The Ligand-binding Function of Hepatic Lipase Modulates the Development of Atherosclerosis in Transgenic Mice*

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To investigate the separate contributions of the lipolytic versus ligand-binding function of hepatic lipase (HL) to plasma lipoprotein metabolism and atherosclerosis, we compared mice expressing catalytically active wild-type HL (HL-WT) and inactive HL (HL-S145G) with no endogenous expression of mouse apoE or HL (E-KO × HL-KO, where KO is knockout). HL-WT and HL-S145G reduced plasma cholesterol (by 40 and 57%, respectively), non-high density lipoprotein cholesterol (by 48 and 61%, respectively), and apoB (by 36 and 44%, respectively) (p < 0.01), but only HL-WT decreased high density lipoprotein cholesterol (by 67%) and apoA-I (by 54%). Compared with E-KO × HL-KO mice, both active and inactive HL lowered the pro-atherogenic lipoproteins by enhancing the catabolism of autologous 131I-apoB very low density/intermediate density lipoprotein (VLDL/IDL) (fractional catabolic rates of 2.87 ± 0.04/day for E-KO × HL-KO, 3.77 ± 0.03/day for E-KO × HL-WT, and 3.65 ± 0.09/day for E-KO × HL-S145G mice) and 125I-apoB-48 low density lipoprotein (LDL) (fractional catabolic rates of 5.67 ± 0.34/day for E-KO × HL-KO, 18.88 ± 1.72/day for E-KO × HL-WT, and 9.01 ± 0.14/day for E-KO × HL-S145G mice). In contrast, the catabolism of apoE-free, 131I-apoB-100 LDL was not increased by either HL-WT or HL-S145G. Infusion of the receptor-associated protein (RAP), which blocks LDL receptor-related protein function, decreased plasma clearance and hepatic uptake of 131I-apoB-48 LDL induced by HL-S145G. Despite their similar effects on lowering pro-atherogenic apoB-containing lipoproteins, HL-WT enhanced atherosclerosis by up to 50%, whereas HL-S145G markedly reduced aortic atherosclerosis by up to 96% (p < 0.02) in both male and female E-KO × HL-KO mice. These data identify a major receptor pathway (LDL receptor-related protein) by which the ligand-binding function of HL alters remnant lipoprotein uptake in vivo and delineate the separate contributions of the lipolytic versus ligand-binding function of HL to plasma lipoprotein size and metabolism, identifying an anti-atherogenic role of the ligand-binding function of HL in vivo.

Hepatic lipase (HL) is a 64-kDa lipolytic enzyme that plays a major role in lipoprotein metabolism. It is synthesized and secreted primarily by hepatocytes (1, 2) and is anchored to the vascular endothelium via heparin sulfate proteoglycans. HL functions both as an acylglycerol hydrolase and a phospholipase, hydrolyzing triglycerides (TG) and phospholipids (PL) in chylomicron remnants and intermediate density lipoproteins (IDL). In humans, HL plays a major role in determining low density lipoprotein (LDL) subclass distribution, which may in turn modulate atherogenic risk (3–7). HL is also an important determinant of high density lipoprotein (HDL) concentration and subclass distribution, converting the phospholipid-rich HDL2 to HDL3 (3, 4, 8–14).

In addition to its function as a lipolytic enzyme, HL has a separate role in lipoprotein metabolism by directly facilitating the uptake of lipoproteins and lipoprotein lipids by cell-surface receptors and/or proteoglycans. In this respect, HL could serve as a ligand that facilitates these interactions or, alternatively, could function as a coreceptor. In vitro studies have demonstrated that HL enhances the binding and/or uptake of chylomicrons, chylomicron remnants, β-very low density protein (VLDL), and LDL (15–20) as well as HDL cholesterol (HDL-C) (18, 20–22) in a variety of cell types. Cell-surface receptors, including the LDL receptor (LDLR), low density lipoprotein receptor-related protein (LRP), and SR-BI, as well as cell-surface proteoglycans, have been implicated in this process (15–17, 20, 22). Initial evidence for a ligand-binding function of HL in cellular lipid uptake and lipoprotein metabolism, independent of the lipolytic function of the lipase, came from studies using heat-inactivated HL (18) and anti-HL antibodies (19, 23). These data were confirmed and extended by in vivo experiments that either infused the receptor-associated protein (RAP) and anti-HL/LDLR antibodies (24) or expressed a catalytically inactive form of HL, HL-S145G, in various mouse models. Adenoviral and transgenic overexpression of HL-S145G in apoE knockout (E-KO) mice demonstrated that the ligand-binding function of HL lowers the plasma concentrations of apoB-containing lipoproteins (apoB-Lp) in the absence of apoE (25, 26). Decreased apoB-Lp levels are also observed in transgenic mice that overexpress catalytically inactive HL in LDLR knockout (KO) mice (27). Finally, adeno viral expression of HL-S145G in mice with no endogenous expression of HL (HL-KO) decreased the plasma concentrations of HDL-C by mechanisms independent of lipolysis (28). However, these in vivo reports are limited by the confounding effects of having density lipoprotein(s); HDL, high density lipoprotein(s); VLDL, very low density lipoprotein(s); HDL-C, high density lipoprotein cholesterol; LDLR, low density lipoprotein receptor(s); LRP, low density lipoprotein receptor-related protein; SR-BI, scavenger receptor class B, type I; RAP, receptor-associated protein; E-KO, apoE knockout; apoB-Lp, apoB-containing lipoprotein(s); KO, knockout; WT, wild-type; TC, total cholesterol; FC, free cholesterol; FPLC, fast protein liquid chromatography; FCR, fractional catabolic rate(s); CE, cholesterol ester; RCD, regular chow diet.

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§ The abbreviations used are: HL, hepatic lipase; TG, triglyceride(s); PL, phospholipid(s); IDL, intermediate density lipoprotein(s); LDL, low density lipoprotein(s); HDL, high density lipoprotein(s); VLDL, very low density lipoprotein(s); HDL-C, high density lipoprotein cholesterol; LDLR, low density lipoprotein receptor(s); LRP, low density lipoprotein receptor-related protein; SR-BI, scavenger receptor class B, type I; RAP, receptor-associated protein; E-KO, apoE knockout; apoB-Lp, apoB-containing lipoprotein(s); KO, knockout; WT, wild-type; TC, total cholesterol; FC, free cholesterol; FPLC, fast protein liquid chromatography; FCR, fractional catabolic rate(s); CE, cholesterol ester; RCD, regular chow diet.

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fully active, endogenous mouse HL present along with HL-S145G in the adenovirus and transgenic studies in E-KO mice and by non-steady-state expression of catalytically inactive HL-S145G in the adenovirus studies (25, 26, 28). More recently, Dichek et al. (29) reported that overexpression of catalytically inactive HL in LDLR-KO, LDLR-KO × apoB-100, and LDLR-KO × apoB-48 (HL-KO) mice facilitates the clearance of apoB-48- and apoB-100-containing lipoproteins. Recent studies in human patients with HL deficiency also support an important role of the ligand-binding function of HL in vivo. Zambon et al. (30) showed that the presence or absence of HL protein in HL-deficient subjects leads to significant differences in the cholesterol content of apoB-Lp. These combined data support an important physiological role for the ligand-binding function of HL in lipoprotein metabolism in vivo.

These multiple functions of HL, which facilitate not only plasma lipid metabolism, but also cellular lipid uptake, can be anticipated to have a major and complex impact on atherosclerosis. Indeed, human and animal studies support both pro-atherogenic and anti-atherogenic roles for HL (14, 31–33). In humans, low or absent HL activity has been associated with increased risk of coronary artery disease (31, 34, 35). Other studies have concluded that decreased HL activity does not influence susceptibility to coronary artery disease (8). Finally, increased HL activity has been reported in patients with coronary artery disease (36, 37). A pro-atherogenic role for HL has been suggested from the inverse correlation between increased HL activity and the plasma levels of anti-atherogenic HDL and the positive correlation with small, dense, pro-atherogenic LDL (37, 38).

Analyses of transgenic and knockout animal models have also provided conflicting data regarding the role that HL plays in atherosclerosis. HL overexpression beneficially alters the plasma lipid profile of mice and rabbits by reducing the amount of cholesterol present in apoB-Lp (26, 27, 39). In addition, overexpression of human HL reduces the aortic cholesterol content in cholesterol-fed mice (40). However, in both species, HL also leads to the formation of potentially small, dense, anti-atherogenic lipoproteins. A pro-atherogenic role for HL has been suggested from the inverse correlation between increased HL activity and the plasma levels of anti-atherogenic HDL and the positive correlation with small, dense, pro-atherogenic LDL (37, 38).

Mice and Diets—The generation of pCMV vectors containing wild-type and catalytically inactive human HL cDNAs has been described previously (25, 28). A 1.6-kb fragment of human HL cDNA was excised from the pCMV vector and subcloned into the expression plasmid pLIV.11 (39) modified by the addition of NotI linkers. After digestion with Sall and SpeI, a fragment containing the human apoE promoter, the human HL cDNA, the poly(A) signal from the human apoE gene, and the hepatic and macrophage control regions of the apoE/apoC-I locus was isolated and injected into the male pronucleus of fertilized eggs from superovulating C57BL/6N females (Charles River Laboratories, Wilmington, MA). Integration of the human HL cDNA in newborn mice was determined by dot blot and Southern blot hybridization of genomic tail DNA using the full-length human HL cDNA as a probe. Human HL transgenic mice were crossed with HL-KO (47) and E-KO (48) mice with a C57BL/6J background to generate HL transgenic mice homozygous for both mouse HL and apoE deficiency. Animals were fed a rodent autoclaved chow diet (NIH-07 chow diet, 0.025% cholesterol and 4.5% fat; Ziegler Brothers, Inc., Gardner, MA).

Northern and Western Blot Analyses—Total mouse RNA was isolated from mouse liver, spleen, heart, lung, fat, ovary, brain, kidney, and livers and pancreas using TRIzol (Invitrogen, Carlsbad, CA), and human liver poly(A)+ RNA was purchased from Ambion Inc. (Austin, TX). Northern blot analysis was performed as described (46). The membrane was hybridized with a digoxigenin-labeled human or mouse HL riboprobe and detected using a digoxigenin chemiluminescent detection kit (Roche Applied Science, Indianapolis, IN). The human HL riboprobe was generated by transcription of a 545-bp SmaI-EcoRI fragment of mouse HL cDNA and subcloned into pBluescript II (Stratagene, La Jolla, CA). The mouse riboprobe was generated by transcription of a HindIII-EcoRI fragment of mouse HL cDNA and subcloned into pBluescript II. A 32P-labeled 693-bp cyclophilin cDNA probe (Ambion Inc.) was used for standardization. For protein expression studies, HL protein was isolated from pre- or post-heparin plasma using a HiTrap heparin column (Amersham Biosciences, Uppsala, Sweden), and liver homogenates were prepared from 100 mg of tissue as described (46).

Post-heparin plasma HL and liver receptors were analyzed by electro- nary and lecithin:cholesterol acyltransferase-overexpressing trans- genic mice significantly reduces aortic atherosclerosis despite the increase in cholesterol content in apoB-Lp (44, 45). These conflicting data in mice have been explained in part by the recent discovery of HL expression in mouse macrophages (46) and by bone marrow transplantation studies demonstrating that macrophage HL expression in the arterial wall enhances early lesion formation in E-KO and lecithin:cholesterol acyl- transferase-overexpressing transgenic mice, without modification of plasma lipoprotein lipids or HL activities (45). Despite these recent advances, the independent roles of the ligand- binding versus lipolytic function in lipoprotein metabolism and the development of atherosclerosis are not well defined.

Here, we have investigated the effects of the lipolytic versus the ligand-binding function of HL on plasma lipoprotein metabolism and atherosclerosis. In this study, we utilized mice with stable, steady-state expression of catalytically active HL-WT or inactive HL-S145G in liver in a background lacking endogenous HL or apoE to circumvent the limitations encountered in previous studies. We demonstrate that whereas the ligand function of HL significantly contributes to plasma HDL metabolism, the ligand-binding function of HL is responsible for lowering the plasma concentrations of pro-atherogenic apoB-Lp by enhancing their catabolism via the LRP pathway. Despite their similar effects on lowering pro-atherogenic apoB-Lp, expression of HL-WT enhances atherosclerosis, whereas expression of HL-S145G markedly reduces aortic atherosclerosis in E-KO mice. These data define a major receptor pathway by which the ligand-binding function of HL alters apoB-Lp catabolism in vivo and identify divergent roles for the lipolytic versus ligand-binding function of HL in lipoprotein metabolism and atherosclerosis.
reaction (Supersignal WestPico kit) using horseradish peroxidase-conjugated secondary antibody (46) and quantitated by densitometry (Amersham Biosciences personal densitometer). The mouse anti-apoB antibody was used with both mouse apoB-48 and apoB-100.

For FPLC plasma lipoprotein analysis, 10μl of pooled mouse plasma (n = 5) were separated by gel filtration using two Superose 6 HR 10/30 columns (Amersham Biosciences) connected in series (45). Lipids in the recovered fractions were analyzed using enzyme kits as described (25, 28). The elution volumes of the plasma lipoproteins separated by FPLC were as follows: VLDL, 15.0–17.0 ml; IDL/LDL, 19.0–23.0 ml; and HDL, 29.0–31.0 ml. For native agarose gel electrophoresis, 2μl of total plasma from three to four control and transgenic mice each were loaded onto a Titan gel (Helena Laboratories, Beaumont, TX) and electrophoresed for 40 min at 60 V. The content of cholesterol was quantified by staining with fat red B (Helena Laboratories, TX).

Lipoprotein Particle Lipid Composition and Size Analysis—Lipoproteins were isolated from 2.5 ml of mouse plasma by sequential ultracentrifugation in KBr solutions at 100,000 rpm for 5 h at 5 °C (mouse VLDL/LDL, d < 1.019 g/ml; mouse LDL, d = 1.019–1.063 g/ml; mouse HDL, d = 1.063–1.21 g/ml; and human LDL, d = 1.050–1.030 g/ml) using a TLA-100.2 rotor in a TL-100 ultracentrifuge (Beckman Instruments, Palo Alto, CA). After centrifugation, samples were extensively dialyzed against phosphate-buffered saline, and their lipid and apolipoprotein content was measured as described above. To determine lipoprotein particle size, VLDL/LDL, LDL, and HDL fractions from each mouse group were analyzed by non-denaturing polyacrylamide gradient gel electrophoresis (52). VLDL/LDL and LDL were electrophoresed at 35 V for 17 h at 4 °C using Tris/glycine-polyacrylamide (4–12%) gradient gels (Invitrogen). HDL fractions were separated by electrophoresis at 90 V for 90 min using Tris borate-polyacrylamide (3–30%) gradient gels (Invitrogen). Gels were fixed and stained either with 0.04% oil red O in 60% ethanol for lipids or with 0.1% Coomassie Brilliant Blue R-250, 50% methanol, and 9% acetic acid for proteins, and then they were scanned with a densitometer Fluorchem™ 8800 imaging system (Alpha Innotech, Imgen Technologies, Alexandria, VA). The migration distance for each particle was obtained from the densitometer, and the molecular diameter was calculated from a calibration curve using known standards.

Metabolic Studies—Lipoproteins were isolated from 2.5 ml of mouse or 50 μl of human plasma by sequential ultracentrifugation in KBr solutions. Mouse 125I-labeled apoB VLDL/LDL and 125I-labeled apoB LDL and human 125I-labeled apoB LDL were prepared by a modification of the iodine monochloride method (53). VLDL/LDL and LDL (1–1.2 mg each) were labeled with 125I or 123I at an efficiency of 29–37%. Mouse 125I-labeled apoB VLDL/LDL and 125I-labeled apoB LDL and human 125I-labeled apoB LDL were then re-isolated by ultracentrifugation and diazoyl overnight against 1× phosphate-buffered saline and 0.01% EDTA. Radiolabeled lipoproteins were analyzed by FPLC and agarose gel electrophoresis to ensure the integrity of the particles. Mouse 125I-labeled apoB VLDL/LDL (1 × 10^6 dpm) and 125I-labeled apoB LDL (1 × 10^6 dpm) and human 125I-labeled apoB LDL (1 × 10^6 dpm) were injected into the saphenous veins of the three different mouse groups (n = 6 per group). Mice were bleed at different time points, and 125I-labeled apoB and 123I-labeled apoB radioactivity were analyzed. For quantification of human 123I-labeled apoB remaining in plasma in the human LDL study, the total plasma was analyzed for its radioactivity content. In mouse VLDL/LDL and LDL studies, 2–5 μl of mouse plasma were fractionated by SDS-PAGE using 4–12% gradient gels; the apoB bands were excised; and 123I-labeled apoB was quantitated using a γ-counter (Cobra Autogama, Packard Instruments Co.) (25). The fractional catabolic rate (FCR) was determined from the area under the apoB radioactivity curves using a multi-exponential curve fitting technique in the WinSAAAM program (Version 3.0.1) (54).

RAP Studies—We first determined the minimal concentration of RAP required for partial in vivo LRP inhibition by measuring the uptake of α2-macroglobulin (Research Diagnostics, Inc.) after injection of RAP protein (Research Diagnostics, Inc.). α2-Macroglobulin was activated with 200 μM methylamine for 1 h at room temperature, 125I-radiolabeled as described (55, 56), and injected (3–5 μg, 10 × 10^6 dpm/30 g of body weight) 1 min after a bolus infusion of 0.3 mg of RAP/30 g of body weight. This RAP dose, which resulted in 36% inhibition of 125I-labeled α2-macroglobulin liver uptake in RAP-injected (n = 3) versus saline-injected (n = 3) mice, was then used for the actual studies. Mouse 125I-labeled 64KDa (30 × 10^6 dpm/30 g of body weight), isolated as described above from E-KO × HL-KO mice, was injected into HL-S145G mice 1 min after either RAP or saline injection. Blood samples were obtained at 1, 5, 10, 20, and 30 min. After flushing, the liver was removed and weighed, and a small portion was removed for assay for 125I at 30 min. Radioactivity remaining in plasma 125I-apoB-48 LDL was determined as described above.

Analysis of Aortic Lesions—The heart and the attached section of the ascending aorta were prepared and analyzed as described (45). The proximal aorta was collected after saline perfusion, and the aorta was placed in 4% phosphate-buffered formaldehyde solution for 24 h and then transferred to 10% phosphate-buffered formaldehyde solution for 5 days. Specimens were embedded in 25% gelatin and sectioned with a cryostat at −25 °C. The aortic root and ascending aorta were sectioned at a thickness of 10 μm, and alternate sections were saved on slides and stained with oil red O for neutral lipids and hematoxylin. Five sections/animal were evaluated for the cross-sectional area of lesions from the aortic root for a distance of 350 mm at 80-mm intervals as reported previously (57).

Statistical Analysis—Data are presented as the means ± S.E. Unpaired Student’s t test was performed for analysis of the plasma lipid concentrations and lipase activities. Non-parametric data were analyzed using the Mann-Whitney test (Instat software, GraphPad, Inc., San Diego, CA).

RESULTS

Expression of Human HL in E-KO × HL-KO Mice—Northern blot hybridization of total liver RNA using the HL riboprobe demonstrated expression of the 1.6-kb HL-WT and mutant HL-S145G mRNAs in the liver (Fig. 1A) but not in spleen, heart, lung, fat, ovary, brain, kidney, and macrophages (data not shown) of E-KO × HL-WT and E-KO × HL-S145G transgenic mice. The absence of mouse HL mRNA was verified by Northern blot analysis using a mouse-specific HL riboprobe (data not shown). Following partial purification by heparin-Sepharose affinity chromatography, immunoblot analysis using a monoclonal anti-HL monoclonal antibody identified the 64-kDa HL-WT and mutant HL-S145G protein bands in post-heparin plasma of E-KO × HL-WT and E-KO × HL-S145G transgenic mice and normal human controls, but not in that of E-KO × HL-KO mice (Fig. 1B). Densitometric scans of Northern (Fig. 1A) and Western (Fig. 1B) blots revealed that compared with human samples, HL expression in liver and post-
heparin plasma was increased by ~3–5-fold in E-KO × HL-WT and E-KO × HL-S145G transgenic mice (data not shown). Post-heparin HL activity was present in E-KO × HL-WT mice (325 ± 34 nmol/min/ml), but was undetectable in E-KO × HL-KO and E-KO × HL-S145G transgenic mice (<20 nmol/min/ml), confirming the lack of functional activity of the human HL-S145G enzyme. Analysis of post-heparin plasma revealed that lipoprotein lipase activity was decreased by 23% (p < 0.003) in both E-KO × HL-WT (940 ± 45 nmol/min/ml) and E-KO × HL-S145G (929 ± 54 nmol/min/ml) mice compared with E-KO × HL-KO mice (1223 ± 56 nmol/min/ml).

**Catalytically Active HL-WT and Inactive HL-S145G Lower the Plasma Concentrations of ApoB-Lp in E-KO × HL-KO Mice**—Previous studies have shown that HL deficiency significantly raises the plasma TC, non-HDL-C, and apoB levels in E-KO mice (44, 45, 58). Consistently, compared with male and female E-KO × HL-KO mice, male and female E-KO × HL-WT mice that expressed fully active HL had reduced plasma levels of TC (by 40% (p < 0.01) and 42% (p < 0.0001), respectively), TG (by 27% (p = 0.04) and 52% (p < 0.0001), respectively), PL (by 43 and 50% (p < 0.0002), respectively), cholesterol ester (CE; by 47% (p < 0.002) and 45% (p < 0.0001), respectively), HDL-C (by 67% (p < 0.0001) and 40% (p < 0.02), respectively), non-HDL-C (by 48% (p < 0.002) and 44% (p < 0.0001), respectively), apoB (by 36 and 35% (p < 0.0001), respectively), and apoA-I (by 54 and 46% (p < 0.0001), respectively) (Fig. 2A). In contrast to HL-WT, catalytically inactive HL-S145G did not significantly alter plasma HDL-C or apoA-I levels in E-KO × HL-KO mice (Fig. 2A). As in E-KO mice expressing HL-WT, analysis of plasma lipoproteins in E-KO × HL-S145G mice by FPLC (Fig. 2B) and native agarose gel electrophoresis (data not shown) revealed that most of the cholesterol reduction occurred in pro-atherogenic apoB-Lp, especially VLDL.

**Fig. 2. Plasma lipid, lipoprotein, and apolipoprotein analysis.** A, plasma lipids, lipoproteins and apolipoproteins of female (upper panel) and male (lower panel) E-KO × HL-KO (black bars), E-KO × HL-WT (white bars), and E-KO × HL-S145G (gray bars) mice. B, FPLC cholesterol profiles of pooled plasma from female (upper panel) and male (lower panel) E-KO × HL-KO (#, n = 5), E-KO × HL-WT (●, n = 5), and E-KO × HL-S145G (□, n = 5) mice. Immunoblots of apoB-100, apoB-48, and apoA-I present in FPLC fractions are shown in the inset. Data are expressed as the means ± S.E. †, p < 0.05; ††, p < 0.02; *, p < 0.0002 (compared with E-KO × HL-KO mice).
mice expressing lipolytically active HL-WT had lower plasma TG (by 18%, p ≤ 0.05), PL (by 22%, p = 0.01), HDL-C (by 73%, p < 0.0001), and apoA-I (by 56%, p < 0.00001) levels (p < 0.01, all) than male E-KO × HL-S145G mice (Fig. 2A). Similarly, female E-KO mice expressing lipolytically active HL-WT had lower plasma TG (by 16%, p < 0.05), PL (by 17%, p < 0.05), HDL-C (by 44%, p < 0.0001), and apoA-I (by 52%, p < 0.00001) levels (p < 0.03, all) than female E-KO × HL-S145G mice (Fig. 2A). The marked decrease in plasma lipid and lipoprotein levels in mice expressing only catalytically inactive HL-S145G demonstrates that the ligand-binding function of HL plays a major role in reducing the plasma levels of apoB-Lp in the E-KO mouse model. In contrast, the lipolytic function of HL appears to be necessary for the effect of HL on HDL-C in this mouse model.

Catalytically Active HL-WT and Inactive HL-S145G Differentially Alter Lipoprotein Particle Lipid Content and Size—HL can significantly alter not only the plasma levels but also the composition of different plasma lipoproteins (7–9, 11–16, 19, 23, 24, 27, 28, 31, 41, 42). However, to date, the separate contributions of the lipolytic versus ligand-binding functions of HL to VLDL, IDL, LDL, and HDL lipid content and particle size have not been investigated. In this study, we isolated VLDL/IDL (d < 1.019), LDL (d = 1.019–1.063), and HDL (d = 1.063–1.21) by sequential density ultracentrifugation from each study mouse group and determined the lipid and apolipoprotein concentrations (Table I) as well as lipoprotein particle size (Fig. 3) in each density fraction.

As expected, from the combined effects of both the lipolytic and ligand-binding functions of HL present in mice expressing HL-WT, the TC, CE, FC, TG, and PL as well as apoB concentrations were decreased in the VLDL/IDL density fractions isolated from E-KO × HL-WT mice compared with E-KO × HL-KO mice (Table I). Most of the reduction in the total plasma lipids of E-KO × HL-WT mice reflected changes in the lipid concentration of the VLDL/IDL fraction; however, HL-WT also decreased the TC, CE, FC, TG, PL, and apoB concentrations in LDL density fractions and the TC, CE, FC, TG, PL, and apoA-I concentrations in HDL density fractions isolated from E-KO × HL-WT mice compared with E-KO × HL-KO mice (Table I).

To determine which of the changes in the lipid content of the lipoprotein fractions was due to the ligand-binding functions of HL, a similar analysis was performed for particles isolated from E-KO × HL-WT mice expressing catalytically inactive HL-S145G. Compared with E-KO × HL-KO mice, HL-S145G also reduced the concentrations of TC, CE, FC, TG, PL, and apoB in the VLDL/IDL density fractions from E-KO × HL-S145G mice (Table I). These findings, including the decrease in VLDL/IDL PL and TG concentrations, are consistent with the function of HL-S145G as a ligand that facilitates the cellular uptake of these lipoprotein particles. In contrast to VLDL/IDL, catalytically inactive HL-S145G had virtually no effect on the concentrations of TC, CE, FC, TG, PL, and apoB in LDL density fractions isolated from E-KO × HL-S145G mice compared with E-KO × HL-KO mice; however, the fraction of plasma TC present in LDL was 2-fold greater in E-KO × HL-S145G mice compared with E-KO × HL-KO mice. Virtually no differences in the concentrations of TC, CE, FC, TG, PL, and apoA-I in the HDL density fractions were evident between E-KO × HL-S145G and E-KO × HL-KO mice.

Direct comparison of the lipid and apolipoprotein concentrations of the various lipoprotein density fractions isolated from mice expressing HL-WT versus HL-S145G (Table I) revealed that except for TG, all lipid (including TC, CE, FC, and PL) and apoB concentrations were lower in VLDL/IDL density fractions isolated from E-KO × HL-S145G mice. In contrast, TC, CE, FC, PL, and TG concentrations were higher in both the LDL and HDL fractions isolated from E-KO × HL-S145G mice compared with E-KO × HL-WT mice. Thus, the metabolic impact of the lipolytic function of HL is greater than that of the ligand-binding function of HL on both LDL and HDL.

The changes in lipoprotein lipid and apolipoprotein content resulting from expression of HL-WT and HL-S145G (Table I) are anticipated to alter lipoprotein particle size. Thus, the sizes of VLDL/IDL, LDL, and HDL isolated from E-KO × HL-KO, E-KO × HL-WT, and E-KO × HL-S145G mice were analyzed by native polyacrylamide gel electrophoresis (Fig. 3, A–C). The major lipoprotein particles in the VLDL/IDL fraction isolated from E-KO × HL-KO mice were > 400, 369, and 348 Å (Fig. 3A). The marked reduction in the 369- and 348-Å particles in the VLDL/IDL fractions isolated from both E-KO × HL-WT and E-KO × HL-S145G mice (Fig. 3A) indicates that the ligand-
binding rather than lipolytic function of HL may be responsible for the clearance of the lipoprotein particles within VLDL/IDL. In contrast, the lipolytic versus ligand-binding function of HL had differential effects on both LDL and HDL. Fig. 3B demonstrates that the complete absence of HL (E-KO × HL-KO mice) led to the accumulation of mainly 259- and 254-A LDL particles (Fig. 3B, upper panel). Expression of HL-S145G, which has only ligand-binding activity, resulted in the accumulation of lipoprotein particles with a broad size distribution (ranging from 320 to 254 Å) in the LDL fraction, whereas HL WT expression, which restores lipolytic activity, dramatically reduced the size and heterogeneity of particles in the LDL fraction (259 and 254 Å) (Fig. 3, B and C). Similarly, a major difference in HDL particle size was observed in mice expressing the active HL WT enzyme versus the inactive HL-S145G enzyme. HDL size (Fig. 3C) and lipid content (Table 1) were similar in the E-KO × HL-KO and E-KO × HL-S145G mice, indicating a minimal effect of the ligand function of HL; however, both were markedly decreased in E-KO × HL-WT mice, consistent with the known role of HL-mediated lipolysis in remodeling HDL (Fig. 3C).

Catalytically Inactive HL-S145G Alters the Plasma Lipid Profile by Enhancing the Catabolism of ApoB-48-containing Lipoproteins in E-KO × HL-KO Mice—We next investigated the in vivo mechanism by which the ligand-binding function of HL alters the metabolism of pro-atherogenic apoB-Lp in E-KO × HL-KO mice. To address this question under steady-state conditions and without the confounding effects of endogenous mouse HL, we performed kinetic studies in E-KO mice with no endogenous expression of mouse HL and stable, long-term expression of catalytically inactive HL-S145G. As most of the cholesterol present in apoB-Lp in E-KO × HL-KO, E-KO × HL-WT, and E-KO × HL-S145G mice accumulates in VLDL/IDL, we first studied the metabolism of VLDL/IDL in these mice by injection of autologous 131I-labeled apoB-48 VLDL (Fig. 4A, inset) into E-KO × HL-KO, E-KO × HL-WT, and E-KO × HL-S145G mice. As shown in Fig. 4A, HL-WT and HL-S145G markedly enhanced the catabolism of 131I-labeled apoB-48 VLDL/IDL (FCR = 3.63 ± 0.09/day and 3.77 ± 0.33/day, respectively; p < 0.02) in E-KO × HL-KO control mice (FCR = 2.87 ± 0.04/day). Interestingly, VLDL/IDL FCR were similar between mice expressing HL-WT and HL-S145G, indicating that the ligand-binding and not the lipolytic function of HL is primarily responsible for enhancing VLDL/IDL catabolism. Both HL-WT and HL-S145G increased the catabolism of 125I-labeled apoB-48 LDL (FCR = 18.88 ± 1.72/day and 9.01 ± 0.14/day, respectively; p < 0.001) in E-KO × HL-KO control mice (FCR = 5.67 ± 0.34/day) (Fig. 3B). However, expression of fully active HL WT further enhanced (by ~2-fold) the clearance of apoB-48 LDL compared with expression of HL-S145G. The differences observed in mouse apoB-48 LDL catabolism were not due to variable expression of the LDLR, as Western blot analysis revealed that hepatic LDLR levels were similar in E-KO × HL-WT and E-KO × HL-S145G mice (Fig. 4C, inset). However, LDLR gene expression was lower in E-KO × HL-KO mice than in E-KO mice expressing HL WT or HL-S145G (Fig. 4C, inset). These studies demonstrate that, in the absence of apoE, catalytically inactive HL-S145G is able to facilitate the catabolism of apoB-48 VLDL/IDL and LDL in vivo; however, effective clearance of LDL requires the lipolytic function of HL.

We also studied the plasma kinetics of human 131I-labeled apoB-100 LDL, which lacks both apoE and apoB-48 (Fig. 4C, inset), in all three mouse models. These particles are expected to be cleared primarily by the LDLR. In contrast to mouse apoB-48 VLDL/IDL and LDL, neither HL-WT nor HL-S145G enhanced the catabolism of human 131I-labeled apoB-100 LDL in E-KO × HL-KO mice (FCR = 3.43 ± 0.08/day, 3.94 ± 0.12/day, and 3.69 ± 0.23/day, respectively; p > 0.05) (Fig. 4C).

RAP Infusion Delays the Clearance of LDL Facilitated by HL-S145G—To further investigate the receptor pathways by which the ligand-binding function of HL facilitates the clearance of apoB-48-containing lipoproteins, we studied the in vivo effects of injecting the LRP inhibitor RAP into mice expressing catalytically inactive HL-S145G. Previous studies have shown that the LRP function, as determined by uptake of 125I-labeled α2-macroglobulin, after infusion of 1–2 mg of RAP/mouse (55, 59). In our studies, we achieved 21.5 ± 4.6% reduction in the plasma clearance of 125I-labeled α2-macroglobulin and 36% inhibition of 125I-labeled α2-macroglobulin uptake by liver LRP after bolus injection of 0.3 mg of RAP/30 g of body weight (p < 0.05). As indicated previously (59), the amount of RAP required to inhibit the LDLR is an order of magnitude greater than that required to inhibit LRP. Since 0.3 mg of RAP achieved only 36% inhibition of LRP, this dose is not likely to inhibit the LDLR. Compared with saline-injected control mice, RAP infusion significantly decreased the plasma clearance of
FIG. 4. In vivo metabolism of apoB-Lp in mice expressing HL-WT and HL-S145G. Shown is the catabolism of mouse autologous 125I-labeled apoB VLDL/IDL (A), mouse autologous 125I-apoB LDL (B), and human 131I-apoB LDL (C) in E-KO × HL-KO (●), n = 6 per group), E-KO × HL-WT (○), n = 6 per group), and E-KO × HL-S145G (●; n = 6 per group) mice. The apoB-100, apoB-48, and apoE content of the radiolabeled injected particles and the hepatic levels of the LDLR in each mouse group were analyzed by Western blotting (insets). FCR (per day (d−1)) are shown. *, p < 0.05 (compared with E-KO × HL-KO mice).

125I-apoB-48 LDL (expressed as percent remaining 125I-apoB-48 plasma counts) at 5 min (87 ± 1.2 versus 96 ± 1.8, p < 0.02), 10 min (80 ± 1.3 versus 89 ± 2.7, p < 0.03), 20 min (70 ± 0.5 versus 80 ± 1.7, p < 0.006), and 30 min (58 ± 1.8 versus 69 ± 0.8, p < 0.02) after 125I-apoB-48 LDL infusion. In addition, the hepatic uptake of 125I-apoB-48 LDL at 30 min after lipoprotein injection (expressed as percent 125I-apoB-48 liver counts/total liver weight 125I-apoB-48 liver weight + total plasma counts) was decreased by ~36% (p < 0.05) in HL-S145G mice infused with RAP (9.5 ± 0.35%) versus saline (15 ± 0.71%). These findings implicate LRP in the catabolism and uptake of apoB-48 LDL from plasma, facilitated by the ligand-binding function of HL.

HL-S145G Significantly Reduces Proximal Aortic Atherosclerosis in E-KO Mice—To investigate the role of catalytically inactive HL-S145G in modulating atherosclerosis, the proximal aortas of male and female E-KO × HL-KO, E-KO × HL-WT, and E-KO × HL-S145G transgenic mice on a regular chow diet (RCD) at 3, 5, and 7 months of age were analyzed (Fig. 5). Consistent with previous reports showing a dissociation between the plasma levels of pro-atherogenic apoB-Lp and development of aortic atherosclerosis in E-KO × HL-KO mice (44, 45), aortic atherosclerosis was significantly increased in female E-KO × HL-WT mice at 3 (3.5-fold, p < 0.001), 5 (9.2-fold, p < 0.0001), and 7 (2.5-fold, p < 0.001) months of age. A trend toward an increased mean aortic lesion area was noted in male E-KO × HL-WT mice at 3 and 5 months of age, which reached statistical significance at 7 months of age (2-fold, p < 0.04) compared with male E-KO × HL-KO mice. In contrast, the mean aortic lesion area was decreased by 78% (p < 0.0001), 84% (p < 0.001), and 78% (p < 0.0001) in female E-KO × HL-S145G mice at 3, 5, and 7 months of age, respectively. Similarly, lesion development was reduced by 74% (p < 0.02),
96% (p < 0.004), and 76% (p < 0.01) in male E-KO × HL-S145G mice at 3, 5, and 7 months of age compared with their respective E-KO × HL-KO control mice.

**DISCUSSION**

HL is a multifunctional protein with ligand-binding and lipolytic activities that significantly alter cellular lipid uptake and lipoprotein metabolism. Data from human (8, 14, 31–34, 36–38) and animal (40, 44, 45) studies indicate that the multiple functions of HL can lead to variable and sometimes opposing effects on lesion development, resulting in unpredictable changes in atherosclerosis. To date, the specific contribution of the ligand-binding function of HL to these various processes and especially to atherosclerosis has not been elucidated.

In this study, we have dissected the effects of the lipolytic *versus* ligand-binding function of HL on plasma lipoprotein metabolism and atherosclerosis using mice with stable, steady-state expression of catalytically active HL-WT or inactive HL-S145G in liver in a background lacking endogenous HL or apoE. We expressed catalytically active HL-WT and inactive HL-S145G primarily in liver since human HL mRNA was not detected in other tissues, including adrenal glands, ovaries, macrophages, and adipose tissue. Thus, any changes in plasma lipoproteins or atherosclerosis observed in the study transgenic mice are a result of increased liver HL transgene expression. The levels of HL overexpression (3–5-fold) achieved in our transgenic mice were within the physiological range reported previously for various metabolic states such as sex, dietary status, diabetes, and hormonal stimulation (6, 10). This contrasts with previous studies in which HL-WT and catalytically inactive HL were overexpressed by either 10-fold (25) or 20-fold (26) in E-KO mice expressing endogenous HL. Thus, the transgenic mice used in this study express HL in liver at physiologically relevant levels compared with mice lacking endogenous HL.

The lipolytic and ligand-binding functions of HL had differential effects on the plasma lipid profile of E-KO × HL-KO mice. HL-WT markedly lowered the plasma concentrations of TC, TG, PL, CE, non-HDL-C, and apoB by up to 52% (p < 0.01) in E-KO mice, indicating that HL will partially but not completely correct the hyperlipidemic effect (44, 58) induced by the absence of apoE in this mouse model. Similarly, even in the absence of lipolysis, catalytically inactive HL-S145G decreased the plasma levels of TC, TG, PL, CE, non-HDL-C, and apoB by up to 61% (p < 0.01). Thus, the lipolytic function of HL is not required for the reduction in apoB-Lp cholesterol in E-KO × HL-KO mice. In contrast, the effects of the lipolytic *versus* ligand-binding function of HL on HDL were quite distinct. HL-WT significantly reduced the plasma concentrations of HDL-C and apoA-I by 40 and 36% (p < 0.01), respectively, whereas the ligand-binding function of HL had no impact on plasma HDL-C or apoA-I levels in E-KO × HL-KO mice. The altered lipid profile of HL-WT and HL-S145G mice was not due to changes in lipoprotein lipase activity since it was reduced by ~23% in both groups of mice compared with E-KO × HL-KO mice; decreased lipolysis resulting from lower lipoprotein lipase activity would increase rather than decrease plasma TC, TG, and PL as well as apoB-Lp and HDL-C levels. These data are consistent with previous adenovirus and transgenic studies in E-KO mice (25, 26) and indicate that whereas the lipolytic function of HL significantly contributes to plasma HDL metabolism, the ligand-binding function of HL accounts for most of the reduction in the cholesterol-rich remnant lipoproteins that accumulate in E-KO × HL-KO mice.

Variability in HL activity contributes to changes not only in the plasma VLDL, IDL, remnant lipoprotein, and HDL levels (8, 9, 11–13, 15, 16, 19, 23, 24, 27, 28, 31), but also in LDL subclasses (7, 14, 41, 42). In humans, some of the pro-atherogenic effects of HL have been ascribed to changes in LDL particle density (7). Reduction in HL activity by pharmacological therapy is associated with an increase in LDL particle buoyancy, decreased levels of small, dense LDL, and reduced coronary atherosclerosis (7). In rabbits, HL overexpression leads to substantial reductions in medium and small VLDL, IDL, and HDL and to the accumulation of small, dense LDL (41, 42). Bergeron et al. (58) have also reported that HL deficiency in E-KO mice results in accumulation of heterogeneous, lipid-rich IDL and LDL as well as vesicular lipoproteins rich in both FC and PL. Thus, HL can change the composition of pro-atherogenic apoB-Lp and anti-atherogenic HDL, which may in turn alter their atherogenic potential. However, the separate effects of the lipolytic *versus* ligand-binding function of HL on VLDL, IDL, LDL, and HDL composition and particle size have not yet been investigated.

Our studies reveal that the lipolytic and ligand-binding functions of HL have differential effects on the particle size and lipid content of different plasma lipoproteins (Fig. 3 and Table 1). Thus, the amount of TC, CE, TG, and PL in VLDL/IDL, LDL, and HDL isolated from E-KO × HL-WT mice was significantly decreased compared with E-KO × HL-KO mice. These data agree with previous reports indicating that HL deficiency leads to the accumulation of lipid-rich VLDL/IDL, LDL, and HDL in E-KO mice (58). Here, we have also demonstrated that even in the absence of HL lipolysis, expression of HL-S145G dramatically reduced the TC, CE, TG, and PL levels in VLDL/IDL. Furthermore, like HL-WT, HL-S145G virtually eliminated the 369- and 348-A particles that accumulated in the VLDL/IDL fraction of E-KO × HL-KO mice. In contrast, LDL from E-KO × HL-S145G mice was more heterogeneous, larger, more lipid-rich, and more abundant than LDL from E-KO × HL-WT mice. Finally, unlike HL-WT, HL-S145G had virtually no effect on plasma HDL levels, lipid content, and particle size in E-KO × HL-KO mice.

The combined effects of HL-WT and HL-S145G on plasma lipoprotein particle lipid content, size, and levels (Figs. 2 and 3 and Table 1) imply different roles for the lipolytic *versus* ligand-binding function of HL in the metabolism of VLDL/IDL, LDL, and HDL. First, the ligand-binding function of HL appears to be primarily responsible for facilitating the clearance of VLDL/IDL in E-KO × HL-WT and E-KO × HL-S145G mice. Second, the accumulation of polydisperse, lipid-rich LDL in E-KO × HL-S145G (but not E-KO × HL-WT) mice indicates that effective clearance of LDL requires the ligand-binding function of HL. Finally, the lipolytic and not the ligand function of HL has a major effect on HDL metabolism in E-KO mice. To test these hypotheses and to gain further insight into the mechanisms by which the lipid-binding function of HL decreases the plasma levels of apoB-Lp, we examined the kinetics of radiolabeled VLDL/IDL and LDL containing only apoB-48 in E-KO × HL-KO, E-KO × HL-WT, and E-KO × HL-S145G mice. E-KO × HL-KO mice accumulate cholesterol-rich remnants that contain apoB-48 as their major apolipoprotein (44, 48, 58). Virtually no apoB-100-containing lipoproteins were present in the plasma of E-KO × HL-KO, E-KO × HL-WT, or E-KO × HL-S145G mice. Thus, injected autologous radiolabeled lipoproteins contained only apoB-48, with no apoE or apoB-100, leaving either HL-WT or HL-S145G to serve as the potential ligand for the two major receptors, the LDLR and LRP.

In the absence of apoE, both HL-WT and HL-S145G enhanced the FCR of 125I-apoB-48 VLDL/IDL by similar amounts (1.26- and 1.31-fold, respectively). These data provide an explanation for the similar reduction in the plasma levels of...
and are presumably cleared primarily by the LDLR. The sim-

apoB-100 LDL particles, which lack both apoE and apoB-48

LDL (by 36%) in HL-S145G-expressing mice. In contrast, nei-

creased it by only 1.59-fold compared with E-KO

apoB-48 LDL by 3.33-fold, whereas inactive HL-S145G in-

macroglobulin, delayed the plasma clearance of apoB-48 LDL

mice. In contrast, HL-WT and HL-S145G differentially altered

ligand-binding and not the lipolytic function of HL is primarily

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cholesterol, TG, and PL observed in Fig. 2 (A and B), as VLDL

and IDL are the principal lipid-containing lipoproteins in the

E-KO × HL-KO mouse model. Fully active HL-WT enhanced the FCR of mouse 125I-
apoB-48 LDL by 3.33-fold, whereas inactive HL-S145G increased it by only 1.59-fold compared with E-KO × HL-KO mice. Thus, HL-WT was more effective in clearing autologous LDL than HL-S145G. These findings provide an explanation for the greater accumulation of LDL lipids and apoB in E-KO × HL-S145G versus E-KO × HL-WT mice (Table I). One possible explanation for the differences in mouse apoB-48 LDL catabo-

ism between the two mouse groups could be variable expres-

sion of catalytically inactive HL in LDLR-KO mice (27) have

anti-LDLR antibodies in Swiss Webster mice (24) and expres-

and proteoglycans (16, 20). In addition, infusion of anti-HL and

ligand-binding function of HL facilitates the cellular

uptake of apoB-Lp via the LRP receptor pathway and are consistent with a recent study demonstrating that RAP blocks endocytosis of HL into rat liver hepatocytes (59).

Although HL-WT lowered the plasma concentrations of pro-

atherogenic apoB-Lp by as much as 42%, it significantly wors-

ened proximal aortic lesion development in both male and

especially female E-KO × HL-WT mice on RCD. The ligand-

binding function of HL does not appear to be responsible for the increased atherosclerosis observed in E-KO × HL-WT mice, as overexpression of the catalytically active form of HL is not pro-atherogenic. Thus, the increased atherosclerosis in E-KO × HL-WT versus E-KO × HL-KO mice can be ascribed to the lipolytic function of liver-expressed HL-WT. Hepatic expression of HL-WT was associated with decreased plasma HDL levels and the formation of smaller, lipid-poor LDL, both associated in humans with increased atherogenic risk (3–7).

In contrast to HL-WT, catalytically inactive HL-S145G signif-

icantly decreased aortic lesion formation (by 65–96%) in both male and female E-KO mice on RCD at 3, 5, and 7 months of age. The decreased atherosclerosis was not attributed to changes in plasma HDL levels. However, the ligand-binding function of HL markedly reduced apoB-containing cholesterol-rich remnants in E-KO × HL-KO mice and, in contrast to E-KO × HL-WT mice, led to the accumulation of heterogeneous, larger, and lipid-rich LDL and LDL. Our combined findings establish a protective role for the ligand-binding function of HL and indicate that in the absence of catalytic activity, HL does not lipolytically remodel apoB-Lp, but rather modulates athero-

sclerosis by enhancing removal of cholesterol-rich remnants via LRP. Since there is evidence that HL can complement and perhaps act synergistically with apoE to facilitate catabolism of apoB-Lp (41), it is possible that the beneficial effects of the ligand-binding function of HL are accentuated by the absence of apoE in this mouse model.

In summary, this study identifies LRP as a major receptor

pathway by which the ligand-binding function of HL alters remnant lipoprotein uptake in E-KO × HL-KO mice and delineates the separate contributions of the lipolytic versus ligand-binding function of HL to plasma lipoprotein composition and metabolism, identifying physiologically relevant but divergent roles for the lipolytic versus ligand-binding function of HL in modulating the development of atherosclerosis in vivo.

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