a control adenoviral vector (Fig. 2A). The HDL preparations were incubated with EL and then separated by nondenaturing GGE. Similar to what occurs with human and normal mouse HDL, EL hydrolysis of SAA-containing HDL does not result in the dissociation of lipid-poor apoA-I (Fig. 2B).

Effect of SAA on EL-mediated HDL metabolism in vivo

We next investigated whether SAA alters EL-mediated metabolism of HDL in vivo. As reported by Maugeais et al. (4), overexpression of EL in mice by adenoviral vector reduces plasma HDL-C in a dose-dependent manner (Fig. 3A). For our studies, we selected a dose of AdEL (0.5 × 10^10 particles) that resulted in a reproducible 30–40% reduction in HDL-C 3 days after viral vector injection. We also selected a dose of AdSAA (2.5 × 10^10 particles) that only modestly enriched HDL particles with SAA (Fig. 2A). C57BL/6 mice were injected with mixtures of AdEL, AdSAA, and AdNull to provide a total viral dose of 3 × 10^10 particles/mouse. Infusion of AdSAA along with AdEL resulted in a modest but not statistically significant reduction in the amount of EL in postheparin plasma compared with AdEL alone (Fig. 3B). Plasma HDL-C concentrations were determined at selected intervals up to 72 h after injection, when adenoviral vector gene expression was maximal. Whereas AdEL resulted in the expected ~35% reduction in HDL-C 72 h after injection, infusion of AdNull or AdSAA had no effect on HDL-C levels at any time point during the course of the experiment (Fig. 3C). In contrast, HDL-C was reduced ~90% when AdSAA and AdEL were administered together, despite comparable levels of EL in plasma. This result suggests a synergistic effect of SAA and EL on HDL metabolism.

Impact of EL and SAA on HDL formation

The marked reduction in apoA-I in AdEL + AdSAA-treated mice prompted us to consider the possibility that HDL biogenesis may be altered when EL and SAA were coexpressed. The initiating step in HDL assembly involves lipidation of apoA-I to form nascent HDL particles. Studies in gene-targeted mice indicate that the major site of
lipidation is the liver, where ABCA1 mediates the efflux of cholesterol and phospholipid from hepatocytes to lipid-free apoA-I (24). To examine the effect of concomitant EL and SAA expression on HDL biogenesis, primary hepatocytes were isolated from C57BL/6 mice 24 h after adenoviral vector infusions, and the impact of EL and/or SAA expression on the ability of hepatocytes to convert lipid-poor apoA-I to nascent HDL was assessed (Fig. 5). As shown previously, the interaction of lipid-free apoA-I with hepatocytes leads to the formation of multiple discretely sized particles ranging from ~7.4 to 20 nm in size (25). These nascent HDLs are known to differ in apoA-I, phospholipid, and cholesterol content (26, 27). Interestingly, compared with hepatocytes from AdNull-treated mice, nascent HDL formation by hepatocytes expressing EL alone was slightly reduced, indicating that EL may alter HDL biogenesis as well as the metabolism of mature HDL. Expression of SAA had no apparent effect on apoA-I lipida
dation. However, concomitant expression of EL and SAA resulted in a marked reduction in the conversion of lipid-poor apoA-I to nascent HDL. This difference in apoA-I lipida
dation was not due to differences in ABCA1 expression (Fig. 5B). Taken together, our data indicate that EL and SAA act synergistically to interfere with HDL formation.

**DISCUSSION**

HDL comprises a polydisperse population of lipoproteins that includes larger spherical particles as well as smaller lipid-poor discoidal HDL. The synthesis of new HDL particles occurs primarily in the liver, where apoA-I is secreted in a lipid-poor/lipid-free form. The addition of phospholipids and cholesterol to apoA-I occurs extracellularly through the action of the lipid transporter ABCA1. The resulting disc-shaped particles, conventionally designated preβ-HDL, are then converted to mature spherical HDL. In plasma, these particles are in a dynamic equilibrium that involves active and continuous remodeling mediated by a number of cellular receptors, lipid transport proteins, and modifying enzymes. It is well documented that EL plays a major role in HDL metabolism through its capacity to remodel circulating HDL particles and hence promote their catabolism (3, 4, 28). In this study, we show for the first time that EL may also modulate HDL metabolism by reducing the extent to which apoA-I is lipida
dated in the liver, an effect that is amplified when hepatic cells secrete SAA. The combined effect of SAA and EL to impede apoA-I lipida
dation may partly explain the reduction in HDL-C that is known to occur during inflammation, when both EL and SAA are induced.

The effect of EL on HDL surface remodeling has been extensively investigated by Rye and colleagues (10, 18). Kinetic studies in vitro indicated that the V_max of phospholipid hydrolysis is significantly greater for rHDLs containing both apoA-I and apoA-II compared with rHDL containing only apoA-I (18), whereas rHDL containing only apoA-II undergoes minimal EL hydrolysis (10, 18). Given that rHDL preparations used in these studies were of similar size and lipid composition, these findings strongly suggested that apolipoproteins on HDL are a major determinant of EL hydrolysis. On the other hand, Broedl et al. (29) reported that adenovirus expression of EL was less effective in altering HDL metabolism in apoA-I/apoA-II double transgenic mice compared with apoA-I single transgenic mice, indicating that apoA-II reduces the ability of EL to alter HDL metabolism in vivo. In the current study, we demonstrated that enrichment with SAA has no discernible effect on the ability of EL to remodel mouse HDL in vitro. We also provide evidence that SAA amplifies the effect of EL on HDL metabolism in vivo, but through a mechanism that appears to be independent of HDL remodeling in the circulation.

Based on in vitro studies using rHDLs, EL-mediated phospholipid hydrolysis leads to the formation of smaller HDL particles without dissociating either lipid-poor apoA-I or apoA-II (10). Thus, the impact of EL on HDL remodeling appears to be analogous to what occurs with phospholipase A_2, where surface hydrolysis leads to the formation of small HDL particles without releasing apoA-I (30). In contrast, the triglyceride lipase activity of hepatic lipase remodels HDL to form smaller particles and generate lipid-poor apoA-I (11). In the current study, we found no evidence that enrichment with SAA promotes the dissociation of lipid-poor apolipoproteins from EL-hydrolyzed HDL either in vitro or in vivo. Thus, it does not appear that SAA impacts EL-mediated intravascular HDL remodeling. Nevertheless, SAA significantly enhanced the ability of EL to reduce HDL-C and apoA-I levels in vivo.
Serum amyloid A and endothelial lipase impede HDL biogenesis

Our data indicate this synergism may be attributed to the combined effect of SAA and EL to interfere with the conversion of lipid-poor apoA-I to nascent HDL at the surface of the liver, thereby reducing the production rate of mature HDL. This conclusion is consistent with the findings of Maugeais et al., (4) who measured the plasma clearance rate of HDL in mice with adenoviral vector-mediated EL overexpression. Although AdEL significantly increased the fractional catabolic rate of mature HDL, the increase in the rate of clearance clearly could not account for the substantial decrease in plasma apoA-I levels that was observed in AdEL-infused mice. This points to an alteration in the rate of HDL production as an important factor contributing to the effect of EL in lowering HDL levels. The authors speculated that EL hydrolyzes nascent HDL phospholipids and consequently reduces the maturation of lipid-poor apoA-I to spherical HDL. Our findings suggest that the entry of nascent HDL into the mature HDL pool may be further impeded during inflammation, when both EL and SAA are present.

The mechanism by which SAA and EL interact to disrupt nascent HDL formation is unclear. ApoA-I lipidation by ABCA1 is known to occur through a multi-step process that is initiated by the high affinity binding of apoA-I to ABCA1 (31). The consequent activation and stabilization of ABCA1 leads to the formation of high capacity binding sites on the plasma membrane where lipid-poor apoA-I can effectively solubilize membrane phospholipids and cholesterol to form nascent HDLs (32, 33). Although both apoA-I binding sites are dependent on ABCA1, the high capacity binding site does not involve a direct interaction between apoA-I and ABCA1. Thus, factors that interfere with ABCA1 activation or the formation of the high capacity binding site would be expected to disrupt apoA-I-mediated cholesterol and phospholipid efflux and HDL biogenesis. There is evidence to suggest that both SAA and EL modulate ABCA1-dependent...
efflux, although both factors are in general thought to promote the removal of cellular cholesterol by ABCA1 (34–36). However, earlier studies did not investigate whether enhanced cholesterol efflux was accompanied by increased apoA-I lipidation, a key event in HDL biogenesis.

In summary, we report the novel finding that coexpression of SAA and EL by adenoviral vector-mediated gene transfer reduces HDL-C in mice to an extent that is significantly more pronounced than when EL is expressed alone. Notably, the cooperative effect of SAA and EL was not accompanied by the appearance of small, lipid-depleted HDLs in the plasma, as would be expected to occur if HDL was undergoing enhanced intravascular remodeling. Our data indicate that apoA-I lipidation by mouse hepatocytes is markedly reduced when EL and SAA are coexpressed. Taken together, our findings suggest that a reduction in nascent HDL formation may be partly responsible for reduced HDL-C during inflammation, when both EL and SAA are known to be upregulated. Current strategies for raising HDL-C to lower cardiovascular disease risk include increasing HDL production (37, 38). Our finding that inflammatory factors may serve to negatively impact nascent HDL formation may be important to consider in order to maximize the success of such strategies.

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