Effects of Nonlipolytic Ligand Function of Endothelial Lipase on High Density Lipoprotein Metabolism in Vivo*

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Endothelial lipase (EL) influences high density lipoprotein (HDL) metabolism in vivo and mediates bridging and uptake of HDL particles independent of its lipolytic activity in vitro. To determine whether EL has a nonlipolytic ligand function in HDL metabolism in vivo, 1 × 10^11 particles of a recombinant adenovirus encoding human EL (AdEL), catalytically inactive human EL (AdELS149A), or control (Adnull) were injected into wild-type, apoA-I transgenic, and hepatic lipase knockout mouse. ELS149A protein was expressed at higher levels compared with preheparin, indicating that both the wild-type and mutant EL were bound to cell-surface heparan sulfate proteoglycans. Overexpression of wild-type EL was associated with a significantly increased postheparin plasma phospholipase activity and dramatically decreased levels of total cholesterol, HDL cholesterol, phospholipids, and apoA-I. Injection of AdELS149A did not result in increased phospholipase activity confirming that ELS149A was catalytically inactive. Expression of ELS149A did not decrease lipid or apoA-I levels in wild-type and apoA-I transgenic mice yet led to an intermediate reduction of total cholesterol, HDL cholesterol, and phospholipids in hepatic lipase-deficient mice compared with control and EL-expressing mice. Our study demonstrates for the first time that EL has both a lipolytic and nonlipolytic function in HDL metabolism in vivo. Lipolytic activity of EL, however, seems to be most important for its effects on systemic HDL metabolism.

Several lipolytic enzymes modulate HDL metabolism, including lipoprotein lipase (LPL) and hepatic lipase (HL), two members of the triglyceride lipase gene family. The activity of LPL is directly associated with HDL cholesterol levels in humans, but the effects of LPL on HDL metabolism are thought to be largely indirect through its effects on triglyceride-rich lipoproteins. HL was shown to directly modulate HDL metabolism by converting larger HDL to smaller HDL particles through hydrolysis of both triglycerides and phospholipids. Independent of their lipolytic activity, LPL and HL can act in cellular lipoprotein metabolism as ligands that mediate the binding and uptake of lipoproteins via proteoglycans and/or receptor pathways. Overexpression of catalytically inactive LPL in transgenic mice resulted in increased triglyceride-rich lipoprotein particle uptake and reduced triglyceride levels. Overexpression of catalytically inactive HL significantly lowered apoB-containing lipoproteins in apoE and LDL receptor knockout mice. Reduction of HDL levels by catalytically inactive HL was only apparent in mice that were HL-deficient.

Endothelial lipase (EL) is a new member of the lipase gene family and mediates cellular binding and uptake of HDL particles and selective uptake of HDL-associated cholesteryl esters independent of its lipolytic properties. We therefore hypothesized that expression of catalytically inactive EL in vivo would result in decreased HDL cholesterol and apoA-I levels. We mutated the putative active site serine 149 of EL to alanine (ES149A). This mutation produces a normally expressed full-length but catalytically inactive enzyme. We then used a recombinant adenovirus to express catalytically inactive ELS149A in wild-type, apoA-I transgenic, and HL-deficient mice. Unexpectedly, in contrast to wild-type EL, overexpression of catalytically inactive ELS149A did not result in reduced HDL and apoA-I levels in wild-type and apoA-I transgenic mice. In HL-deficient mice, however, overexpression of catalytically inactive EL moderately reduced total cholesterol, HDL cholesterol, and phospholipid levels, consistent with the concept that EL, like LPL and HL, can modulate lipoprotein metabolism in vivo independent of its lipolytic function. Catalytic activity of EL, however, appears to be the main mechanism by which EL lowers HDL levels.

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The abbreviations used are: HDL, high density lipoprotein; EL, endothelial lipase; LPL, lipoprotein lipase; HL, hepatic lipase; FPLC, fast protein liquid chromatography; FFA, free fatty acids; DPPC, dipalmitylophosphatidylcholine; HSPGs, heparan sulfate proteoglycans.

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Control

EL

EL S149A

FIG. 1. Expression of EL S149A and wild-type EL in HEK293 cells.

MATERIALS AND METHODS

Construction of Catalytically Inactive EL S149A—The catalytically inactive EL S149A was generated by mutation of the catalytic triad (Ser149→Asp149, His254→His254) replacing the active serine 149 by alanine (EL S149A). EL S149A cDNA was generated by using site-directed mutagenesis kit (primers sense, 5′-GGGCGTCGCTTCTAGCCTGGAAGAGAAAAG-3′; primer antisense, 5′-GCCACGTGGCCTCGAGGGCGTGAGCCGATCAAGTGG-3′) according to the manufacturer’s protocol (Stratagene) and confirmed by sequencing.

In Vitro Transfection—HEK293 cells were transfected with EL, EL S149A, or a control encoding plasmid (green fluorescent protein) using LipofectAMINE (Invitrogen) according to the manufacturer’s protocol. AdELS149A, Adnull were generated as described previously (8). To generate

Cobas Fara II (Roche Diagnostics) using Sigma reagents (Sigma). cholesterol, and phospholipid levels were measured enzymatically on a

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Lipase activity determined in conditioned media of HEK293 cells transfected with control (green fluorescent protein), EL S149A, or wild-type EL

|          | TG-lipase | PL-lipase |
|----------|-----------|-----------|
| Control  | 25 ± 5    | ND a      |
| EL S149A | 25 ± 4    | ND        |
| EL       | 172 ± 5   | 872 ± 68  |

a TG, triglyceride; PL, phospholipid.

Table II

Triglyceride (TG) and phospholipase (PL) activity determined in postheparin plasma at day 3 after injection of wild-type C57BL/6 mice with AdEL, AdELS149A, or control (Adnull)

|          | TG-lipase | PL-lipase |
|----------|-----------|-----------|
| Adnull   | 13,051 ± 980 | 263 ± 135 |
| AdELS149A| 14,231 ± 486 | 324 ± 106 |
| AdEL     | 12,163 ± 547 | 5937 ± 1172 |

RESULTS

Lipolytic activity of wild-type EL and EL S149A were first analyzed in vitro. HEK293 cells were transfected with EL, EL S149A, or control. EL and EL S149A protein were expressed at very similar levels (Fig. 1), but EL S149A had no detectable triglyceride or phospholipase activity (Table I), confirming that EL S149A was catalytically inactive.

To determine the effect of EL S149A on HDL metabolism in vivo, we injected wild-type mice with 1 × 10^11 particles of an adenovirus encoding EL (AdEL), EL S149A (AdELS149A), or no transgene (Adnull). EL was detected with rabbit anti-EL peptide sera (1:3000) and goat anti-rabbit peroxidase-conjugated antisera (1:5000) as the secondary antibody (8).

Lipase Assays—Triglyceride lipase and phospholipase activity were measured as described previously (16). Triglyceride lipase activity was measured according to a modification of the method of Nilsson-Ehle and Schotz (17). The assay tubes contained, in a total volume of 0.3 ml, 0.05 M Tris-HCl, pH 8.0, 0.75% bovine serum albumin, 3.4 mM triolein, ~250 mM phosphatidylcholine, and culture medium or mouse plasma. Samples were incubated for 1 h at 37 °C. Reactions were stopped and products were extracted by the method of Belfrage and Vaughan (18) except that 100 μg of lysopalmitoylphosphatidylcholine per ml was included as carrier in the organic extraction mix. Liberation of more than 2.5 nmol of FFA/ml and more than 1.5 nmol of FFA/ml compared with background for the triglyceride lipase and the phospholipase assay, respectively, are consistently statistically significantly different from background (p < 0.05).

Statistical Analysis—Lipase assay results are presented as means ± S.E. Graphs are presented as means ± S.D.

To determine the effect of EL S149A on HDL metabolism in vivo, we injected wild-type mice with 1 × 10^11 particles of an adenovirus encoding EL (AdEL), EL S149A (AdELS149A), or no transgene (Adnull). Protein expression was determined by Western blot analysis in the pre- and postheparin plasma at day 3 after virus injection (Fig. 2). EL and EL S149A protein were both substantially increased in the postheparin plasma compared with preheparin, indicating that both the wild-type and mutant EL were bound to cell-surface heparan sulfate proteoglycans (HSPGs). Overexpression of EL resulted in significantly increased phospholipase activity in postheparin plasma. In contrast, injection of AdELS149A did not result in altered triglyceride or phospholipase activity (Table II). The changes in plasma total cholesterol, HDL cholesterol, and phospholipids during the course of the study are depicted in Fig. 3, A–C. As
shown previously (8), overexpression of wild-type EL resulted in a marked reduction of all plasma lipids lasting over the course of the study. In contrast, overexpression of ELS149A did not result in decreased total cholesterol or phospholipid levels compared with baseline and showed slight reduction of HDL cholesterol only at day 5 after virus injection. Plasma lipoprotein profiles determined at day 10 after virus injection by FPLC analysis showed no reduction of HDL cholesterol in AdELS149A-injected mice compared with control (Fig. 3D).

In order to determine the effects of this human catalytically inactive EL S149A on human-type HDL particles, we injected human apoA-I transgenic mice with $1 \times 10^{11}$ particles AdEL, AdEL S149A, or Adnull (Fig. 4). Table III summarizes the postheparin triglyceride lipase and phospholipase activities at day 3 after virus injection. As expected, EL-expressing mice showed significantly increased phospholipase activity in postheparin plasma, whereas expression of EL S149A did not result in increased phospholipase activity compared with control. Overexpression of wild-type EL resulted in significantly decreased total cholesterol, HDL cholesterol, phospholipid and apoA-I levels (Fig. 5, A–D). Overexpression of catalytically inactive EL, however, did not result in reduction of any of these parameters over the course of the study.

Dugi et al. (7) reported that expression of catalytically inactive HL resulted in reduced HDL levels only in mice that were HL-deficient, suggesting that the presence of endogenous mouse HL may account for the lack of effect observed in other mouse models (5, 6). We therefore tested the hypothesis that endogenous mouse HL may also mask the effects of catalytically inactive EL, as hepatic EL and HL may have overlapping functions in HDL metabolism. HL knockout mice were injected with $1 \times 10^{11}$ particles of AdEL, AdEL S149A, or Adnull (Fig. 6). As seen in wild-type and apoA-I transgenic mice, EL-expressing mice showed increased phospholipase activity, whereas expression of catalytically inactive EL did not result in increased lipase activity compared with control (Table IV). Overexpression of wild-type EL resulted in a dramatic and sustained reduction of total cholesterol, HDL cholesterol, and phospholipid levels. Interestingly, expression of catalytically inactive EL in HL-deficient mice led to an intermediate reduction of total cholesterol, HDL cholesterol, and phospholipid levels compared with that seen in wild-type EL expressing mice and control (Fig. 7, A–C), lasting over the course of the study. Plasma lipoprotein profiles determined at day 5 after virus injection confirmed the reduction of HDL cholesterol in AdEL S149A-expressing mice compared with control (Fig. 7D).

| Table III |

| Triglyceride (TG) and phospholipase (PL) activity determined in pre- and postheparin plasma at day 3 after injection of apoA-I transgenic mice with AdEL, AdEL S149A, or control (Adnull) |
|-----------------|-----------------|-----------------|
|                  | TG-nmol/h/ml    | PL-ug/ml/h/ml  |
|                  | Preheparin      | Postheparin     | Preheparin | Postheparin |
| Adnull           | 22.11 ± 319     | 16.110 ± 1658   | 348 ± 63   | 232 ± 264   |
| AdEL S149A       | 1889 ± 474      | 13.204 ± 5047   | 461 ± 58   | ND          |
| AdEL             | 2876 ± 418      | 11.433 ± 1450   | 78 ± 149   | 1829 ± 881  |

* ND, not detected.
DISCUSSION

Our study demonstrates the following: 1) mutation of the putative active site serine 149 of wild-type EL to alanine generates a catalytically inactive yet normally expressed full-length EL, confirming serine 149 to be part of the catalytic triad of EL (Ser\(^{149}\)-Asp\(^{173}\)-His\(^{254}\)); 2) EL can be released into the plasma by heparin, suggesting that EL, like LPL and HL, is bound to cell-surface HSPGs; and 3) EL has both a lipolytic and a nonlipolytic function in the metabolism of HDL in vivo, yet lipolytic activity of EL appears to be the main mechanism by which EL modulates systemic HDL levels.

EL is a member of the triglyceride lipase gene family comprising pancreatic lipase, LPL and HL. Recent results from overexpression and loss-of-function studies suggest that EL, like other members of this gene family, plays an important role in lipoprotein metabolism (8, 10–12). LPL and HL are bound to HSPGs and act there to either hydrolyze lipoproteins or mediate uptake of lipoproteins independent of their lipolytic activity. The clusters of positively charged residues in LPL and HL that have been implicated in heparin binding are highly conserved in EL (8, 9). In this study, we demonstrated for the first time that EL can be released into the plasma by heparin in vivo, suggesting that EL, like LPL and HL, is anchored to the luminal endothelial surface via HSPGs. Release of EL from HSPGs is accompanied by significantly increased phospholipase activity levels.

Recent in vitro data suggested that EL can promote HDL binding (13, 14), holoparticle uptake (13), and selective uptake of cholesteryl esters (CE) independent of its lipolytic activity (13). To determine whether lipolytic activity of EL is required for its effects on HDL metabolism in vivo, we used adenoviral vectors to overexpress wild-type and catalytically inactive ELS\(^{149A}\) in several different mouse models. ELS\(^{149A}\) was well expressed and could be released by heparin to the same extent as wild-type EL, suggesting that neither expression nor proteoglycan binding of the mutated EL was impaired. Overexpression of wild-type EL resulted in significantly reduced levels of total cholesterol, HDL cholesterol, and phospholipid, and apoA-I levels in all mouse models examined. Unexpectedly, despite even higher protein expression levels compared with wild-type EL, overexpression of ELS\(^{149A}\) did not result in reduction of total cholesterol, HDL cholesterol, phospholipids, or apoA-I levels in wild-type and apoA-I transgenic mice. Therefore, lipolytic activity of endothelial lipase appears to be very important for its effects on HDL metabolism. However, the possibility that catalytically inactive ELS\(^{149A}\) may serve as a ligand to mediate the binding and uptake of lipoproteins to an extent that does not affect systemic HDL levels in these two mouse models cannot be ruled out. Importantly, overexpression of catalytically inactive EL in HL knockout mice resulted in a significant reduction of lipoprotein metabolism (8, 10–12). LPL and HL are bound to HSPGs and act there to either hydrolyze lipoproteins or mediate uptake of lipoproteins independent of their lipolytic activity. The clusters of positively charged residues in LPL and HL that have been implicated in heparin binding are highly conserved in EL (8, 9). In this study, we demonstrated for the first time that EL can be released into the plasma by heparin in vivo, suggesting that EL, like LPL and HL, is anchored to the luminal endothelial surface via HSPGs. Release of EL from HSPGs is accompanied by significantly increased phospholipase activity levels.

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total cholesterol, HDL cholesterol, and phospholipids that was intermediate between that observed in wild-type EL expressing mice and control, providing evidence that EL has a nonlipolytic ligand function in vivo.

Dugi et al. (7) previously reported that reduction of HDL levels by catalytically inactive HL was only apparent in mice that were HL-deficient. Our data suggest that endogenous mouse HL not only masks the effects of catalytically inactive HL but also accounts for the lack of effect of catalytically inactive EL observed in wild-type and apoA-I transgenic mice. The presence of endogenous active HL may interfere with the effects of hepatic expression of catalytically inactive EL, as EL and HL may have overlapping functions in HDL metabolism. Both EL and HL were shown to mediate binding of HDL to cell-surface HSPGs in vitro (14) and to hydrolyze HDL lipids in vitro (16) and in vivo (5–8, 10, 19, 20). The specific roles of HL versus EL in HDL metabolism are currently not very well understood. We recently proposed that HL may primarily act on triglyceride-enriched HDL particles resulting in reduction in size and increased uptake of cholesteryl esters as well as remnant HDL$_2$ particles, whereas EL may act primarily on HDL phospholipids resulting in dissociation of apolipoproteins and subsequent catabolism (21). The results of the present study, however, indicate that EL and HL have at least partially overlapping functions in HDL metabolism. Studies in EL-deficient and EL/HL double knockout mice may contribute to a better understanding of the specific roles of HL and EL in HDL metabolism.

In summary, in the present study we tested the hypothesis that EL can alter HDL metabolism independent of its lipolytic function. Overexpression of wild-type EL dramatically lowered total cholesterol, HDL cholesterol, phospholipid, and apoA-I levels in all mice models examined. Expression of catalytically inactive EL$^{S149A}$ did not reduce HDL cholesterol in wild-type and apoA-I transgenic mice but resulted in a moderate reduction of total cholesterol, HDL cholesterol, and phospholipids in mice that were HL-deficient. We therefore conclude that EL can modulate HDL metabolism independent of its lipolytic function, yet the lipolytic activity of EL appears to be the main determinant for its effects on HDL metabolism.

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Fig. 7. Total cholesterol (A), HDL cholesterol (B), and phospholipid levels (C) in HL-deficient mice injected with AdEL$^{S149A}$ (diamonds), AdEL (squares), or control (triangles) over the course of the study. D. FPLC profile of mice 5 days after injection of AdEL$^{S149A}$ (diamonds), AdEL (squares) or control (triangles).
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