Hepatic Cholesterol Homeostasis

Is the Low-Density Lipoprotein Pathway a Regulatory or a Shunt Pathway?

Allan D. Sniderman, Yanqin Qi, Cheng-I J. Ma, Rui Hao Leo Wang, Mark Naples, Chris Baker, Jing Zhang, Khosrow Adeli, Robert S. Kiss

Objective—The hypothesis that cholesterol that enters the cell within low-density lipoprotein (LDL) particles rapidly equilibrates with the regulatory pool of intracellular cholesterol and maintains cholesterol homeostasis by reducing cholesterol and LDL receptor synthesis was validated in the fibroblast but not in the hepatocyte. Accordingly, the present studies were designed to compare the effects of cholesterol that enters the hepatocyte within an LDL particle with those of cholesterol that enters via other lipoprotein particles.

Approach and Results—We measured cholesterol synthesis and esterification in hamster hepatocytes treated with LDL and other lipoprotein particles, including chylomicron remnants and very-LDL. Endogenous cholesterol synthesis was not significantly reduced by uptake of LDL, but cholesterol esterification (280%) and acyl CoA:cholesterol acyltransferase 2 expression (870%) were increased. In contrast, cholesterol synthesis was significantly reduced (70% decrease) with other lipoprotein particles. Furthermore, more cholesterol that entered the hepatocyte within LDL particles was secreted within very-LDL particles (480%) compared with cholesterol from other sources.

Conclusions—Much of the cholesterol that enters the hepatocyte within LDL particles is shunted through the cell and resorbed within very-LDL particles without reaching equilivrium with the regulatory pool. (Arterioscler Thromb Vasc Biol. 2013;33:00-00.)

Key Words: cholesterol ■ 3-hydroxy-3-methylglutaryl-coenzyme A ■ high-density lipoproteins ■ sterol O-acyltransferase ■ very–low-density lipoproteins

The low-density lipoprotein (LDL) receptor pathway plays a major role in the regulation of the plasma level of LDL in humans. When clearance of LDL particles through the LDL receptor pathway is markedly impaired or abolished, as in familial hypercholesterolemia, profound elevations in plasma LDL result. At the other extreme, more effective clearance of LDL particles by the LDL receptor pathway is the major mechanism by which statins lower plasma LDL. However, these changes in the activity of the LDL receptor pathway are produced by disease or pharmacological intervention and are not evidence of its physiological role.

The LDL receptor pathway paradigm stipulates that intracellular cholesterol homeostasis is based on a reciprocal relationship between the rate at which cholesterol within an LDL particle enters the cell through the LDL receptor pathway and the rate at which cholesterol and LDL receptors are synthesized within the cell. In brief, cholesterol that enters the cell within an LDL particle is released from the particle in the lysosome and equilibrates with the cholesterol in the endoplasmic reticulum (ER) membrane.
the hepatocyte is at the center of all the major cholesterol fluxes within the organism, with multiple lipoprotein receptor–mediated endocytic pathways that contribute cholesterol to the total hepatocyte cholesterol pool. There are also multiple outputs of cholesterol from the hepatocyte: cholesterol can be metabolized to bile acids, as well as dissolved in bile; cholesteryl and cholesterol ester can be secreted within apolipoprotein B (apoB)–containing very-LDL (VLDL) particles; and finally cholesterol can be transferred from the hepatocyte plasma membranes via ATP-binding cassette transporter A1 and ATP-binding cassette transporter G1 to apolipoprotein A-I and particles of high-density lipoprotein (HDL) as well as secreted directly with HDL.

Although the LDL receptor pathway plays an important role in determining the concentration of plasma LDL, it does not follow that the LDL receptor pathway paradigm accurately describes cholesterol homeostasis in the liver. There is evidence that it does not, in that LDL receptor activity and cholesterol synthesis have been documented to persist in the face of increased delivery of LDL particles to the liver.10–19

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
CR Versus LDL Uptake
Preliminary experiments demonstrated, as anticipated, relatively similar binding but significantly greater uptake of chylomicron remnants (CR) compared with LDL particles (1 hour: 6.86±0.48 versus 12.24±3.74; 4 hours: 20.30±2.80 versus 51.37±3.34; 8 hours: 31.37±2.02 versus 74.47±7.59 cpm/ng cell protein; all significant differences [P<0.05] by Student t test; Figure I in the online-only Data Supplement). Accordingly, the relative concentrations of CR and LDL in all subsequent experiments were adjusted to ensure that the total cholesterol (in LDL or CR) taken up by the cells was equivalent.

Regulatory Mechanisms
Cholesteryl ester (CE) synthesis was measured after uptake of 1H-cholesterol–labeled LDL particles versus uptake of 1H-cholesterol–labeled CR particles in cholesterol-depleted and cholesterol-loaded hamster hepatocytes. Under both conditions, formation of CE was substantially less when cells are incubated with CR than when they are incubated with LDL. As would be expected, formation of CE from CR cholesterol was substantially greater in cholesterol-loaded cells than in cholesterol-depleted hepatocytes. Under both conditions, formation of CE from exogenous cholesterol was substantially greater from LDL than CR (3.44±0.69 versus 2.11±0.21% of total radioactivity in cholesterol-loaded cells, Figure 1A; 3.45±0.43 versus 0.42±0.11% of total radioactivity in cholesterol-depleted cells, Figure 1B). These results indicate that more of the cholesterol within LDL is esterified than the cholesterol within CR. These findings were confirmed by measuring CE formation based on incorporation of [14C]-oleate into CE. Formation of CE in cholesterol-loaded cells was significantly greater with LDL than with CR (14.4±5.6 versus 1.27±0.14 cpm/ng; Figure 1C).

Effects of LDL- and CR-Derived Cholesterol on Synthesis of Cholesterol and CE
One mechanism to regulate total cellular cholesterol levels is the downregulation of endogenous cholesterol synthesis by uptake of exogenous cholesterol. The de novo synthesis of cholesterol was measured by incorporation of [3H]-mevalonate. Equivalent amounts of exogenous cholesterol were taken up by the hepatocytes, and LDL-treated cells synthesized 3-fold more cholesterol than CR-treated cells (2.85±0.30 versus 0.86±0.15 cpm/ng; Figure 1D). In addition, more newly synthesized cholesterol was esterified when cells are incubated with LDL compared with CR particles (6.53±1.11 versus 1.96±0.44%; Figure 1E). These results demonstrate that LDL-derived cholesterol does not downregulate de novo synthesis as CR-derived cholesterol does. Furthermore, more newly synthesized cholesterol is esterified in LDL-treated cells compared with CR-treated cells, suggesting higher ACAT activity in LDL-treated cells.

All these observations are consistent with the hypothesis that more of the CR-derived cholesterol comes into equilibrium with the regulatory pool than the cholesterol that is liberated from LDL particles. These results are consistent with the observation that the intracellular route followed by CR particles differs from that followed by LDL particles.

Fluorescence Microscopy of LDL and CR
Fluorescent hydrophobic dyes (DiO and DiD) are irreversibly incorporated into separate lipoproteins and then incubated with primary hamster hepatocytes for increasing periods of
time. During that time, these hydrophobic dyes traffic through the hepatocyte within the lipoprotein particle into which they are incorporated. Unlike the natural lipid components of the lipoprotein particles, the hydrophobic dyes are reliable guides until they aggregate within the lysosomes (≤1 hour). Within the first 15 minutes, DiO-CR (green) and DiD-LDL (red) are in separate and distinct endosomes (Figure 2). By 1 hour, there was partial overlap of DiO-CR and DiD-LDL in lysosome compartments (as detected by colocalization with lysotracker blue). These results indicate that during the initial phases of internalization, the vast majority of LDL and CR particles take separate endocytic routes.

**LDL-Specific Effect on Hepatic Cholesterol Homeostasis**

The initial series of experiments suggested that metabolic consequences for hepatic cholesterol homeostasis differed after uptake of LDL particles versus CR particles. To test this hypothesis further, we extended the initial observations using a variety of other lipoproteins, including human chylomicrons, CR, HDL, VLDL, and mouse β-VLDL. The effects of lipoprotein-deficient serum (LPDS), LDL and non-lipoprotein–derived free cholesterol were also studied.

Increasing concentrations of LDL, VLDL, chylomicrons, CR, β-VLDL, and HDL were labeled with 2 μCi of [3H]-cholesteryl oleate and added to hepatocytes for 24 hours. Incorporation of radioactivity into the hepatocytes increased over time (Figure IIA in the online-only Data Supplement). The concentration of each lipoprotein where the equivalent amount of radioactivity was incorporated into the hepatocytes after 24 hours was considered to be the equivalent concentration, and mass experiments were conducted to ensure that equivalent cholesterol mass loading was achieved. After loading hepatocytes with the lipoprotein- and non-lipoprotein–derived cholesterol, we measured the total mass of cholesterol within the hepatocyte by 3 different methods: (1) total cholesterol and free cholesterol kits (Roche Diagnostics); (2) high-performance liquid chromatography separation and quantification of free and cholesteryl ester; and (3) gas chromatographic separation and quantification of free and cholesteryl ester. All 3 methods confirmed that the incubation of hepatocytes with lipoprotein and non-lipoprotein–derived cholesterol resulted in equivalent cholesterol loading (Figure 3A; Figure IIB and IIC in the online-only Data Supplement). As a negative control, LPDS-treated hepatocytes contained the lowest mass of cholesterol (Figure 3A).

Incubation with LDL, chylomicrons, CR, VLDL, β-VLDL, HDL, and non-lipoprotein–derived cholesterol resulted in a significant and almost identical increase in cholesterol mass compared with the LPDS control. These results establish that uptake of exogenous cholesterol from multiple lipoprotein and non-lipoprotein sources increases the total mass of cholesterol within the cell to a similar extent. For this reason, we can presume that, under the loading conditions used here for this and subsequent experiments, the hepatocytes were cholesterol...
loaded to an equivalent degree, and any differences noted are not because of differences in the degree of cholesterol loading. Importantly, uptake of non-lipoprotein–derived cholesterol, presumably to the plasma membrane, resulted in the same amount of cholesterol loading, thereby serving as a suitable positive control.

Under the conditions described above, each of these exogenous lipoprotein-derived and non-lipoprotein–derived cholesterol sources was labeled with an equivalent amount of \[^{3}H\]–cholesterol, and a cholesterol esterification assay was performed. LDL-derived cholesterol promoted a significant increase in CE formed compared with other sources of cholesterol (Figure 3B). Interestingly, VLDL-derived cholesterol also led to increased CE synthesis that was significantly greater than human chylomicrons, human CR, and mouse \(\beta\)-VLDL, but not as high as LDL, perhaps caused by contaminant LDL because the VLDL sample was generated by sequential density gradient ultracentrifugation or perhaps because VLDL itself may bind to the LDL receptor. In any case, the appearance of exogenous cholesterol in CE from LDL was considerably greater than for any of the other sources of cholesterol.

Endogenous cholesterol synthesis was measured by incorporation of \[^{3}H\]–mevalonate into free and esterified cholesterol. As a positive control, hepatocytes treated with LPDS, which does not contain significant exogenous cholesterol, had the highest radioactive cholesterol formed (Figure 4). Endogenous cholesterol synthesis was virtually as high when LDL–derived cholesterol was added, and this amount was significantly greater than the amount of endogenous cholesterol synthesis when the other sources of lipoprotein were added to the medium. Interestingly, VLDL also resulted in a small increase in de novo–synthesized cholesterol, suggesting that VLDL–derived cholesterol partially mimics the effect of LDL–derived cholesterol. These results are consistent with the hypothesis that cholesterol released from LDL does not enter the regulatory pool as does the cholesterol from the other lipoprotein particles, and therefore, LDL–derived cholesterol does not downregulate endogenous cholesterol synthesis.

The amount of newly synthesized cholesterol that was incorporated into CE was also determined for each condition. CE synthesis mirrors cholesterol synthesis (Figure 4). For the positive control, treatment with LPDS resulted in the highest percent of CE formed from de novo–synthesized cholesterol. That cholesterol ester synthesis is upregulated in LPDS medium, in which there is no source of exogenous cholesterol and, therefore, endogenous cholesterol synthesis is increased, is anticipated. LDL treatment also resulted in significantly increased CE formation. However, addition of chylomicrons, CR, VLDL, \(\beta\)-VLDL, HDL, and non-lipoprotein–derived cholesterol resulted in a significantly lower percentage of CE formed. These results are consistent with the hypothesis that
Figure 4. Total de novo–synthesized cholesterol was increased with low-density lipoprotein (LDL) cholesterol loading. Hepatocytes grown in lipoprotein-deficient serum (LPDS) were treated with the different lipoproteins and an equivalent amount of [3H]-mevalonate for 24 h. Then, cellular lipids were extracted and separated by thin layer chromatography. Radioactivity associated with the free cholesterol band (gray bar) and the cholesteryl ester band (black bar) was measured by scintillation counting, and the results are presented as the mean±SD of quadruplicate measures of 3 independent experiments. *P<0.05, **P<0.001, compared with LPDS condition. †[V] indicates [very-low-density lipoprotein; Chylo, human chylomicrons; CR, chylomicron remnants; HDL, high-density lipoprotein; and VLDL, very–low-density lipoprotein.

exogenous cholesterol released from LDL particles does not downregulate endogenous cholesterol synthesis as effectively as other lipoprotein and non-lipoprotein sources of cholesterol, and incorporation of both exogenous and endogenous cholesterol into cholesterol ester under conditions of LDL-derived cholesterol loading is substantially greater than with other lipoprotein particles.

mRNA Expression of HMGCоА Reductase and LDL Receptor
SREBP2 responds to intracellular cholesterol levels in the regulatory pool in the ER membrane by controlling the mRNA expression of HMGCоА reductase and the LDL receptor. To confirm the measured activities of endogenous cholesterol synthesis, we measured the mRNA expression by quantitative polymerase chain reaction. Incubation with LDL resulted in an mRNA expression level of HMGCоА reductase similar to treatment with LPDS (Figure III in the online-only Data Supplement). Incubation with other lipoproteins or with non-lipoprotein–derived cholesterol resulted in a significantly lower mRNA expression level, suggesting that the cholesterol derived from these treatments did enter the regulatory pool. LDL receptor mRNA was similar to HMGCоА reductase mRNA with regard to treatments, except to a lesser extent. Therefore, these results indicate that LDL-derived cholesterol does not enter the regulatory pool to diminish the SREBP2-mediated expression of HMGCоА reductase and LDL receptor mRNA.

ACAT Activity and ACAT1 and ACAT2 Expression
The enzyme responsible for esterification of cholesterol intracellularly is ACAT (also known as SOAT). In hepatocytes, there are 2 independent enzymes (ACAT1 and ACAT2) with putative separate physiological functions and location. These experimental results to date are consistent with a model that posits that a substantial portion of the cholesterol in LDL that is internalized, trafficked to, and released from the lysosome tends to be rapidly exposed to ACAT, whereas cholesterol from other lipoprotein and non-lipoprotein sources is not. Accordingly, to further explicate the role of ACAT activity in these different responses, under the same conditions as in Figure 1A, CE synthesis was measured after treatment with [3H]-cholesterol–labeled LDL or CR without and with a total ACAT inhibitor (58035). As observed previously, CE synthesis was substantially greater on addition of LDL compared with CR (Figure IV in the online-only Data Supplement). Importantly, addition of the ACAT inhibitor resulted in a significant decrease in CE formed to the same background level for LDL and CR, demonstrating that the increase in CE synthesis on LDL treatment is entirely dependent on ACAT-mediated newly esterified cholesterol.

These findings suggest that LDL might induce the activity of either or both ACAT1 and ACAT2. To determine whether this was the case, Western blots of cell lysates from LDL-, CR-, and other lipoprotein-treated hepatocytes were performed. There was no difference in ACAT1 expression between cells treated with different lipoproteins (Figure V; Figure V in the online-only Data Supplement). However, ACAT2 expression was significantly upregulated in LDL-treated cells (8.7-fold) compared with LPDS-treated cells. ACAT2 expression was also significantly upregulated in VLDL-treated cells (4.4-fold) although not to the same degree as LDL. These results suggest that LDL treatment particularly upregulates ACAT2 expression, despite the fact that an equivalent amount of cholesterol was delivered by other treatments.

Figure 5. Acyl CoA:cholesterol acyltransferase 2 (ACAT2) expression is upregulated by low-density lipoprotein (LDL) treatment. Hepatocytes were treated with lipoprotein-deficient serum (LPDS), very–low-density lipoprotein (VLDL), chylomicrons, LDL, chylomicron remnants (CR), [very-low-density lipoprotein (β-VLDL), high-density lipoprotein (HDL), or non-lipoprotein–derived cholesterol for 24 h, and then cell lysates were prepared and Western blots performed for acyl CoA:cholesterol acyltransferase 1 (ACAT1), ACAT2, LDL receptor, proprotein convertase subtilisin/kexin type 9 (PCSK9), and inducible degrader of LDL receptor (IDOL). GAPDH was used as a loading control. Western blots from 3 separate experiments were performed, and a representative Western blot is shown here. Quantification is shown in Figure V in the online-only Data Supplement.
Important regulators of LDL receptor activity and expression on the cell surface of the hepatocyte are proprotein convertase subtilisin/kexin type 9 (PCSK9) and inducible degrader of LDL receptor (IDOL). Both proteins, although they are regulated differently and act mechanistically differently, contribute to the downregulation, internalization, and degradation of LDL receptors. We performed Western blots of the LDL receptor, PCSK9, and IDOL of cell lysates from hepatocytes treated with different lipoproteins (Figure 5; Figure V in the online-only Data Supplement). LDL receptor expression was relatively increased in VLDL- and LDL-treated hepatocytes (2.8- and 6.3-fold, respectively) compared with LPDS, consistent with the lack of an effect of LDL- or VLDL-derived cholesterol on the cholesterol regulatory pool to downregulate LDL receptor expression. However, chylomicrons, HDL, and non-lipoprotein-derived cholesterol had lower LDL receptor expression, as expected (1.1-, 0.7-, and 0.9-fold, respectively, compared with LPDS control). Interestingly, CR- and β-VLDL-treated hepatocytes had increased LDL receptor expression compared with the LPDS control (5.6- and 5.8-fold, respectively). We postulated that expression levels of PCSK9 and IDOL may determine the LDL receptor expression levels (Figure 5; Figure V in the online-only Data Supplement). Interestingly, PCSK9 levels increased moderately in VLDL- and LDL-treated cells compared with LPDS (1.1- and 3.7-fold, respectively), despite increased LDL receptor levels. However, PCSK9 protein levels were inversely correlated with LDL receptor levels in chylomicron-, CR-, β-VLDL-, HDL- and non-lipoprotein-derived cholesterol-treated hepatocytes, explaining LDL receptor expression levels. IDOL was significantly upregulated in all lipoprotein-treated cells in comparison with LPDS (Figure 5; Figure V in the online-only Data Supplement), demonstrating that IDOL is not a factor explaining the increased LDL receptor expression levels. However, expression of PCSK9 does explain the expression level of the LDL receptor in most lipoprotein-treated hepatocytes but not in LDL- and VLDL-treated hepatocytes. Therefore, LDL-induced changes in hepatocytes represent a unique homeostatic mechanism not explained by PCSK9 or IDOL.

**Double Labeling**

Because experiments with LDL and CR were performed in separate primary cell cultures and then compared, it was important to extend these results by performing LDL and CR treatment experiments in the same cells. For this reason, we used double labeling of the same hepatocytes with [3H]-cholesterol and [14C]-cholesterol labels. Assuring similar labeling efficiency, the results were that 14.98±2.92% of [3H]-cholesterol derived from LDL was incorporated into CE, whereas 4.69±0.99% of [14C]-cholesterol derived from CR was incorporated into CE (Figure 6A). In a second experiment, reverse labeling of [3H]-cholesterol-CR and [14C]-cholesterol-LDL was performed. Once again assuming similar labeling efficiency, results showed that 24.54±1.73% of [14C]-cholesterol derived from LDL was incorporated into CE, whereas 8.35±1.95% of [3H]-cholesterol derived from CR was incorporated into CE (Figure 6A). These results confirm that LDL-derived cholesterol is preferentially esterified compared with CR-derived cholesterol.

**ApoB Secretion**

To determine the fate of delivered cholesterol, VLDL secretion was measured. In this experiment, [3H]-cholesterol-labeled LDL and [4C]-cholesterol-labeled CR were incubated with hepatocytes for 24 hours in the presence of an LCAT inhibitor. The cell medium was collected, and the CE lipids were collected by thin layer chromatography and submitted for scintillation counting. More than 3-fold LDL-derived cholesterol was secreted as CE into the medium than CR-derived cholesterol (152.3±21.1 versus 46.6±16.3 cpm/μg cell protein; Figure 6B). This experiment was repeated using anti-apoB antibodies to immunoprecipitate secreted apoB and its cholesterol-associated radioactivity. Immunoprecipitation of media from cells grown in fetal bovine serum with [3H]-cholesterol or LPDS with [14C]-cholesterol (representing the positive and negative controls, respectively) showed 23927±3551 and 4159±420 cpm/mg cell protein, indicating that apoB secretion increases in cholesterol-loaded compared with cholesterol-depleted cells. By comparison, LDL-treated cells had 27548±3319 cpm/mg cell protein, and CR had 5710±1714 cpm/mg cell protein (Figure 6C). These results demonstrate that LDL-derived cholesterol is preferentially esterified and secreted as part of an apoB-containing particle (VLDL) compared with CR-derived cholesterol.

**Efflux Results**

Hepatocytes were dually labeled by adding separately labeled LDL and CR for uptake, and then an efflux assay was performed. To be complete and to confirm the effect observed, we labeled the LDL and CR in 3 different ways: (1) [3H]-cholesterol-LDL and [14C]-cholesterol-CR; (2) [3H]-cholesterol-LDL and [3H]-cholesterol-CR; and (3) [3H]-cholesterol-LDL and [14C]-cholesterol-LDL. In each case, the cholesterol-associated LDL label was preferentially transported to HDL by cholesterol efflux (Figure 6D). The results demonstrate that LDL-derived cholesterol, no matter how labeled and delivered, is preferentially transported to an efflux-accessible site for mediated cholesterol efflux.

**Discussion**

The conventional model states that cholesterol, which enters the cell within LDL particles, rapidly equilibrates with the cholesterol within the regulatory pool and, by doing so, induces a coordinated, physiologically purposeful series of responses, which restore cholesterol balance by reducing its synthesis and uptake. Increased input leads to decreased input. Alterations in output are not part of the equation. But the conventional model, which was based on studies in fibroblasts, does not take into account that cholesterol can be secreted from the hepatocyte either as bile acids or dissolved in bile acids or within VLDL or HDL particles and that cholesterol esterification within the hepatocyte can be driven by either ACAT1 or ACAT2, whereas only ACAT1 is present in other cells.
Lange et al demonstrated that the pools of cholesterol in the plasma membrane, the mitochondria, and the ER are in rapid reversible equilibrium. Cholesterol delivered to the plasma membrane moves first to the ER and mitochondria where both immediate and delayed adaptive responses are stimulated. Only after equilibration in these compartments does cholesterol move to the site of ACAT activity so that cholesterol esterification, which renders cholesterol bioinactive, can occur. Our results suggest that more of the cholesterol released from the CR lysosome is delivered to the plasma membrane-ER-mitochondria compartment than from the LDL lysosome. In contrast, compared with CR-containing lysosomes, a greater portion of the cholesterol released from LDL-containing lysosomes is delivered to ACAT2 and rapidly esterified and incorporated into nascent apoB lipoproteins.

Our data suggest that much of the cholesterol taken up within LDL particles is so rapidly esterified that it does not gain access to the regulatory pool but rather is almost immediately resecreted within apoB or nascent HDL particles. However, this pattern seems to be characteristic of cholesterol from LDL particles but not of cholesterol from CR, VLDL, and β-VLDL particles or of cholesterol from non-lipoprotein particle sources. Cholesterol from all these sources seems to equilibrate rapidly with the cholesterol regulatory pool rather than primarily and initially interacting with ACAT. In fibroblasts, by contrast, ACAT activity is less, cholesterol ester cannot exit the cell, and, therefore, cholesterol derived from LDL will eventually equilibrate with the regulatory pool and deactivate the SREBP2 pathway. These differences may also be related to differences in endosomal pathways for lipoprotein particles because our fluorescent microscopic studies demonstrated that LDL and CR seem to enter the cell by separate pathways. These observations are supported by other reports of a delay before lysosomes containing CR particles reach the perinuclear region, whereas lysosomes containing LDL particles seemed to move rapidly to the perinuclear region.

Given that the mass of CE seems to be a major determinant of the rate of secretion of apoB particles from hepatocytes, linkage of the LDL endocytosis pathway to the apoB secretion pathway via esterification of cholesterol creates a shunt pathway for cholesterol, which enters and leaves the hepatocyte without interacting biologically with the cholesterol within the regulatory pools. Interestingly, entry of LDL cholesterol upregulates ACAT2 expression in this model system.
and future studies are required to determine how LDL-derived cholesterol upregulates ACAT2 expression and how LDL-derived cholesterol may be specifically targeted to ACAT2-mediated esterification.

Involvement of PCSK9 and IDOL in the hepatic processing of LDL-derived cholesterol is still uncertain. PCSK9, synthesized as a 72-kDa zymogen, which autocatalytically cleaves itself into a mature 60-kDa protein, is secreted and binds to the LDL receptors promoting their uptake and lysosomal degradation.23 PCSK9 is regulated by SREBP2, in that low intracellular cholesterol levels stimulate PCSK9 expression and high intracellular cholesterol levels reduce PCSK9 levels (confirmed in Figure 8). The role of PCSK9 is paradoxical, in that under low intracellular cholesterol conditions, HMGCoA reductase, the rate-limiting enzyme of endogenous cholesterol biosynthesis, and LDL receptor expression are also stimulated (to increase intracellular cholesterol levels), whereas PCSK9 downregulates the LDL receptor achieving the opposite. Western blotting showed that the LDL receptor and PCSK9 are inversely correlated, providing evidence that SREBP2-mediated upregulation of PCSK9 is the strongest determinant of LDL receptor expression levels.29 However, this is not the case in LDL- and VLDL-treated hepatocytes, as these cells have high PCSK9 (presumably because of the shunting effect on LDL-derived cholesterol away from the regulatory pool and, therefore, increased SREBP2) and increased LDL receptor. This result suggests there is a complexity in the regulation of the LDL receptor by LDL and VLDL treatment that has not been previously appreciated. This can also be demonstrated by the inverse relationships between PCSK9 and the LDL receptor depending on the lipoprotein treatment: chylomicron, HDL, and non-lipoprotein–derived cholesterol treatment increased PCSK9 expression and decreased LDL receptor expression, whereas β-VLDL and CR decreased PCSK9 expression and increased LDL receptor expression. Thus, it seems that the precise intracellular location of delivery of cholesterol is an important determinant of the homeostatic response.

It also seems that all cholesterol pools are not equivalent and that cholesterol that enters hepatocytes does not automatically enter the regulatory pool (by which we mean the actual mass of cholesterol that regulates the SREBP2 pathway). Interestingly, the immature 72-kDa form of PCSK9 is observed in the LDL-treated cells but not in the CR-treated cells, suggesting a level of regulation of PCSK9 that controls the processing and secretion of apoB lipoprotein particles.27,28 Newly synthesized intracellular PCSK9 might interact with apoB in the lumen of the ER, preventing intracellular VLDL degradation and enhancing VLDL secretion,29 and this may be responsible for the lack of downregulation of the LDL receptor, despite SREBP2-mediated stimulation of PCSK9 expression. In any case, these results imply more complicated regulatory pathways for PCSK9 in hepatocytes than are usually understood to exist.30–32

IDOL is a liver X receptor nuclear receptor–regulated target, which mediates ubiquitination and targeted degradation of the LDL receptor.31 IDOL is stimulated under conditions of high intracellular cholesterol but operates independently of SREBP2 regulation. Therefore, the observation that all cholesterol treatment resulted in increased IDOL expression is not inconsistent with the understanding of regulation of IDOL.34,35 In this case, we do not see any difference between LDL- and CR-treated cells16 and do not predict that IDOL plays a role in the observed experimental results.

These results in hepatocytes contrast with those in fibroblasts, in which uptake of an LDL particle produces acute downregulation of cholesterol and LDL receptor synthesis, observations that constitute core evidence for the LDL receptor paradigm. In the fibroblast, however, hydrolysis of lysosomal CE is more rapid than esterification.8,37 Because ACAT activity is relatively low in the fibroblast compared with the hepatocyte8 and because cholesterol cannot be secreted from the fibroblast, cholesterol released from the lysosome does enter the regulatory pool in the fibroblast, whereas it does not in the hepatocyte.

There are important limitations to our study. Although there are many similarities with regard to cholesterol homeostasis and apoB metabolism, both in vivo and in vitro, we cannot assume that these results in hamster hepatocytes apply without reservation to human hepatocytes. Furthermore, our study of cholesterol homeostasis even in this system is incomplete because we did not estimate the impact on bile acid synthesis and secretion of cholesterol within bile. It also seems that published results in macrophages may be contrary to our results in hepatocytes. In the macrophage, the cholesterol within CR is rapidly reesterified notwithstanding that there seems to be a similar pause of CR particles just after uptake.23,24 This difference may relate to differences in mechanisms of uptake, with syndecan-1 playing a more prominent role in uptake in the hepatocyte membrane compared with the macrophage.39 Alternatively, the intracellular distribution of ACAT may differ in macrophages versus hepatocytes.40,41 The high expression of ACAT2 in hepatocytes is the most likely cause of the physiological differences in handling LDL-derived cholesterol.

In summary, we demonstrate that LDL- and VLDL-derived cholesterol is handled differently than cholesterol derived from other lipoproteins by the hepatocyte. LDL-derived cholesterol upregulates ACAT2 expression, leading to increased cholesterol esterification, enhanced secretion of cholesterol within VLDL particles, and increased efflux of cholesterol to HDL particles. Consequently, entry of cholesterol into the regulatory pool is decreased, and endogenous cholesterol synthesis is not downregulated. These results are consistent with in vivo apoB kinetic data obtained in humans, demonstrating that the LDL receptor and endogenous cholesterol synthesis remain active in the face of large amounts of LDL cholesterol delivered to the liver and that increased apoB secretion is associated with increased delivery of cholesterol to the liver.8,9,20

These findings do not invalidate the importance of the LDL pathway, but they do point to a more multifaceted and complex model of cholesterol homeostasis in the liver than the fibroblast. Furthermore, they indicate that intracellular cholesterol homeostasis is dependent, at least in part, on site-specific metabolic linkages that create micrometabolic domains within the cell. Thus, cholesterol released from a remnant particle at one site within the cell will tend to enter the regulatory compartment more easily than cholesterol released from an LDL particle at another site. In contrast, if cholesterol is released
in close proximity to active ACAT2, the result will be a shunt pathway of cholesterol through the cell without that cholesterol ever entering the regulatory compartment.

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Disclosures

None.

References


The low-density lipoprotein receptor paradigm demonstrates the exquisite homeostasis of intracellular cholesterol regulation. The liver is central to total body cholesterol regulation and, therefore, represents the most important cell type to understand cholesterol homeostasis. Here, we demonstrate that this paradigm is incomplete when describing cholesterol homeostasis in hepatocytes. We also demonstrate that the cholesterol derived from low-density lipoprotein does not enter the normal intracellular regulatory pool but rather is shunted out as part of a very–low-density lipoprotein particle. These findings have enormous clinical relevance not only on the accumulation of cholesterol in hepatocytes as part of fatty liver disease but also on the plasma concentration of low-density lipoprotein as a contributing factor to atherosclerosis.
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Supp. Figure I: Binding and internalization studies.  A) 125I-labeled LDL (100 ng; black bar) and CR (100 ng; white bar) were added to hepatocytes at 4°C to measure cell surface receptor binding.  Results are expressed as cpm/μg cell protein ±SD.  B) 3H-cholesteryl oleate (2μCi), incorporated into 50 μg of LDL (black bar) and CR (white bar), were added for increasing times to hepatocytes at 37°C to measure internalization rates.  Results are expressed as the average cpm/ng cell protein ±SD. **p<0.001 compared to the LDL condition.
Supp. Figure IIA: Uptake and loading studies. $^3$H-cholesteryl oleate (2 μCi) was incorporated into the different lipoproteins: VLDL (closed circles); Chylomicrons (open circles); LDL (closed inverted triangles); CR (open inverted triangles); βVLDL (closed squares); HDL (open squares); and non-lipoprotein derived cholesterol (closed diamond). Lipoproteins were added to hepatocytes for 24 hours at increasing concentrations of lipoprotein with the same amount of radioactivity. After 24 hours, lipids were extracted and cellular radioactivity was determined by scintillation counting. Results are expressed as cpm/μg cell protein as a mean of quadriplicates±SD. We chose the concentration of lipoproteins that would be utilizable for all loading conditions and was at a point of non-saturation (at 80 cpm/μg).
Supp. Figure IIB: Total cellular cholesterol mass increases with lipoprotein loading. Hepatocytes were cholesterol loaded with the different lipoproteins (LDL, human chylomicrons (Chylo), CR, β-VLDL, VLDL, HDL and non-lipoprotein associated free cholesterol), and then the total cholesterol mass was measured using the enzymatic kit assay method. Results are presented as the mean ±SD of quadruplicate measurements. Cholesterol loading increased total cholesterol mass, free cholesterol (grey bar) and cholesteryl ester (black bar), and hepatocytes were almost identically cholesterol loaded with the different lipoproteins. *p<0.05, compared to the LPDS condition.
Supp. Figure IIC: Total cellular cholesterol mass increases with lipoprotein loading. Hepatocytes were cholesterol loaded with the different lipoproteins (LDL, human chylomicrons (Chylo), CR, β-VLDL, VLDL, HDL and non-lipoprotein associated free cholesterol), and then the total cholesterol mass was measured using the GC method. Results are presented as the mean ±SD of quadruplicate measurements. Cholesterol loading increased total cholesterol mass, free cholesterol (grey bar) and cholesteryl ester (black bar), and hepatocytes were almost identically cholesterol loaded with the different lipoproteins. *p<0.05, compared to the LPDS condition.
Supp. Figure III: Hepatocytes were loaded with lipoprotein derived and non-lipoprotein derived cholesterol for 24 hours. mRNA was extracted using the RNeasy mini RNA extraction kit (Qiagen). Analysis of mRNA expression of HMGCoA reductase, LDL receptor and β-tubulin by real-time quantitative PCR from 200 ng total RNA was carried out as described in Methods. Values shown are means ± SD of triplicate experiments. The expression of each gene was normalized to β-tubulin expression, and mRNA fold changes relative to LPDS control were determined. *p < 0.05, **p < 0.01 by Student's t-test.
Supp. Figure IV: ACAT inhibitor drastically reduces CE formation in LDL-treated hepatocytes. To determine the relative contribution of ACAT activity to the accumulation of CE in LDL and CR-treated hepatocytes, the ACAT inhibitor (ACATi) 58035 was added at the same time as lipoprotein. Lipoproteins were labeled with [³H]-cholesterol and treated as before and the radioactive CE was presented as the mean ±SD of quadruplicates of three identical experiments. *p<0.05, compared to the CR (no ACATi) condition. **p<0.001, compared to the LDL (no ACATi) condition. † p<0.001, comparing the LDL (no ACATi) condition to the CR (no ACATi) condition.
Supp. Figure V: Quantification of western blots. Cell lysates of hepatocytes treated with different lipoproteins from three independent experiments were prepared and western blots were performed for ACAT1, ACAT2, LDL receptor, PCSK9 and IDOL. GAPDH was used as a loading control. Bands were scanned with the AlphalImage scanning program, normalized to the LPDS control and average ±SD was computed and graphed for each protein. *p<0.05 compared to the LPDS condition. **p<0.01 compared to the LPDS condition.
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. Na$^{125}$I was from Amersham Biosciences, and $[^3]H$cholesterol, $[^14]C$cholesterol, $[^3]H$mevalonate, $[^14]C$oleate, and $[^3]H$cholesteryl oleate were from PerkinElmer Life Sciences. $[^3]H$cholesteryl oleate and 3, 3-dioctyloxacarbocyanine 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 ($\beta$-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 $^{125}$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). \(^{3}\text{H}\)cholesteryl oleate radiolabeled lipoproteins (25 \(\mu\)g) were added to the cells for 1 h, 4 h, or 8h 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 \(^{3}\text{H}\)-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 \(^{3}\text{H}\)-cholesterol label was used. Importantly, \(^{3}\text{H}\)-cholesteryl oleate and \(^{3}\text{H}\)-cholesterol labeled the cells to an equivalent degree over the concentration curve, demonstrating that both labels are reliable indicators of cholesterol uptake. \(^{3}\text{H}\)-cholesteryl oleate labeling of lipoproteins results in a 95-100% incorporation into internal compartments (non-methyl \(\beta\)-cyclodextrin accessible) while \(^{3}\text{H}\)-cholesterol labeling of lipoproteins 65-70% incorporation into internal compartments (non-methyl \(\beta\)-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 \(\mu\)g/mL; CR 12 \(\mu\)g/mL; \(\beta\)-VLDL 18 \(\mu\)g/mL; Chylomicrons 8 \(\mu\)g/mL; VLDL 25 \(\mu\)g/mL; HDL 100 \(\mu\)g/mL; non-lipoprotein-derived cholesterol 100 \(\mu\)g/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: isoctane (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 isoctane: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; $\beta$-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 RNeasy 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: $\beta$-actin 5$'$-GCACCAAGGTGTGATGGTG-3$'$ and antisense primer 5$'$-CGGTTGGCCTTCAGGGTTC-3$'$; HMGCoA reductase 5$'$-CGAGGAAAGACTGTGTTGTTT-3$'$ and antisense primer 5$'$-CACGTTCCCTGAAGATCTTG-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 $\beta$-actin expression. Fold changes relative to controls were determined using the $\Delta\Delta$Ct 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


