Materials and Methods

Materials—Plastic cell culture dishes were obtained from Corning (Primaria for primary cell cultures) and culture media from Invitrogen. Fetal bovine serum, lipoprotein deficient serum, and bovine serum albumin were purchased from Sigma. NaI was from Amersham Biosciences, and [3H]cholesterol, [14C]cholesterol, [3H]mevalonate, [14C]oleate, and [3H]cholesteryl oleate were from PerkinElmer Life Sciences. [3H]cholesteryl oleate and 3, 3-diocyloxacarbocyanine perchlorate (DiO) and 1, 1’-dioctadecyl-3,3,3’,3’-tetramethylindodicarbocyanine perchlorate (DiD) labeling dyes (Invitrogen) were incorporated into lipoproteins by microinjection according to Vassiliou et al. \(^1\), \(^2\)

Isolation of Lipoproteins – Chylomicrons, VLDL, LDL, and HDL were isolated from a human peripheral blood sample by sequential density gradient ultracentrifugation. Chylomicrons were treated with hepatic lipase to produce lipolyzed chylomicron remnants (CR; \(^3\)) and then reisolated by density gradient ultracentrifugation. Mouse chylomicrons were isolated from the plasma of apoE deficient mice, then re-enriched by incubation with bacterially expressed apoE (β-VLDL) as previously described \(^4\), and then reisolated by density gradient ultracentrifugation. The concentration of lipoproteins was calculated using the Markwell Lowry protein assay.

Isolation and assay of hepatocytes - All experiments performed were in accordance with protocols approved by the McGill University Health Centre Animal Care Committee. Male Syrian Golden hamsters were housed (3 animals/cage) under controlled temperature (72°F) and lighting (12 h light/dark cycle). Animals had free access to autoclaved water and food. Primary hepatocytes from Syrian Golden hamsters were isolated as described previously \(^5\) and seeded onto Primaria plates in DMEM containing 10% FBS or 10% lipoprotein deficient serum (LPDS). After allowing 4 h for the cells to adhere, cells were incubated with 10% LPDS for 16 h prior to the uptake and binding assays. For the binding assay, lipoproteins (LDL and CR) were radiolabeled with I using iodobeads (Pierce; according to manufacturer’s instructions). The binding assay was performed at
4°C for 1 h before extensive washing and determination of bound radioactivity (normalized to non-specific binding and total cell protein). [3H]cholesteryl oleate radiolabeled lipoproteins (25 μg) were added to the cells for 1 h, 4 h, or 8 h and after extensive washing, the amount of radiolabel taken up by the cells was normalized for non-specific uptake and total cell protein.

Due to the differences in the size and composition of the lipoprotein particles, we compared the uptake of cholesterol from each with increasing concentrations of total lipoprotein cholesterol. These experiments were first performed with 3H-cholesteryl-oleate labeled lipoproteins (as above except that a range of concentrations was used at a 24 h time point). Next, the same experiment was performed except that a 3H-cholesterol label was used. Importantly, 3H-cholesteryl oleate and 3H-cholesterol labeled the cells to an equivalent degree over the concentration curve, demonstrating that both labels are reliable indicators of cholesterol uptake. 3H-cholesteryl oleate labeling of lipoproteins results in a 95-100% incorporation into internal compartments (non-methyl β-cyclodextrin accessible) while 3H-cholesterol labeling of lipoproteins 65-70% incorporation into internal compartments (non-methyl β-cyclodextrin accessible) with the remaining radioactivity in the plasma membrane (as previously shown by Zheng et al. 6). Experiments with both labels were included in order to find a concentration of each lipoprotein that gave a similar degree of radioactive labeling. The concentration of each lipoprotein (and non-lipoprotein derived cholesterol) served as a guide for the mass assays (LDL 50 μg/mL; CR 12 μg/mL; β-VLDL 18 μg/mL; Chylomicrons 8 μg/mL; VLDL 25 μg/mL; HDL 100 μg/mL; non-lipoprotein-derived cholesterol 100 μg/mL). We then performed the same experiment with unlabeled lipoproteins at these specific concentrations for 24 h and performed mass measurements. Total cellular cholesterol mass was measured by three different methods: 1) total cholesterol and free cholesterol kits (Roche Diagnostics; according to manufacturer’s protocol); 2) high performance liquid chromatography (HPLC) separation and quantitation of free and cholesteryl ester; 3) gas chromatographic separation and quantitation of free and cholesteryl ester.

Chromatography of lipids – Gas chromatography experiments were performed as previously described 7. HPLC was performed as follows. Cellular lipids were extracted
by a one hour incubation with hexane. The effluent was evaporated under a stream of nitrogen and re-dissolved in 100 μL of chloroform: iso-octane (1:1). The solution was transferred to Agilent HPLC vials and stored at -20°C until the chromatography. Samples were analyzed using an Agilent 1100 series equipped with a quaternary pump and an Alltech ELSD 2000 evaporative light-scattering detector. The column used was an Onyx monolithic Si (Phenomenex) and the solvent system was based on the method of Graeve and Janssen. Solvent A consisted of iso-octane:ethylacetate (99.8:0.2); solvent B was acetone:ethylacetate (2:1) with 0.02% acetic acid; solvent C was isopropanol:water (85:15) with acetic acid and ethanolamine each at 0.05%. Gas flow was 3.0 L/min and drift tube temperature was set at 60 degrees C. Peaks were analyzed using Agilent Chemstation software and quantified using calibration curves prepared with commercial lipid standards (Sigma-Aldrich, Avanti Polar Lipids). All three methods confirmed that the incubation of hepatocytes with lipoprotein and non-lipoprotein-derived cholesterol resulted in equivalent cholesterol loading. Therefore, for all subsequent experiments, we achieved the equivalent loading and labeling of hepatocytes for each lipoprotein (including non-lipoprotein-derived cholesterol).

For the ACAT assay, hepatocytes were incubated with [3H]-cholesterol (5 μCi) incorporated into the predetermined concentrations of lipoproteins, FBS or LPDS for 24 h to uniformly label the cells. Then the radioactive lipids were extracted with hexane. Lipids were separated by thin layer chromatography using hexane/diethyl ether/acetic acid (105:45:1.5, v/v/v) as a solvent system. Cholesterol and cholesteryl ester (CE) spots were scraped from the plate and radioactivity determined by liquid scintillation. Results are expressed as percent CE of the total radioactive cholesterol (free and esterified) ±SD. Alternatively, cells incubated with unlabeled lipoproteins for 24 h were incubated with [14C]oleate (1 μCi) for the last 12 h and then the radioactive lipids quantified as above. Results are expressed as cpm of radioactive CE/ng total cell protein ±SD. For de novo synthesis, cells were incubated with unlabeled lipoproteins for 24 h in the presence of [3H] mevalonate (5μCi) and then lipids were quantified as above. Results are expressed as cpm of radioactive cholesterol or cholesteryl ester/ng total cell protein ±SD. For de novo synthesized CE, the amount of CE was taken as a percent of total radioactive cholesterol ±SD, to account for the significantly increased amount of newly synthesized
cholesterol in LDL-treated cells. Typical specific activities for $[^3]H$ cholesterol labeling are (in cpm/ng lipoprotein protein): chylomicrons 15803 cpm/ng; CR 10280 cpm/ng; β-VLDL 6905 cpm/ng; VLDL 5086 cpm/ng; HDL 1122 cpm/ng; LDL 2543 cpm/ng.

**mRNA Quantitation** - Total RNA was prepared from primary hamster hepatocytes cells using the RNasey mini RNA extraction kit (Qiagen), according to the manufacturer's instructions. Total RNA (200 ng) was reverse-transcribed using the QuantiTect Reverse Transcription kit (Qiagen). Real-time quantitative PCR was carried out using the Quantitect SYBR Green PCR kit and QuantiTect Primer assays (Qiagen) using the primers: β-actin 5′-GCACCAAGGTGATGATGTG-3′ and antisense primer 5′-CGGTTGGCCTTCAGGGTTC-3′; HMGCoA reductase 5′-CGAGGAAAGACTGTGGTG-3′; LDL receptor 5′-GTG TGA AGA TAT TGA CGA GTG-3′, and antisense primer 5′-AGT AGA TTC TAT TGT TGG TCA-3′. All reactions were performed on an ABI PRISM 7300 Sequence Detection System (Applied Biosystems). Amplifications were carried out in a 96-well plate with 50 μl reaction volumes and 40 amplification cycles (94°C, 15s; 55°C, 30s; 72°C, 34s). Experiments were carried out in triplicate, and the mRNA expression was taken as the mean of three separate experiments. The expression of each gene was normalized to β-actin expression. Fold changes relative to controls were determined using the ΔΔCt method.

**Double Labeling** - We labeled hepatocytes with $[^3]H$cholesterol-LDL and $[^14]C$cholesterol-CR. To assure completeness, we converted and normalized cpm values to dpm values based on the efficiency of detection in a scintillation counter (according to Beckman protocols). Total radioactivity incorporated into the same hepatocyte wells of $[^3]H$cholesterol-LDL and $[^14]C$cholesterol-CR were 277860±62550 and 260540±39110 dpm/mg cell protein, respectively. Therefore, essentially the same radioactivity of each isotope was incorporated. The converse labeling of $[^3]H$cholesterol-CR and $[^14]C$cholesterol-LDL was also performed. Total radioactivity incorporated was 253690±41820 and 267390±43980 dpm/mg cell protein, respectively. Results are expressed as percent radioactive CE of the total radioactivity ±SD, for each isotope.
**Fluorescence Microscopy**— Images were collected on a Zeiss LSM-510 Meta laser scanning microscope with either 63x or 40x oil immersion lens. Cells were placed on coverslip culture dishes (MatTek Corp., Ashland, MA) at ~70% confluence. For live cell imaging, cells were maintained at 37 °C in a heated chamber and incubated with Hepes-buffered complete media, pH 7.4.

**ACAT1, ACAT2, LDL receptor, PCSK9, IDOL and GAPDH Expression** – Western blotting of hepatocyte samples treated with different lipoproteins were probed with antibodies to ACAT1, ACAT2 and LDL receptor (Santa Cruz), PCSK9 (kind gift from Dr. Jingwen Liu, VA Palo Alto Health Care System), IDOL and GAPDH (Santa Cruz). Cells treated with FBS or LPDS alone were used as positive and negative controls, respectively.

**ApoB Secretion** - We labeled hepatocytes with [³H]cholesterol-LDL (50 μg with 5μCi) or [³H]cholesterol-CR (12 μg with 5μCi) for 24 h in the presence of an LCAT inhibitor, washed cells extensively, then added media with no serum. The media was collected after 8 h, concentrated by a spin column, lipids were extracted and CE quantified by TLC and liquid scintillation. To assure specificity, alternatively, we immunoprecipitated apoB (rabbit anti apoB (Sigma)) from the media samples and then performed the lipid isolation and quantification (validated and confirmed according to 9-11). Immunoprecipitation of media from cells grown in FBS with [³H]-cholesterol or LPDS with [³H]-cholesterol (representing the positive and negative controls, respectively) showed 23927±3551 and 4159±420 cpm/mg cell protein.

**HDL-mediated cholesterol efflux** - We performed an efflux assay according to Kiss et al. 12-14 Briefly, we labeled hepatocytes with: a) [³H]cholesterol-LDL and [¹⁴C]cholesterol-CR; b) [³H]cholesteryl ester-LDL and [¹⁴C]cholesterol-CR; c) [³H]cholesteryl ester-CR and [¹⁴C]cholesterol-LDL for 24 h. We washed the cells extensively and then we added HDL for 4 h and measured the amount of radioactivity that associated with the HDL.
References


