Impact of Combined Deficiency of Hepatic Lipase and Endothelial Lipase on the Metabolism of Both High-Density Lipoproteins and Apolipoprotein B–Containing Lipoproteins

Robert J. Brown, William R. Lagor, Sandhya Sankaranaravanan, Tomoyuki Yasuda, Thomas Quertermous, George H. Rothblat, Daniel J. Rader

Rationale: Hepatic lipase (HL) and endothelial lipase (EL) are extracellular lipases that both hydrolyze triglycerides and phospholipids and display potentially overlapping or complementary roles in lipoprotein metabolism.

Objective: We sought to dissect the overlapping roles of HL and EL by generating mice deficient in both HL and EL (HL/EL-dko) for comparison with single HL-knockout (ko) and EL-ko mice, as well as wild-type mice.

Methods and Results: Reproduction and viability of the HL/EL-dko mice were impaired compared with the single-knockout mice. The plasma levels of total cholesterol, high-density lipoprotein (HDL) cholesterol, non–HDL cholesterol, and phospholipids in the HL/EL-dko mice were markedly higher than those in the single-knockout mice. Most notably, the HL/EL-dko mice exhibited an unexpected substantial increase in small low-density lipoproteins. Kinetic studies with [3H]cholesteryl ether–labeled very-low-density lipoproteins demonstrated that the HL/EL-dko mice accumulated counts in the smallest low-density lipoprotein–sized fractions, as assessed by size exclusion chromatography, suggesting that it arises from lipolysis of very-low-density lipoprotein. HDL from all 3 lipase knockout models had an increased cholesterol efflux capacity but reduced clearance of HDL cholesteryl esters versus control mice. Despite their higher HDL cholesterol levels, neither HL-ko, EL-ko, nor HL/EL-dko mice demonstrated an increased rate of macrophage reverse cholesterol transport in vivo.

Conclusions: These studies reveal an additive effect of HL and EL on HDL metabolism but not macrophage reverse cholesterol transport in mice and an unexpected redundant role of HL and EL in apolipoprotein B lipoprotein metabolism. (Circ Res. 2010;107:00-00.)

Key Words: lipase ■ lipoprotein ■ knockout mouse ■ low-density lipoprotein metabolism ■ reverse cholesterol transport

Hepatic lipase (HL) and endothelial lipase (EL) are members of an extracellular lipase family that includes lipoprotein lipase (LPL).1–6 These 3 heparin-binding lipases are anchored to the endothelial surface and mediate the hydrolysis of triglycerides (TGs) and phospholipids (PLs) within circulating lipoproteins. Although all 3 lipases have both TG and PL hydrolytic activity, LPL is predominantly a TG lipase, whereas EL is predominantly a phospholipase; HL shares an intermediate TG lipase and phospholipase activity.7,8 In addition to their catalytic functions, these lipases are capable of “bridging” lipoproteins with cell surface proteoglycans independently of hydrolytic activity.9–12

Both HL and EL influence the metabolism of high-density lipoproteins (HDLs). Overexpression of HL in mice reduces plasma HDL levels13–16; in contrast, HL-ko mice have significantly elevated plasma levels of HDL.17 Likewise, the overexpression of EL in mice substantially reduces plasma HDL,5,18 whereas the loss of EL activity in mice significantly raises plasma HDL.18,19 Furthermore, numerous human studies have demonstrated that plasma HDL cholesterol (HDL-C) levels are strongly associated with variations in the genes encoding HL (LIPC)20–24 and EL (LIPG).23–26 However, the interaction between HL and EL on influencing HDL metabolism and its key function of reverse cholesterol transport (RCT) is unknown.

HL also modulates the metabolism of apolipoprotein (apo)B-containing lipoproteins (LpBs). Overexpression of HL in mice significantly reduces plasma levels of LpBs13,15,16; in contrast,
HL-knockout (ko) mice fed a high-fat diet exhibit a modest increase in plasma LpB levels, which is exacerbated in the absence of apoE27 or the low-density lipoprotein (LDL) receptor. Genetic variation in LIPC is associated with TG and LpB levels, and human deficiency of HL causes elevated LpB levels. In contrast to HL, the role of EL in the metabolism of plasma lipoproteins is uncertain. In vitro, EL is capable of hydrolyzing very-low-density lipoprotein (VLDL) triglyceride (TG) and phospholipid (PL). Moderate transgenic overexpression of EL does not affect LpB levels, but adenoviral-mediated overexpression of EL in mice reduces LpB levels. Furthermore, very modest elevations of LpBs in the plasma were observed in EL-ko mice lacking either apoE35,36 or the LDL receptor. Human genetic studies to date have not observed significantly reduced litter sizes on breeding the HL/EL-dko mice versus both HL-ko and EL-ko mice, which were similar to each other. We also noted from the HL/EL-dko litters that 2 pups were deceased at birth because of a lack of abdominal organs and 3 mice died before 12 weeks of age because of an infection that was secondary to poor healing of the umbilicus. Studies with HL-ko, EL-ko, and wild-type (WT) mice have shown that both the HL-ko and EL-ko mice have elevated plasma lipids beyond those observed with the individual knockouts. A gene dosage effect with the removal of HL raised plasma lipids beyond those observed with the individual knockouts. The deletion of EL has a more pronounced effect on plasma lipids versus the deletion of HL, likely because of the loss of the greater phospholipase activity of EL versus HL. The deletion of both HL and EL from mouse models, the HL/EL-dko mice, was viable. However, we observed significantly reduced litter sizes on breeding the HL/EL-dko mice versus both HL-ko and EL-ko mice, which were similar to each other. We also noted from the HL/EL-dko litters that 2 pups were deceased at birth because of a lack of abdominal organs and 3 mice died before 12 weeks of age because of an infection that was secondary to poor healing of the umbilicus. No such observations were recorded with the HL-ko and EL-ko litters. These observations raise the possibility that HL or EL may have a novel function in development and vascular repair that warrants future exploration.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Non-standard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>apo</td>
<td>apolipoprotein</td>
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<tr>
<td>CEI</td>
<td>cholesteryl ether</td>
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<td>CETP</td>
<td>cholesteryl ester transfer protein</td>
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<td>EL</td>
<td>endothelial lipase</td>
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<tr>
<td>FCR</td>
<td>fractional catabolic rate</td>
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<td>FPLC</td>
<td>fast-performance liquid chromatography</td>
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<td>HDL</td>
<td>high-density lipoprotein</td>
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<td>HDL-C</td>
<td>high-density lipoprotein cholesterol</td>
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<td>HL</td>
<td>hepatic lipase</td>
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<tr>
<td>HL/EL-dko</td>
<td>hepatic lipase/endothelial lipase double-knockout</td>
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<tr>
<td>ko</td>
<td>knockout</td>
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<td>LDL</td>
<td>low-density lipoprotein</td>
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<td>LPL</td>
<td>lipoprotein lipase</td>
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<tr>
<td>PL</td>
<td>phospholipid</td>
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<tr>
<td>RCT</td>
<td>reverse cholesterol transport</td>
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<td>sLDL</td>
<td>small low density lipoprotein</td>
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<td>TC</td>
<td>total cholesterol</td>
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<tr>
<td>TCB</td>
<td>tyramine cellobiose</td>
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<tr>
<td>TG</td>
<td>triglyceride</td>
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<tr>
<td>VLDL</td>
<td>very-low-density lipoprotein</td>
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<td>WT</td>
<td>wild type</td>
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HL/EL-double heterozygous mice were generated by crossing HL-ko and EL-ko mice. The double heterozygous mice were used to obtain the first generations of mice lacking both HL and EL (HL/EL-dko). The HL/EL-dko mice were subsequently obtained by breeding either the HL/EL-dko mice or the HL/EL-double heterozygous mice. Lipids from fasted plasma and fast performance liquid chromatography (FPLC) samples were quantified using commercially available kits. Apolipoproteins were detected by immunoblot analyses. Human and mouse lipoproteins were isolated by density gradient ultracentrifugation. Human VLDL was radiolabeled with [3H]cholesteryl ether (CEi), and human HDL3 was radiolabeled with [3H]CEi and [125I]tyramine cellobiose (TCB). Cholesterol efflux in vivo RCT studies were performed using established methods. For metabolic studies, radiolabeled HDL3 was injected intravenously and plasma were collected at 2 minutes and at 1, 2, 4, 6, 9, and 24 hours postinjection for analyses. Radiolabeled VLDL was injected IV and plasma were collected at 2 and 20 minutes and at 5 and 24 hours postinjection for analyses. All studies were approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Results

Reproduction and Viability of HL/EL-dko Mice

To dissect the roles of HL and EL in lipoprotein metabolism, we generated HL/EL-dko mice for comparison and contrast with HL-ko, EL-ko, and WT mice. Like the HL-ko and EL-ko mouse models, the HL/EL-dko mice were viable. However, we observed significantly reduced litter sizes on breeding the HL/EL-dko mice versus both HL-ko and EL-ko mice, which were similar to each other. We also noted from the HL/EL-dko litters that 2 pups were deceased at birth because of a lack of abdominal organs and 3 mice died before 12 weeks of age because of an infection that was secondary to poor healing of the umbilicus. No such observations were recorded with the HL-ko and EL-ko litters. These observations raise the possibility that HL or EL may have a novel function in development and vascular repair that warrants future exploration.

Plasma Lipids and Lipoproteins of HL/EL-dko Mice

This is the first time HL-ko and EL-ko mice have been examined side-by-side together with the HL/EL-dko lipid profiles. Using sex- and age-matched mice, our data show that both the HL-ko and EL-ko mice have elevated plasma levels of total cholesterol (TC), HDL-C, and PL compared to control mice (Figure 1). The deletion of HL has a more pronounced effect on plasma lipids versus the deletion of HL, likely because of the loss of the greater phospholipase activity of EL versus HL. The deletion of both HL and EL from mice raised plasma lipids beyond those observed with the individual knockouts. A gene dosage effect with the removal of HL or EL alleles leading to the double knockout was observed for the increase of plasma TC, HDL-C, and PL (Online Figure I). Plasma TG levels were comparable among all groups of mice (Figure 1D; Online Figure I, D).

We next assessed the plasma levels of apolipoproteins from the 4 groups of mice by immunoblot analyses (Figure 2) and densitometry of the immunoblots (Online Figure II). We observed a very striking 19-fold increase of apoB-100 in the HL/EL-dko plasma versus WT plasma. Plasma from both HL-ko and EL-ko mice displayed a less profound 7-fold increase of apoB-100 versus control mice. In contrast, only...
modest increases of apoB-48 were observed in the plasma from HL-ko, EL-ko, and HL/EL-dko mice. Plasma levels of apoE were 2-fold greater in the HL/EL-dko mice versus the control mice and 40% higher in both HL-ko and EL-ko mice. We observed no difference in the plasma levels of apoA-I between the HL-ko and WT mice and only a modest 13% and 18% increase in the plasma levels of apoA-I within the EL-ko and HL/EL-dko mice, respectively. The trends for plasma levels of apoB-100 and apoA-I correlate with the plasma particle concentrations of total LDL and HDL, respectively, as determined by nuclear magnetic resonance (Online Figure III). Notably, plasma very small LDL levels in the HL/EL-dko mice were substantially elevated compared to those within WT, HL-ko, and EL-ko mice.

We separated lipoproteins within pooled plasma from our 4 groups of mice by FPLC (Figure 3). HDL-C was elevated in the EL-ko (n=16) compared to WT and individual lipase knockout mice. Fasted plasma from 2- to 3-month-old sex-matched WT, HL-ko, EL-ko, and HL/EL-dko (DKO) were assessed for total cholesterol (A), HDL-C (B), PL (C), and TG (D). The number of mice assessed (n) are indicated. *P<0.001 vs WT. All errors are ± SD.

Figure 1. The HL/EL-dko mice exhibit elevated plasma cholesterol and PL compared to WT and individual lipase knockout mice. Fasted plasma from 2- to 3-month-old sex-matched WT, HL-ko, EL-ko, and HL/EL-dko (DKO) were assessed for total cholesterol (A), HDL-C (B), PL (C), and TG (D). The number of mice assessed (n) are indicated. *P<0.001 vs WT. All errors are ± SD.

Figure 2. Plasma levels of apoB, apoE, and apoA-I are elevated in the HL/EL-dko mice. Fasted plasma proteins from 2- to 3-month-old sex-matched WT, HL-ko, EL-ko, and HL/EL-dko mice were separated by denaturing polyacrylamide gel electrophoresis, transferred to nitrocellulose, and subjected to chemiluminescent immunoblot analyses for apoB100, apoB48, apoE, and apoA-I. Apolipoproteins were quantified by scanning densitometry from 4 different sets of mice and data were graphed (see Online Figure II).

Figure 3. Elevated cholesterol in the HDL and sLDL fractions of plasma from the HL/EL-dko mice. Fasted plasma samples from six 2- to 3-month-old sex-matched WT (diamonds), HL-ko (squares), EL-ko (triangles), and HL/EL-dko mice (circles) were pooled (total of 100 μL) and fractionated (500 μL of each fraction) by FPLC. Total cholesterol from each fraction was quantified. IDL/LDL indicates intermediate-density lipoprotein and LDL. The sLDL cholesterol peak identified from the plasma of HL/EL-dko mice is indicated (arrow).
in the 3 knockout groups versus control mice, in the order of control<HL-ko<EL-ko<HL/EL-dko. Unexpectedly, we observed a distinct peak from the HL/EL-dko plasma that was present in the smallest (s)LDL fractions; this peak was not observed from the plasma of either the HL-ko or EL-ko mice. A notable presence of apoB-100 (relative to apoB-48) and apoE was observed with this peak from the HL/EL-dko profile versus the WT profile (Online Figure IV). When the HL/EL-dko mice were placed on a Western diet for 6 months, this peak was greatly exacerbated (Online Figure V, A). In an attempt to determine whether the lipase bridging function alone would remove the sLDL from plasma, we expressed a catalytically inactive EL at supraphysiological levels in the HL/EL-dko mice by using adenoviruses. However, after 7 days of expression, catalytically inactive EL only modestly reduced cholesterol levels across all fractions relative to preinfection levels (Online Figure V, B); thus, it is likely that the activities of EL or HL are required for the clearance of sLDL.

We also isolated LDL and HDL within plasma from the 4 groups of mice by ultracentrifugation and determined their TC, PL, TG, and protein compositions as a mass percentage (Online Figure VI). Compared to LDL from control mice, the LDL from HL/EL-dko mice had no appreciable change of TC content but had notable differences for TG (81% lower), PL (33% higher), and protein (16% lower) (Online Figure VI, B). The composition of HDL isolated from HL-ko and EL-ko mice also differed versus HDL from control mice.

Identifying the Precursor of the sLDL Within HL/EL-dko Mice

The combination of markedly elevated plasma apoB-100 levels, LDL particle concentrations, FPLC profiles, and LDL composition are all consistent with the accumulation of sLDL that may be derived from VLDL within the plasma of HL/EL-dko mice. To determine whether the sLDL is derived from abnormal VLDL catabolism, we injected [3H]CEt-labeled VLDL into HL/EL-dko and control mice and followed the progression of the [3H]CEt in the plasma over 24 hours by FPLC analyses (Figure 4). As expected, the association of [3H]CEt progressed from VLDL to LDL throughout the study. Some [3H]CEt was associated with HDL, but this may be attributable, in part, to de novo lipoprotein formation driven by the association of endogenous apoA-I with lipids shed from VLDL. At 5 hours after injection, [3H]CEt was associated with the LDL fractions in both WT and HL/EL-dko mice (Figure 4C). By 24 hours, most of the [3H] label had cleared, but there was a clear difference in the LDL fractions between the 2 groups of mice: the control animals accumulated [3H]CEt in the normal-sized LDL range, whereas this peak was shifted toward sLDL in the HL/EL-dko mice (Figure 4D). These data confirm that this lipoprotein is derived from VLDL and it accumulates when LDL cannot be removed from the circulation in the HL/EL-dko mice.

Cholesterol Efflux Ex Vivo and RCT In Vivo

The differences observed in HDL composition among the control, HL-ko, EL-ko, and HL/EL-dko mice suggested that the abilities of HDL to efflux cholesterol and affect RCT may also vary. We tested the ability of both plasma and isolated HDL from the 4 groups of mice to efflux [3H]cholesterol from J774 macrophages ex vivo (Online Figure VIII). The efflux capacities of equal quantities of plasma from HL-ko and
HL-ko mice were comparable but significantly greater than control plasma, whereas the HL/EL-dko plasma had a significantly greater capacity versus the individual knock-out plasma (Online Figure VIII, A). The efflux capacities of equal quantities of HDL from HL-ko and EL-ko mouse plasma were comparable but significantly greater than HDL from control plasma, whereas the HDL from HL/EL-dko plasma had a significantly greater capacity versus the control and individual knockout HDL (Online Figure VIII, B).

The ex vivo efflux data combined with the HDL composition data suggest that a higher HDL-PL content attributable to the loss of phospholipase activity within the circulation would allow for greater efflux of peripheral cholesterol to HDL. To determine whether this increased efflux capacity would be beneficial in vivo, we measured macrophage-to-feces RCT in the lipase knockout mice. To accomplish this, \( [^3H] \)cholesterol-labeled J774 macrophages were injected into the intraperitoneal cavity, and the appearance of macrophage-derived \( [^3H] \)cholesterol in the plasma and feces was monitored for 48 hours (Figure 5). Over 48 hours, the amount of \( [^3H] \)sterol in the plasma of HL-ko mice was higher than that observed in the plasma of control mice (Figure 5A). The \( [^3H] \)sterol levels in the plasma from the EL-ko mice were significantly greater than those in the HL-ko mice (Figure 5A). The plasma \( [^3H] \)sterol levels in the HL/EL-dko mice trended higher than those for the EL-ko plasma (Figure 5A) but were not statistically significant. Despite higher HDL-C levels and increased \( [^3H] \)sterol in the plasma of knockout mice, the fecal excretion of \( [^3H] \)sterol among control, HL-ko, EL-ko, and HL/EL-dko mice was not significantly different (Figure 5B).

**Catabolism and Selective Uptake of HDL\(_3\)**

Although HL-ko, EL-ko, and HL/EL-dko mice all had higher HDL-C levels and increased plasma levels of macrophage-derived \( [^3H] \)sterol in our RCT studies, the overall rate of macrophage-to-feces RCT, as assessed by fecal excretion of macrophage-derived \( [^3H] \)sterol, was surprisingly unaffected. We hypothesized that this might be attributable to impaired clearance of HDL from the plasma. To address this possibility, we injected \( [^{125}I] \)TCB/[\( ^{3H} \)]CEt-labeled HDL\(_3\) into the 4 groups of mice and compared their plasma clearance. The fractional catabolic rates (FCRs) for the clearance of plasma \( [^3H] \)CEt were significantly slower for both the EL-ko and HL/EL-dko mice versus control mice but not significantly different from each other (Table). The FCRs for the clearance of plasma \( [^{125}I] \)TCB/[\( ^{3H} \)]CEt-labeled HDL\(_3\) was comparable between HL-ko and control mice but was significantly lower in the EL-ko and HL/EL-dko mice versus the control mice (Table). These data clearly show the importance of EL in the selective uptake of cholesteryl ester from HDL\(_3\) and it solidifies the notion that EL is the preferential lipase associated with whole-body HDL\(_3\) catabolism compared with HL.

**Discussion**

Although HL and EL display overlapping catalytic functions and lipoprotein substrate specificities, important physiological distinctions exist between these lipases. The HL/EL-dko mice share some similarities with the individual knockout mice, but they also have some remarkable differences. The greatly increased HDL in these mice improves cholesterol efflux capacity but does not increase RCT because of reduced whole-body uptake of HDL-derived cholesterol. Our study also uncovered unexpected redundant roles for both HL and EL in the metabolism of LpBs and that a combined deficiency of these lipases results in an accumulation of sLDL.
The HL/EL-dko mice are viable, which is in contrast to the LPL-ko mice, which die as neonates. However, our data suggest that both HL and EL share overlapping roles in reproduction and survival. Wade et al previously addressed the impaired reproduction ability of HL-ko mice. Litter sizes for HL-ko mice in our study exactly match those of previous reported data; litter sizes of HL-ko mice and EL-ko mice are comparable, which suggests that a reproductive impairment may exist in the absence of EL. The HL/EL-dko mice exhibit a significant impairment compared to both HL-ko and EL-ko mice, which is manifested by markedly reduced litter sizes from dko-dko breeding, as well as early neonatal lethality. The placenta was identified to have one of the highest levels of EL expression, yet its function in this tissue remains to be investigated. Although the causes for reduced litter sizes in EL-ko and HL/EL-dko mice remain to be determined, it may be similar to the reduced progesterone levels, leading to impaired ovulation in HL-ko mice, or it may be attributable to the loss of the undefined role of placental EL, which is augmented by reduced ovulation in the absence of HL. The roles of HL and EL in these processes are poorly understood and require further future studies.

The unexpected presence of a PL-rich sLDL in the HL/EL-dko mice clearly shows that HL and EL play a redundant role in the normal catabolism of LDL, which is otherwise masked in the HL-ko and EL-ko mice. Hepatic HL and EL mRNA from the HL-ko and EL-ko mice showed that HL and EL, respectively, were not elevated versus control mice (data not shown); thus, it is unlikely that a compensatory mechanism of transcriptional upregulation of the other lipase in the single-knockout models prevented the observation of sLDL. Our data point to a mechanism whereby the phospholipase activities of either HL or EL are necessary for the effective processing of LpBs and prevention of the accumulation of sLDL. Although LPL has a low phospholipase activity relative to HL and EL,4 the activity appears to be insufficient in the HL/EL-dko mice to properly hydrolyze LDL lipids to effectively promote catabolism.

We postulate that sLDL accumulates in the HL/EL-dko mice as a result of aberrant LDL catabolism mediated by LPL. Previous studies have demonstrated that elevated activities of LPL or HL contribute to the formation of sLDL. This may be attributable to the increased TG lipase activity within the plasma relative to the combined phospholipase activities by HL and EL. The HL/EL-dko mouse model eliminates the major phospholipase activities toward plasma lipoproteins, thus dramatically shifting the balance of plasma lipase activity toward TG lipase because of the remaining activity by LPL. Effective hydrolysis of PL by HL and EL in LpBs undergoing TG lipolysis by LPL may be required for the efficient metabolism of LDL, and in the absence of both HL and EL, a PL-rich and TG-depleted sLDL particle is the result. Another potential possibility to account for the presence of sLDL is an upregulation of LPL activity to compensate for the loss of TG lipase activities from both HL and EL. Although epidemiological studies support a proatherogenic role for sLDL, the causality of this relationship has not been directly and cleanly examined in an animal model. The HL/EL-dko mice may be a useful model to study the effects of sLDL on atherogenesis, particularly given that when these mice are fed a Western diet, the levels of sLDL are further increased.

The additive effect of HL and EL deficiency on plasma levels of HDL-C demonstrates that these enzymes work in concert to modulate HDL remodeling and metabolism. We performed additional studies to assess the effects on HDL function with regard to cholesterol efflux and RCT. The deficiency of HL, EL, or both increased the capacity of HDL to accept cholesterol from macrophages, likely because of the increased HDL-PL content and a larger HDL particle number. However, the increased efflux capacity is offset by a slower clearance of HDL-C from the circulation; thus, macrophage RCT is ultimately unaffected. Pharmacological inhibition of EL, with or without additional HL inhibition, might be expected to raise HDL-C levels and improve cholesterol efflux capacity, but the effect of this approach on RCT and atherogenesis remains uncertain.

Unlike humans, mice do not have cholesteryl ester transfer protein (CETP), which exchanges TG from LpB with CE from HDL. Hydrolysis of TG-enriched HDL by lipases enhances HDL clearance, whereas shedding apoA-I from the particle. ApoA-I could accept cholesterol from macrophages and thus contribute to enhanced RCT in mice expressing CETP. We speculate that overexpressing CETP in HL-ko or EL-ko mice might improve RCT; however, no enhancement may be observed in the HL/EL-dko mice. We further hypothesize that the expression of CETP in HL/EL-dko mice would result in a significant accumulation of sLDL, which might amplify any atherogenic phenotype. Thus, in humans, EL inhibition might have an adverse effect on the metabolism of LpBs, particularly if HL is also partially inhibited.

In summary, deletion of both HL and EL in mice yielded a novel phenotype of extremely high HDL-C without an overall increase in macrophage RCT but with an unexpected substantial increase in plasma apoB-100 and sLDL. This demonstrates the complementary effects of HL and EL on the metabolism of both HDL and LpBs. It also raises important questions about the advisability of pharmacological inhibition of EL, particularly if HL were also partially inhibited.

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Disclosures

D.J.R. is a coinvestigator of a patent related to EL as a target for pharmacological inhibition and is a consultant to several companies with an interest in novel HDL-targeted therapies.

References


Hepatic lipase (HL) and endothelial lipase (EL) are extracellular heparin-binding lipases that hydrolyze the triglycerides and phospholipids associated with circulating lipoproteins.

- HL and EL appear to have overlapping roles in modulating the metabolism of apolipoprotein B-containing lipoproteins (LpBs) and high-density lipoproteins (HDLs).
- Targeted deletion of either HL or EL in animal models raises HDL levels, which might be predicted to reduce atherosclerosis by promoting reverse cholesterol transport.

**What New Information Does This Article Contribute?**

- HL and EL share a redundant role in LpB metabolism; in the absence of both enzymes in mice, a very small low-density lipoprotein particle accumulates in plasma, likely because of impaired phospholipid hydrolysis coupled with increased triglyceride lipase activity of lipoprotein lipase.

- HL and EL share a redundant role in HDL metabolism; a combined deficiency of both enzymes results in a substantial increase in HDL cholesterol (HDL-C) levels, but does not improve in vivo macrophage reverse cholesterol transport likely because of a slow clearance of HDL from the plasma.

- HL inhibition cannot be excluded.

**Novelty and Significance**

Both HL and EL are members of a family of extracellular lipases that also includes lipoprotein lipase. Each of these lipases can modulate the levels of plasma lipoproteins by hydrolyzing their triglycerides and phospholipids. Previously published in vitro and in vivo studies suggest that HL and EL share a significant overlap in the metabolism of HDL, thus making either of the 2 lipases desirable candidates for pharmacological inhibition to raise plasma HDL levels and, thereby, reducing atherosclerotic burden.

We attempted to dissect the physiological roles of HL and EL by generating mice deficient in both lipases (HL/EL-dko) for comparison with single HL-knockout (ko) and EL-ko mice, as well as wild-type control mice. We observed elevated HDL-C in both single-knockout models. The elevated HDL-C in the plasma all-knockout models results in increased cholesterol efflux from macrophages, but this did not translate into improved reverse cholesterol transport in vivo. Unexpectedly, we also observed a significant accumulation of potentially proatherogenic sLDL particles in the plasma of HL/EL-dko mice but not in the plasma of single-knockout mice. Overall, our study questions the advisability of EL inhibition as a therapeutic target, particularly if HL inhibition cannot be excluded.

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The impact of combined deficiency of hepatic lipase and endothelial lipase on the metabolism of both high density lipoproteins and apoB-containing lipoproteins

Brown: HL and EL double-knockout mice

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Subject codes: [90] Lipid and lipoprotein metabolism, [130] Animal models of human disease, [145] Genetically altered mice
Non-standard Abbreviations and Acronyms

BSA, bovine serum albumin
CE, cholesteryl ester
CET, cholesteryl ether
EL, endothelial lipase
FCR, fractional catabolic rate
FBS, fetal bovine serum
FPLC, fast performance liquid chromatography
HDL, high density lipoprotein
HDL-C, high density lipoprotein cholesterol
HL, hepatic lipase
HL/EL-dko, hepatic lipase / endothelial lipase double-knockout
ko, knockout
LDL, low density lipoprotein
PL, phospholipid
RPMI, Roswell Park Memorial Institute
sLDL, small low density lipoprotein
TC, total cholesterol
TCB, tyramine cellobiose
TG, triglyceride
VLDL, very low density lipoprotein
WT, wild-type
Methods

Animals
C57BL/6 (or wild-type, WT) mice and hepatic lipase (HL)-knockout (ko) mice on a C57BL/6 background were obtained from Jackson Laboratories – (stock numbers 000664 and 002056, respectively). Endothelial lipase (EL)-ko mice were previously described. The EL-ko mice were further backcrossed at least 4 additional generations onto the C57BL/6 background. HL/EL-double heterozygous mice were generated by crossing HL-ko and EL-ko mice. The double heterozygous mice were used to obtain the first generations of mice lacking both HL and EL (HL/EL-dko). The HL/EL-dko mice were subsequently obtained by breeding either the HL/EL-dko mice or the HL/EL-double heterozygous mice. All mice were maintained on a normal chow diet and a 12 h light/12 h dark cycle. At eight weeks of age, some HL/EL-dko litters (as described under Results), data obtained from the HL/EL-dko mice were compared to age- and sex-matched HL-ko, EL-ko, and C57BL/6 mice unless otherwise indicated. All studies were approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Adenoviral expression
Adenoviruses expressing catalytically inactive human EL were generated as previously described. Briefly, a cDNA of human EL containing the S149A mutant (thus rendering the protein enzymatically inactive) was generated by site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to manufacturer instructions, to convert the codon expressing serine-149 of human EL (TCC) into GCC (to express alanine). Adenoviruses expressing catalytically inactive EL cDNA were generated propagated in HEK293 cells as described.

Mouse plasma lipids and lipoproteins
Mice were fasted for 6 h and bled via the retroorbital plexus (while under anaesthesia using isofluorane) for the analysis of plasma lipids and lipoproteins. The concentrations of plasma and lipoprotein total cholesterol (TC), high density lipoprotein (HDL)-cholesterol (HDL-C), phospholipids (PL), and triglycerides (TG) were assessed using commercially available colourimetric assays (Roche). The low density lipoprotein (LDL) and HDL particle concentrations from three sets of pooled plasma (with plasma from four mice per set) were assessed by nuclear magnetic resonance by Liposcience; LDL size was assessed as ‘large’, ‘medium-small’, ‘small’, or ‘very small’ in the same analyses. The protein concentration of lipoprotein preparations was determined using a bicinchoninic acid protein assay. Plasma apolipoproteins A-I, B, and E were detected by immunoblot analyses using polyclonal antibodies raised in rabbits (Biodesign), as previously described. For the isolation of mouse LDL and HDL by ultracentrifugation, mouse plasma was adjusted to a density of 1.019 g/ml with KBr and an initial ultracentrifugation to float lipoproteins with a density less than 1.019 g/ml was performed using a Ti70.7 rotor (Beckman) for 3 h at 99,000 rpm. The top layer (containing chylomicrons, very low density lipoproteins (VLDL), and intermediate density lipoproteins) was removed gently with a pipet. The bottom layer was adjusted to a density of 1.063 g/ml with KBr; LDL was floated by ultracentrifugation and isolated as above. The supernatant was adjusted to a density of 1.21 g/ml with KBr; HDL was floated by ultracentrifugation and isolated as above. Lipoproteins were dialyzed against 0.9% w/v NaCl containing 10 mM EDTA, pH 7.4. Lipid and protein content of the isolated lipoproteins were determined in triplicate. For the separation of lipoproteins by FPLC, a total of 100 μl pooled undiluted or diluted plasma from WT, HL-ko, EL-ko, and HL/EL-dko mice were loaded onto two in-series Superose-6 columns that had been equilibrated with phosphate-buffered saline. Five hundred microlitre fractions were collected, and TC from each fraction was measured. For the analysis of apolipoproteins from FPLC fractions, 8 μl of 3.8% sodium citrate solution containing 10x Complete Protease Inhibitor Cocktail

S3
(Roche) was added to 160 μl of freshly isolated pooled plasma from WT or DKO mice. Samples were immediately separated by FPLC and fractions were immediately subjected to immunoblot analyses.

**Triglyceride lipase assays**

Post-heparin plasma was collected from the retroorbital plexus of WT and HL/EL-dko mice at 5 min after tail vein injection of 500 U/kg heparin. The TG lipase activity of the post-heparin plasma was assessed using a glycerol-stabilized [3H]triolein substrate as described previously.5

**Ex vivo cholesterol efflux assays**

Free cholesterol efflux assays from J774 monocyte-derived macrophages were performed as previously described.7 Briefly, 1 x 10^5 J774 cells were plated per well of 24-well plates and cultured at 37°C for 24 h in Roswell Park Memorial Institute (RPMI)-1640 media containing 10% fetal bovine serum (FBS) and 50 μg/ml gentamycin. After 24 h, media were replaced with fresh RPMI-1640 containing 1% FBS, 50 μg/ml gentamycin, 1 μCi/ml [3H]cholesterol, and 2 μg/ml of the acyl CoA:cholesterol acyltransferase inhibitor Sandoz-58035. Cells were incubated for 24 h, then media were replaced with RPMI-1640 containing 0.2% bovine serum albumin (BSA), 50 μg/ml gentamycin, and 2 μg/ml Sandoz-58035. After 18 h, cells were washed and media were replaced with Minimum Essential Medium containing 2 μg/ml Sandoz-58035 and either 2% mouse plasma or 25 μg/ml mouse HDL. Following a 4 h incubation, cells and media were collected and assessed by liquid scintillation counting for [3H]cholesterol. Fractional [3H]cholesterol efflux was calculated as a percentage of [3H]cholesterol released into media per amount of total cell and media [3H]cholesterol.

**In vivo reverse cholesterol transport (RCT) assays**

Macrophage RCT experiments were performed as previously described.8 Briefly, J774 monocyte-derived macrophages cultured in RPMI-1640 containing 10% FBS and 50 μg/ml gentamycin were radiolabelled by incubating with RPMI-1640 containing 1% FBS, 5 μCi/ml [3H]cholesterol, and 50 μg/ml acetylated LDL for 48 h. Radiolabelled cells (5 x 10^6 cells with ~6 x 10^6 cpm) were injected into mice intraperitoneally. Mice were individually caged in metabolic cages with unlimited access to food and water. Plasma were collected via the retroorbital plexus at 0, 2, 4 (or 6), 24, and 48 h post-injection from mice under anaesthesia using isoflurane. Total feces were collected at 48 h post-injection. The plasma and fecal [3H]sterol levels were measured by liquid scintillation counting and expressed as a percent of total [3H]cholesterol injected.

**Metabolic studies with human HDL3**

HDL3, within the density range between 1.125 g/ml and 1.21 g/ml, was isolated from human plasma by sequential ultracentrifugation.9 The HDL3 protein was radiolabelled with [125I]tyramine cellobiose (TCB) and the HDL3 lipid core was radiolabelled with [3H]cholesteryl hexadecyl ether (CEt) using established methods.10 Radiolabelled HDL3 (~1 x 10^6 cpm [3H]CEt and ~1 x 10^6 cpm [125I]TCB) was injected intravenously into mice. Plasma were collected via the retroorbital plexus at 2 min. and at 1, 2, 4, 6, 9, and 24 h post-injection from mice under anaesthesia using isofluorane; plasma were assessed for [3H] by liquid scintillation counting and [125I] by γ counting. The fractional catabolic rates (FCR) of [3H]CEt and [125I]TCB were calculated from the area under plasma decay curves fitted to a bicompartamental model using WinSAAM software,11 assuming a plasma volume equivalent to 3.5% of body mass. Plasma radioactivity were expressed as a percent of total radioactivity in plasma at 2 min.

**Metabolic studies with human VLDL**

VLDL from human plasma was isolated free of chylomicrons by rate floatation ultracentrifugation.12 Agarose gel electrophoresis was performed to verify that no chylomicron contamination was present in the isolated VLDL. One milligram (of protein) of VLDL was radiolabelled with 1 mCi of [3H]CEt as previously described.13 Radiolabelled VLDL (~2 x 10^6 cpm [3H]CEt) was injected intravenously into 4-6
month sex-matched WT (n=2) and HL/EL-dko (n=3) mice. Plasma were collected via the retroorbital plexus at 2 and 20 min., and at 5 and 24 h post-injection from mice under anaesthesia using isofluorane. Plasma were pooled and lipoproteins were separated by FPLC as described above. Fractions were assessed for $^3$H by liquid scintillation counting. The radioactivity from each fraction was expressed as a percent of total radioactivity in plasma at 2 min.

**Statistical analyses**

Where statistical values are provided, the data were analyzed using a one-way analysis of variance with a 95% confidence interval, followed by correction with a Bonferroni Multiple Comparison Test for the number of groups assessed using GraphPad Prism software. Error bars indicate ± standard deviation (SD), as indicated for each figure and supplemental figure.
References

### Supplemental Table I. Litter sizes of lipase knockout mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Number of Litters</th>
<th>Litter size (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL-ko</td>
<td>22</td>
<td>5.59 ± 2.15</td>
</tr>
<tr>
<td>EL-ko</td>
<td>31</td>
<td>5.06 ± 2.19</td>
</tr>
<tr>
<td>HL/EL-dko</td>
<td>13</td>
<td>3.31 ± 1.65*</td>
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*, $p$<0.01 versus HL-ko and $p$<0.05 versus EL-ko.
**Supplemental Figure I (panels A and B).** Figure legend for all panels: The removal of HL and EL alleles from mice results in a gene-dose effect on plasma total cholesterol and phospholipid levels. Fasted plasma from 2-3 month old sex-matched mice were assessed for (A) total cholesterol, (B) HDL cholesterol, (C) phospholipid, and (D) triglyceride. The data presented for HL+/+ EL+/+ (or wild-type), HL-/ EL+/+ (or HL-ko), HL+/+ EL-/ (or EL-ko), and HL-/ EL-/ (or HL/EL-dko) are the same as from Figure 1 of the main manuscript. The number of mice assessed (n) are indicated. *, p<0.01 versus WT. **, p<0.001 versus WT. All errors are ±SD.
Supplemental Figure I (panels C and D). Figure legend for all panels: The removal of HL and EL alleles from mice results in a gene-dose effect on plasma total cholesterol and phospholipid levels. Fasted plasma from 2-3 month old sex-matched mice were assessed for (A) total cholesterol, (B) HDL cholesterol, (C) phospholipid, and (D) triglyceride. The data presented for HL+/+ EL+/+ (or wild-type), HL-/- EL+/+ (or HL-ko), HL+/+ EL-/- (or EL-ko), and HL-/- EL-/- (or HL/EL-dko) are the same as from Figure 1 of the main manuscript. The number of mice assessed (n) are indicated. *, p<0.01 versus WT. **, p<0.001 versus WT. All errors are ±SD.
Supplemental Figure II. Plasma levels of apoB, apoE, and apoA-I are elevated in the HL/EL-dko mice. Fasted plasma proteins from 2-3 month old sex-matched wild-type (WT), HL-ko, EL-ko, and HL/EL-dko (DKO) mice were separated by denaturing polyacrylamide gel electrophoresis and subjected to immunoblot analyses for (A) apoB-100, (B) apoB-48, (C) apoE, and (D) apoA-I. Fold increases relative to WT were semi-quantified by scanning densitometry of immunoblots (insets) from four different sets of mice. *, p<0.001 versus WT. All errors are ±SD.
Supplemental Figure III. LDL and HDL particle numbers are elevated in lipase knockout mice. Three sets of pooled fasted plasma (with plasma from four mice per set) from wild-type (WT), HL-ko, EL-ko, and HL/EL-dko (DKO) were subjected to nuclear magnetic resonance to quantify the plasma particle concentrations of (A) total LDL and its subsets based on size, and (B) total HDL. *, $p<0.01$ versus WT. **, $p<0.001$ versus WT. All errors are ±SD.
Supplemental Figure IV. ApoB, apoE, and apoA-I co-fractionate with the small LDL fractions. (A) Lipoproteins from pooled plasma obtained from three 4-6 month sex-matched wild-type (WT) mice and HL/EL-dko (DKO) mice were separated by FPLC to identify the location of the sLDL peak (arrow). VLDL, fractions 3-8. IDL/LDL, fractions 10-24. sLDL, fractions 25-30. HDL, fractions 31-43. FPLC fractions from (B) WT, and (C) DKO plasma were immunoblotted for apoB, apoE, and apoA-I.
**Supplemental Figure V.** The small LDL cholesterol from HL/EL-dko mice can be modulated by feeding a Western diet or expressing catalytically inactive lipase. (A) Two male and two female HL/EL-dko mice were fed a Western diet from 6 weeks of age until 6 months of age. Fasted plasma at 6 months were collected, pooled (total of 100 μl) and fractionated (500 μl of each fraction) by FPLC. Total cholesterol from each fraction was quantified. IDL/LDL, intermediate and low density lipoproteins. The peak of the small LDL (sLDL) identified from the plasma of HL/EL-dko mice is indicated (arrow). (B) Three 4–6 month old male HL/EL-dko mice were infected with adenovirus expressing a catalytically inactive EL. Prior to infection (day 0) and post-infection (day 7), fasted plasma were collected, pooled (total of 100 μl) and fractionated (500 μl of each fraction) by FPLC. Total cholesterol from each fraction was quantified. The peak of the sLDL identified from the plasma of HL/EL-dko mice is indicated (arrow).
Supplemental Figure VI. LDL and HDL from lipase deficient mice have different lipid and protein compositions. (A) LDL, and (B) HDL, from wild-type (WT), HL-ko, EL-ko, and HL/EL-dko (DKO) mice were isolated from fasted plasma by ultracentrifugation as described within Methods. Total cholesterol, PL, TG, and protein contents were measured in triplicate and are expressed as a percent of total mass (of the four components assessed).
**Supplemental Figure VII.** Post-heparin plasma triglyceride lipase activity is elevated in HL/EL-dko mice. Post-heparin plasma from 2-3 month old sex-matched WT ($n=6$) and DKO mice ($n=7$) were assessed for hydrolysis of triolein. Data are expressed as nmol free fatty acid (FFA) released/min/ml plasma. Errors are ±SD.
**Supplemental Figure VIII.** Plasma and HDL from lipase knockout mice have an increased capacity to promote macrophage cholesterol efflux. J774 monocyte-derived macrophages loaded with [³H]cholesterol were incubated with acceptors of cholesterol of either (A) 2% v/v plasma, or (B) 25 μg/ml HDL (equal protein) from wild-type (WT), HL-ko, EL-ko, or HL/EL-dko (DKO) mice, as described within Methods. Data are expressed as a percentage of fractional efflux to the acceptor during 4 h. Data are representative of two independent experiments with triplicate samples. *, p<0.01 versus WT; **, p<0.001 versus WT. Errors are ±SD.