MATERIALS AND METHODS

Animals and Diet
HSKO mice in the C57BL/6 background (>99%) were generated as described previously \(^1,2\) and crossed with LDLrKO mice in the C57Bl/6 background (Jackson Laboratories) to generate heterozygous HSKO/LDLrKO. Mice used for atherosclerosis studies were generated by crosses of heterozygous HSKO/LDLrKO mice; genotypes of offspring were determined by PCR\(^1\). Female and male HSKO/LDLrKO and LDLrKO littermate control mice were fed a chow diet until 7-9 wks of age, and then switched to an atherogenic diet containing 10% palm oil and 0.2% cholesterol for 16 wks in most experiments, unless otherwise indicated. Diet composition has been published previously \(^3\). Mice were maintained on a 12h light/dark cycle. All protocols and procedures were approved by the Institutional Animal Care and Use Committee.

Lipid and lipoprotein analysis
Plasma was collected by tail bleeding or cardiac puncture from mice fasted for 4 hr. TPC, free cholesterol (FC), and triglycerides (TG) concentrations were determined by enzymatic assays using commercial kits\(^4\). Cholesterol distribution among lipoproteins was determined after fractionation of plasma by gel filtration chromatography using a Superose 6 10/300 GL column (GE Healthcare). An aliquot of plasma containing approximately 15 µg total cholesterol was injected onto the column and eluted with 0.9% saline containing 0.01% EDTA and 0.01% sodium azide at a flow rate of 0.4 ml/min. The column effluent was mixed with a commercially available enzymatic total cholesterol reagent (Pointe Scientific, Inc., Canton, MI) delivered at 0.125 ml/min. After passing through a knitted reaction coil maintained at 37°C, the absorbance of the reaction mixture was read at 500 nm using a UV-VIS detector. The area under the VLDL, LDL, and HDL peaks was calculated using Chrom perfect Spirit (Justice Laboratory Software) chromatography software. To calculate the cholesterol concentration in each lipoprotein fraction, the ratio of the respective peak area to total peak area was multiplied by the total plasma cholesterol. Liver lipid analysis was performed by enzymatic assay of detergent-extracted liver\(^5\).

Analysis of atherosclerotic lesions
Mice were sacrificed after 16 wks of atherogenic diet consumption. First they were anesthetized with ketamine/xylazine, and the vasculature then was perfused with cold PBS. Aortas were isolated and fixed in 10% buffered formalin. After fixation, aortas were cleaned of adventitial fat and pinned open for measurement of surface lesion areas. Images of en face aortas were analyzed using WCIF Image J software. Aortas were then lipid extracted for quantification of total and FC content using gas-liquid chromatography, as described previously \(^3\). Aortic roots were embedded in Optimal Cutting Temperature (Tissuetek) media in a plastic mold, frozen, and cut at 8 µm intervals. Sections were collected from the aortic region moving toward the apex of the heart and sequentially placed on 8 slides, such that each slide had sections 64 µm apart. The sections were fixed in 10% buffered formalin, stained in 0.5% Oil Red O for 25 minutes and counterstained with hematoxylin. Stained sections were photographed and Image-Pro software (Media Cybernetics Inc., Rockville, Md.) was used to quantify lesion area. The lesion areas of three sections representing different regions were averaged for each mouse.

In vivo Macrophage RCT
Macrophage RCT assays were conducted as described by Rader and colleagues \(^6\) with minor modifications \(^7\). J774 mouse macrophages or bone marrow-derived macrophages (BMM) from LDLrKO mice were radiolabeled and cholesterol-loaded with \(^3\)H-cholesterol and acetylated LDL,
respectively. Cells were then injected into the peritoneal cavity of recipient mice fed the atherogenic diet. Plasma samples were collected at 6h, 24h, and 48h after injection. Feces were collected throughout the 48h study. At necropsy, tissues were harvested and ³H-tracer levels in plasma, liver, bile, and feces were then quantified after lipid extraction and liquid scintillation counting. Aliquots of plasma were also fractionated by FPLC and cholesterol mass, and radiolabel distribution among lipoprotein fractions was quantified.

**Real-time PCR Analysis**

At sacrifice, tissues were harvested and snap frozen in liquid nitrogen. Total RNA was isolated from livers of male mice using TRizol (Invitrogen), and real-time RCR was performed as reported previously. Primer sequences were the same as described previously. GAPDH was used as the endogenous control.

**Cellular cholesterol efflux**

Cholesterol efflux from J774 macrophages to plasma of mice fed the atherogenic diet was measured as previously reported, with minor modifications. Briefly, 350,000 J774 cells were plated into each well of a 24-well plate and incubated in labeling medium (RPMI 1640 medium containing 1% FBS and 2 µCi ³H-cholesterol) for 24 hours. Cells were then washed once and incubated with efflux medium (MEM-HEPES media containing 2.8% apoB lipoprotein-depleted plasma). Four hours later, medium was harvested and cellular lipid was extracted with isopropanol. A 100 µl aliquot of medium and cellular lipid extract was taken for scintillation counting to determine percentage cholesterol efflux during incubation. Aliquots of efflux medium were fractionated on a Superdex 200HR column (1X30 cm), and radioactivity of each fraction was determined by scintillation counting.

**Biliary lipids**

A measured volume of gallbladder bile collected from mice (n=5-10) was subjected to neutral lipid extraction. 5-α cholestane (5 µg) was added to each extraction tube as an internal standard. Aliquots of the bottom organic phase were used to determine total cholesterol (TC) content by gas-liquid chromatography and phospholipid (PL) content by enzymatic assay. Bile acid (BA) was measured as reported previously using the top phase of the lipid extract.

**Fecal cholesterol excretion**

Two-day fecal collections were subjected to lipid extraction, and cholesterol content was measured as described previously.

**Macrophage cholesterol content**

Peritoneal cells were harvested by lavage from 4h-fasted mice. Cells were suspended in RPMI-1640 medium containing 1% Nutridoma-SP (Roche Applied Science), 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine and cultured at 37°C for 2 hours. Then, non-adherent cells were removed by washing with PBS and adherent macrophages were extracted with isopropanol at room temperature overnight. TC and FC content was determined by gas-liquid chromatography and cellular protein was measured by Lowry protein assay after NaOH digestion, as previously reported.
In vivo VLDL TG secretion rate determination

Tyloxapol (500 mg/kg body weight) was injected intravenously into 4h-fasted, anesthetized, atherogenic diet-fed mice (n=4-6). Blood was collected before (0 min), 30, 60, and 90 min after injection for measurement of plasma triglyceride (TG) concentration by enzymatic assay. VLDL TG secretion rate was determined by calculating the slope of the time vs. plasma TG concentration plot for each animal using linear regression analysis.

VLDL/IDL composition and size analysis

Plasma was collected from LDLrKO mice (n=4/group) fed an atherogenic diet for 16 wk after they were fasted for 4h. VLDL/IDL were isolated by ultracentrifugation at d=1.019 g/ml (100,000 rpm for 4h, Beckman Coulter TLA100.2 rotor) and chemical composition was determined by enzymatic assay and chemical assays. Lipid and protein content were expressed as percentage of total mass. Aliquots of VLDL/IDL were used to measure particle size using a Zetasizer Nano S dynamic light scattering instrument (Malvern Instruments Ltd., Worcestershire, UK). Particle sizes are reported as the major peak mean by volume analysis.

In vivo VLDL turnover

VLDL were separated from plasma of fasted LDLrKO mice fed an atherogenic diet for 16 wk by ultracentrifugation at d=1.006 g/ml. The VLDL preparation was refloated at d=1.006 gm/ml and radiolabeled with $^{125}\text{I}$ using the iodine monochloride method. Ninety-seven percent of the radioactivity was trichloroacetic acid-precipitable. VLDL tracer (0.25 x $10^6$ cpm) was diluted to 200 µl with saline for retro-orbital injection into 4h-fasted, anesthetized recipient mice (n=5/genotype) fed an atherogenic diet for 16 wk. Blood samples were collected at 2 and 30 min and 1, 3, 5, 8 and 24 h after injection and $^{125}\text{I}$ radiolabel in plasma was determined by gamma radiation counting. ApoB was precipitated from plasma using isopropyl alcohol and $^{125}\text{I}$ radiolabel in apoB was quantified by gamma radiation counting. Turnover curves were plotted as the percentage of injected tracer remaining in plasma or as the percentage of $^{125}\text{I}$-apoB remaining in plasma relative to the injected dose vs. time.

Gel electrophoresis

Plasma (1 µl) from LDLrKO or HSKO/LDLrKO mice fed an atherogenic diet for 19 wk was fractionated by 0.7% agarose gel electrophoresis. Following a 2h capillary transfer of the fractionated plasma from the agarose gel to a 0.2 µm nitrocellulose membrane, Western blot analysis was performed using anti-mouse apoA-I (Meridian Life Science, Inc. K23600G). SDS-PAGE and 4-30% non-denaturing gradient gel electrophoresis and Western blot analysis was performed as previously described.

Statistical analysis

Results are reported as mean ± standard error of the mean. Data were analyzed using two-tailed Student’s t test (with Welch’s correction in case of unequal variance) using Graphpad Prism software. $P <0.05$ was considered statistically significant.
References


8. de la Llera-Moya M, Drazul-Schrader D, Asztalos BF, Cuchel M, Rader DJ, Rothblat GH. The ability to promote efflux via ABCA1 determines the capacity of serum specimens with similar high-density lipoprotein cholesterol to remove cholesterol from macrophages. *Arterioscler Thromb Vasc Biol.* 2010;30:796-801.


