High density lipoprotein metabolism in low density lipoprotein receptor-deficient mice

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Abstract The LDL receptor (LDLR) and scavenger receptor class B type I (SR-BI) play physiological roles in LDL and HDL metabolism in vivo. In this study, we explored LDL metabolism in LDLR-deficient mice in comparison with WT littermates. Murine HDL was radiolabeled in the protein ([125I]) and in the cholesteryl ester (CE) moiety ([3H]). The metabolism of [125I]/[3H]HDL was investigated in plasma and in tissues of mice and in murine hepatocytes. In WT mice, liver and adrenals selectively take up HDL-associated CE ([3H]). In contrast, in LDLR−/− mice, selective HDL CE uptake is significantly reduced in liver and adrenals. In hepatocytes isolated from LDLR−/− mice, selective HDL CE uptake is substantially diminished compared with WT liver cells. Hepatic and adrenal protein expression of lipoprotein receptors SR-BI, cluster of differentiation 36 (CD36), and LDL receptor-related protein 1 (LRP1) was analyzed by immunoblot. The respective protein levels were identical both in hepatic and adrenal membranes prepared from WT or from LDLR−/− mice. In summary, an LDLR deficiency substantially decreases selective HDL CE uptake by liver and adrenals. This decrease is independent from regulation of receptor proteins like SR-BI, CD36, and LRP1. Thus, LDLR expression has a substantial impact on both HDL and LDL metabolism in mice.—Rinninger, F. M. Heine, R. Singaraja, M. Hayden, M. Brundert, R. Ramakrishnan, and J. Heeren. High density lipoprotein metabolism in low density lipoprotein receptor-deficient mice. J. Lipid Res. 2014. 55: 1914–1924.

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Specific plasma membrane receptors play critical roles in lipoprotein metabolism (1). A well-established molecule is the LDL receptor (LDLR) (2). This protein mediates the cellular internalization of cholesterol-rich LDL particles, for instance by the liver (3). Another membrane protein, scavenger receptor class B type I (SR-BI), has a dominant role in the physiology of HDL (4). This receptor facilitates the selective uptake of HDL-associated cholesteryl esters (CEs) by liver and adrenals (i.e., cellular lipid uptake without holo-particle internalization) (5, 6).

The physiological significance of these receptors is illustrated by mutations in the respective genes. In mice, LDLR deficiency is associated with an increase in LDL cholesterol as well as accelerated atherosclerosis (7, 8). With respect to HDL, induced mutations in the murine gene encoding SR-BI induce an increase in plasma HDL cholesterol and accelerated atherosclerosis (9, 10). However, besides an increase in LDL cholesterol, a deficiency of the LDLR can modulate non-LDL lipoproteins in plasma as well. For instance, a rise in plasma HDL cholesterol has been reported in LDLR-deficient mice in some studies but not in others (7, 11, 12). These observations suggest that an LDLR deficiency can affect the metabolism of more than one lipoprotein fraction, at least to some extent. However, with respect to the mechanism, it is not known how an LDLR deficiency modifies HDL cholesterol.

Abbreviations: CD36, cluster of differentiation 36; CE, cholesteryl ester; CET, cholesteryl oleyl ether; CETP, cholesteryl ester transfer protein; FPLC, fast performance LC; LDL, LDL receptor; LRP1, LDL receptor-related protein 1; organ-FCR, organ fractional catabolic rate; plasma-FCR, plasma fractional catabolic rate; SR-BI, scavenger receptor class B type I; TC, tyramine cellobiose.

1 This study is dedicated to Professor Heiner Greten, MD.
2 To whom correspondence should be addressed.
3 The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of five figures and three tables.

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In the current study, HDL metabolism was explored in LDLR-deficient mice and compared with WT animals that express functional LDLR (7). To explore the fate of distinct lipoprotein components, HDL particles were radiolabeled in both the protein as well as in the lipid moiety (13). In metabolic studies using double radiolabeled HDL, liver and adrenals of WT mice selectively take up HDL-associated CE. In LDLR\(^{-/-}\) mice, however, selective HDL CE uptake is significantly reduced in liver and adrenals. In parallel, uptake of radiolabeled HDL by isolated primary hepatocytes in vitro was explored. Compared with WT and in line with the in vivo studies, an LDLR deficiency is associated with a decrease in selective HDL CE uptake. Notably, SR-BI expression in liver and adrenal membranes was identical in mice with and without LDLR even though HDL selective CE uptake was reduced. In summary, we demonstrate that LDLR expression has a substantial impact on HDL metabolism in vivo and in vitro. Remarkably, regulation of selective HDL CE uptake occurs independent from SR-BI protein expression in tissues.

MATERIALS AND METHODS

Materials

Primers were purchased from Metabion. Taq-DNA polymerase, culture media, sera, and supplements for cell culture were supplied by Invitrogen. Six-well tissue culture plates were obtained from Ssniff. Culture media, sera, and supplements for cell culture were supplied from plasma of LDLR\(^{-/-}\) mice. Male LDLR\(^{-/-}\) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) (7). Mice were fasted 4 h before blood harvest. Murine HDL (d = 1.063–1.21 g/ml) was isolated from WT (WT-HDL) plasma or LDLR-deficient (LDLR\(^{-/-}\) HDL) plasma by sequential ultracentrifugation (14). LDL (d = 1.020–1.050 g/ml) was prepared from plasma of LDLR\(^{-/-}\) mice.

Lipoprotein preparation

Mice lacking a functional LDLR gene (LDLR\(^{-/-}\)) were purchased from The Jackson Laboratory (Bar Harbor, ME) (7). Male LDLR\(^{-/-}\) mice on a C57BL/6J genetic background and the respective male littermate controls (WT) were used. The genotype of each mouse was analyzed by PCR from genomic DNA isolated from tail biopsies (7). Mice were maintained on a standard laboratory diet and water ad libitum. Animals were fasted throughout the 24 h study period but had unlimited access to water. Plasma aliquots were directly assayed for \(^{125}\text{I}\) radioactivity, and \(^{3}\text{H}\) was analyzed after lipid extraction (16). Computer modeling was used to fit (by method of least squares) multieponential curves, arising from a common two-pool model, simultaneously to both tracers’ plasma decay data, and to calculate plasma fractional catabolic rates (plasma-FCRs) for each tracer (17). The modeling was done separately for the data from each mouse, so that individual plasma-FCRs for both tracers were calculated for each animal. In some cases, HDL metabolism in mice was explored for a 2 h interval only.

Tissue sites of uptake of HDL-associated tracers were determined 2 h or 24 h after injection of radiolabeled WT-HDL or LDLR\(^{-/-}\) HDL (5). Finally, animals were anesthetized, the abdomen and chest were opened, and a catheter was inserted into the heart. The inferior vena cava was cut, and the mice were perfused extensively with saline (50 ml per animal). After perfusion, liver, adrenals, kidneys, brain, heart, lungs, spleen, stomach, intestine, and carcass from each mouse were harvested and homogenized. Homogenates from each tissue and from carcass were directly assayed for \(^{125}\text{I}\) radioactivity, and aliquots were analyzed for \(^{3}\text{H}\) after lipid extraction (16). Total radioactivity recovered from all tissues and from the carcass of each mouse was calculated (5). The fraction of total tracer uptake attributed to a specific organ was calculated as the radioactivity recovered in that organ divided by the total radioactivity recovered from all tissues and carcass. Thus the % of recovered extravascular radioactivity in tissues is determined 2 h or 24 h after injection of labeled HDL.

To allow comparison of the specific activities of various tissues in HDL internalization and to directly compare the rates of uptake of the apo component and the CE moiety of HDL, the data are expressed as organ fractional catabolic rates (organ-FCRs) (5). These rates are calculated as follows: (Organ-FCR in Tissue \(X\) = (Plasma-FCR) × (Fraction [%] of Total Body Tracer Recovery in Tissue \(X\)). This organ-FCR represents the fraction of the plasma pool of either HDL tracer cleared by an organ per hour. \(^{125}\text{I}\)-TC represents the uptake of HDL holo-particles by tissues (18). Selective HDL CE uptake is calculated as the difference in organ-FCR between \(^{3}\text{H}\)CEt and \(^{125}\text{I}\)-TC.

HDL metabolism in mice

\(^{125}\text{I}\)-TC-LDL metabolism in plasma and tissue sites of uptake of this lipoprotein were investigated in mice analogously as outlined for radiolabeled HDL.

Preparation of murine hepatocytes

Primary hepatocytes were isolated from murine liver by perfusion (37°C, 18 min) with Hanks’ balanced salt solution.
supplemented with collagenase (0.3 mg/ml, type 1), HEPES (10 mM), and protease inhibitor mixture "complete" (19). Thereafter, these cells were seeded (37°C, 2.0 h) in DMEM containing FBS (5%, v/v), penicillin (100 µg/ml), and streptomycin (100 µg/ml). Finally, the culture medium was aspirated, and the cells were washed (PBS, 3x). Hepatocytes were used for 125I-TC-/[3H]CEt-WT-HDL uptake or 125I-TC-WT-HDL binding assays.

Uptake and binding assay for radiolabeled HDL
To determine uptake of radiolabeled HDL, hepatocytes were incubated (37°C, 2.0 h) in DMEM containing BSA (5 mg/ml), penicillin (100 µg/ml), streptomycin (100 µg/ml), and 125I-TC-/[3H]CEt-WT-HDL (20). Finally, cells were harvested by trypsin/EDTA (1x, trypsin 0.05%, EDTA 0.53 mM) treatment, and cellular uptake of HDL tracers was measured. 125I was directly radioassayed, and [3H] was analyzed after lipid extraction (16). Uptake of 125I-TC-/[3H]CEt-WT-HDL by cells is shown in terms of apparent HDL particle uptake, expressed as HDL protein (5, 20). This is done to compare the uptake of both tracers on a common basis. The uptake of HDL holo-particles is represented by 125I-TC, and the difference between [3H]CEt and 125I-TC yields apparent selective HDL CE uptake by cells (18).

To investigate binding of radiolabeled HDL, hepatocytes were incubated (4°C, 2.0 h) in medium containing 125I-TC-WT-HDL (21). Thereafter, the medium was removed, and the cells were washed (PBS, 4°C). 125I-TC-WT-HDL binding to the cells was finally analyzed as outlined (21).

Immunoblots
Membrane fractions from murine liver or adrenals were prepared (22). Protease inhibitor cocktail "complete" was present during the entire preparation. Membrane fractions were boiled (93°C, 10 min) and separated by SDS polyacrylamide gel (10%) electrophoresis under reducing conditions (mercaptoethanol) and thereafter transferred to a nitrocellulose membrane (6). Finally, the blots were probed with anti-SR-BI (rabbit polyclonal antibody to murine SR-BI, Novus Biologicals), anti-LDL receptor-related protein 1 (LRP1) (sheep anti-LRP1 antibody, raised against a synthetic peptide from human LRP1) (23), anti-cluster of differentiation 36 (CD36) (rabbit polyclonal antiserum against murine CD36) (24), anti-ABCA1 (rabbit polyclonal antibody, Novus Biologicals), or anti-β-actin (monoclonal anti-β-actin, mouse, Sigma Aldrich) as primary antibodies. β-Actin was used as loading control. Finally, the blots were incubated with respective horseradish peroxidase-conjugated secondary antibodies (Sigma Aldrich) and developed with ECL. Blots were exposed to Bio Max MR films and quantified using Image Quant software, version 5.2 (GE Healthcare).

Miscellaneous
Routinely, all mice were fasted for 4 h before blood harvest for analytical or preparative purposes. Total cholesterol, HDL cholesterol, and triglycerides from plasma were measured using enzymatic assays. Plasma lipoproteins were fractionated by fast performance LC (FPLC) (25). In order to measure VLDL production, the nonionic detergent Tyloxapol (Triton WR 1339) was used to inhibit VLDL catabolism (26). Protein was analyzed as outlined (27).

Statistics
Values are means ± SEM. All statistical analyses were performed using Student’s t test. Probability values <0.05 were considered statistically significant.

RESULTS
As expected, total plasma cholesterol was significantly higher in male LDLR-/- mice (284%) compared with their WT littermates (100%) (Table 1). HDL cholesterol was elevated (111%) in LDLR-deficient male mice compared with WT animals (100%); however, this difference was not statistically significant. Triglycerides were significantly higher (235%) in LDLR-/- mice compared with WT (100%). Plasma lipids were also analyzed in female mice (Table 1). In female LDLR-/- animals, total cholesterol and triglycerides increased to a similar extent compared with male LDLR-/- mice, whereas no increase in HDL cholesterol was detected.

To determine the distribution of cholesterol with respect to lipoprotein fractions, murine plasma was fractionated by FPLC (Fig. 1) (25). The major change due to the LDLR deficiency was an increase in cholesterol corresponding to particles of the LDL/IDL fractions. However, in LDLR-/- mice, a small increase in plasma cholesterol in the size range of HDL could be detected. These results on plasma lipids and on HDL cholesterol in LDLR-/- mice are in line with previous studies (7, 12). The compositional analysis of HDL isolated by sequential ultracentrifugation from murine plasma showed that LDLR-/- HDL is significantly enriched in triglycerides and depleted in phospholipids compared with WT-HDL (Table 2).

The metabolism of 125I-TC-/[3H]CEt-WT-HDL was investigated in WT and in LDLR-/- mice (Fig. 2). This murine HDL preparation was injected intravenously in mice, and thereafter, blood samples were harvested during a 24 h interval (5). In these studies, [3H]CEt represents the metabolism of HDL-associated CE, and 125I-TC shows HDL holo-particle clearance (18). The difference between both tracers ([3H]CEt - 125I-TC) represents selective CE removal. In WT mice, the plasma decay of HDL-associated [3H]CEt is faster compared with 125I-TC; the difference in decay between both tracers yields selective CE removal from the HDL plasma pool by tissues in WT animals. In parallel, 125I-TC-/[3H]CEt-WT-HDL was injected in LDLR-/- mice. In this case, no difference in plasma decay between both HDL tracers can be detected. These data suggest that selective CE removal from the HDL plasma pool does not occur in LDLR-/- mice.

From decay curves shown in Fig. 2, plasma-FCRs for both HDL-associated tracers were calculated (Fig. 3) (17). In WT animals, the plasma-FCR for WT-HDL-associated [3H]CEt is substantially higher compared with 125I-TC; the difference between both rates ([3H]CEt - 125I-TC) yields selective CE removal from the plasma HDL pool by tissues. In LDLR-/- mice, no significant difference in plasma-FCR for 125I-TC is detected compared with WT. In contrast, in LDLR-/- mice, a substantial reduction in plasma-FCR for [3H]CEt is observed. Calculation of the difference between [3H]CEt and 125I-TC yields no difference, again showing that there is no selective CE removal from plasma WT-HDL by tissues in LDLR-/- mice.
TABLE 1. Plasma cholesterol, HDL cholesterol, and triglycerides of WT or LDLR−/− mice

|        | Total Cholesterol | HDL Cholesterol | Triglycerides |
|--------|-------------------|-----------------|--------------|
| Males  |                   |                 |              |
| WT     | 93.3 ± 3.8 (30)   | 83.8 ± 3.6 (27) | 105.8 ± 7.0 (20) |
| LDLR−/−| 264.8 ± 16.9 (31)| 92.7 ± 4.7 (28) | 249.1 ± 26.1 (23) |
| P      | <0.0001           |                 | <0.0001      |
| Females|                   |                 |              |
| WT     | 89.9 ± 4.4 (30)   | 78.2 ± 3.0 (30) | 122.5 ± 8.2 (24) |
| LDLR−/−| 229.7 ± 8.8 (22)  | 75.1 ± 3.3 (22)| 161.2 ± 8.5 (17) |
| P      | <0.0001           |                 | 0.251        |

Male or female WT or LDLR−/− mice were fasting for 4 h. Thereafter, blood was harvested and plasma was analyzed as outlined in Materials and Methods. Values are means ± SEM; the number of mice is given in parentheses.

Organ-specific HDL catabolism was determined. Twenty-four hours after 125I-TC/[3H]CEt-WT-HDL injection, tracer content of each tissue was analyzed, and HDL uptake was calculated and expressed in terms of organ-FCRs (5). HDL tracer uptake by the liver is shown in Fig. 3. In WT mice, the hepatic organ-FCR for [3H]CEt is higher compared with 125I-TC; the difference between both rates ([3H]CEt - 125I-TC) yields selective CE uptake by the liver from WT-HDL. This result is in line with earlier studies in WT mice in which similar levels of selective CE uptake from WT-HDL were observed (6, 31). In LDLR−/− mice, the hepatic uptake rate for 125I-TC was similar to the respective rate in WT animals (Fig. 3). However, in LDLR−/− mice, the hepatic organ-FCR for [3H]CEt decreased significantly compared with WT, indicating that although holo-particle uptake was similar in both LDLR−/− and WT murine livers, selective CE uptake from WT-HDL ([3H]CEt - 125I-TC) was decreased significantly in livers of LDLR−/− mice.

Uptake of 125I-TC/[3H]CEt-WT-HDL by adrenal glands was explored (Fig. 5). In WT adrenals, the organ-FCR for [3H]CEt is substantially higher compared with 125I-TC, and the difference between both rates yields substantial selective CE uptake from HDL ([3H]CEt - 125I-TC) in glands with LDLR protein expression (5, 6). In adrenals from LDLR−/− mice, the organ-FCR for 125I-TC was not significantly different compared with WT, indicating normal holo-particle uptake. Similar to the results for liver, adrenal organ-FCR for [3H]CEt decreased significantly in LDLR−/− glands compared with WT. This decrease in HDL lipid internalization by LDLR−/− glands yielded a significant reduction in selective CE uptake ([3H]CEt - 125I-TC). Uptake of 125I-TC/[3H]CEt-WT-HDL by murine kidneys was investigated (Fig. 3). The kidney organ-FCR for HDL-associated 125I-TC is higher compared with the respective rate for [3H]CEt, and this result is consistent with a physiological role of the kidneys in HDL apo catabolism (28). Kidney organ-FCRs for both HDL-associated tracers did not differ between WT and LDLR−/− mice.

The composition of WT-HDL and LDLR−/− HDL is different (Table 2). Therefore, the metabolism of 125I-TC/[3H]CEt-LDLR−/− HDL was explored by the identical approach as with labeled WT-HDL (Fig. 4; supplementary Fig. 1). In WT mice, the plasma-FCR for LDLR−/− HDL-associated [3H]CEt is substantially higher compared with 125I-TC; the difference between both tracers ([3H]CEt - 125I-TC) yields selective CE removal from the plasma HDL pool by tissues (17). Comparing WT and LDLR−/− mice, no significant difference in plasma-FCR for 125I-TC is detected. In LDLR−/− mice, however, a significant reduction in plasma-FCR for [3H]CEt is observed. The difference between [3H]CEt and 125I-TC yields a significant reduction in selective CE removal from the plasma LDLR−/− HDL pool by tissues in LDLR−/− mice.

To explore the metabolism of LDLR−/− HDL by tissues, tracer content was analyzed 24 h after injection of 125I-TC/[3H]CEt-LDLR−/− HDL (5). In WT mice, the hepatic organ-FCR for [3H]CEt is higher than the one due to 125I-TC; the difference between both rates ([3H]CEt - 125I-TC) yields selective CE uptake by the liver from LDLR−/− HDL (Fig. 4). In LDLR−/− mice, the hepatic uptake rate for 125I-TC was similar to the respective rate in WT animals. However, in these LDLR-deficient mice, the hepatic organ-FCR for [3H]CEt decreased significantly compared with WT. Although holo-particle uptake was similar in livers of both genotypes, selective CE uptake from LDLR−/− HDL ([3H]CEt - 125I-TC) decreased significantly in the livers of LDLR−/− mice.

HDL-associated apos can exchange in plasma between lipoprotein fractions (1). In some species, CE can be transferred from HDL to more buoyant lipoproteins, and this reaction is mediated by CETP (15). However, mice have no CETP activity in the circulation, and therefore, no
and LDL is reduced in LDLR−/− mice. Consequently, the LDLR-dependent uptake of VLDL lipoproteins (supplementary Table II). We speculated that this mechanism exists that mediates a transfer of HDL-associated lipids to more buoyant lipoproteins (36). To address the question of whether initially HDL-associated lipids can be transferred into non-HDL lipoproteins in plasma during a 24 h period, [125I]-TC/[^3H]CET-LDLR−/− HDL was injected in WT and in LDLR−/− mice (supplementary Table I). After 40 min, 8 h, and 24 h, blood was harvested and lipoproteins were separated using FPLC. Tracer in fractions corresponding to triglyceride-rich lipoproteins, LDL, and HDL were analyzed. In WT mice, during the 24 h time course both LDLR−/− HDL-associated tracers remained predominantly in the HDL fraction. In LDLR−/− mice, however, [3H]CET tracer initially associated with LDLR−/− HDL was detected after 8 h and after 24 h to a substantial extent in fractions with a lower density than HDL. Qualitatively identical results were obtained with [125I]-TC/[^3H]CET-WT-HDL in both groups of mice (data not shown). Because there is no CETP in murine plasma, we investigated the mechanism of this [3H]CET recovery in non-HDL lipoproteins (supplementary Table II). We speculated that HDL-derived radiolabeled lipoproteins are partially reused for VLDL assembly and secretion.

Table 2. Chemical composition of murine HDL

|                | % of Total Mass | % of Total Mass | P     |
|----------------|-----------------|-----------------|-------|
| Cholesterol    | 21.1 ± 0.3      | 20.4 ± 0.4      | 0.25  |
| Phospholipids   | 28.0 ± 0.6      | 24.2 ± 0.5      | 0.0059|
| Triglycerides   | 3.4 ± 0.07      | 7.9 ± 0.1       | <0.0001|
| Protein        | 47.5 ± 0.9      | 47.6 ± 1.0      | 0.98  |

Blood was harvested in parallel from fasted (4 h) WT or LDLR−/− male mice. Subsequently, from plasma, HDL (d = 1.063–1.21 g/ml) was isolated by sequential ultracentrifugation. Thereafter, cholesterol, phospholipids, triglycerides, and protein were analyzed. Values are means ± SEM of n = 5 independent determinations; four independent preparations yielded qualitatively identical results.
HDL metabolism in LDL receptor-deficient mice

A less complex system compared with an animal, hepatocytes were isolated from WT and LDLR−/− mice. Twenty-four hours after tracer injection, the animals were euthanized, and tissues were analyzed for both tracers. Liver (B), adrenal (C), and kidney (D) organ-FCRs for 125I-TC (125I) and [3H]CEt ([3H]) and the difference in organ-FCRs between [3H]CEt and 125I-TC were calculated. All calculations were done as described in Materials and Methods. A: Values are means ± SEM of n = 7 (WT) or n = 5 (LDLR−/−) mice. An independent experiment yielded qualitatively identical results.

compared with WT. In LDLR−/− mice, a significant reduction in initial plasma-FCR for [3H]CEt and a significant decrease in selective CE removal ([3H]CEt - 125I-TC) from plasma LDLR−/− HDL by tissues are detected during the 2 h period. In WT mice, 2 h after 125I-TC/[3H]CEt-LDLR−/− HDL injection we observed selective CE uptake ([3H]CEt - 125I-TC) by the liver. In LDLR-deficient mice, however, selective CE uptake from LDLR−/− HDL ([3H]CEt - 125I-TC) is decreased significantly. In summary, plasma decay and liver uptake of HDL-associated tracers during a 2 h period are qualitatively consistent with the results for the 24 h experiments.

The liver is composed of distinct cell types; however, hepatocytes quantitatively dominate in this organ (29). To investigate the role of the LDLR in cellular HDL uptake in a less complex system compared with an animal, hepatocytes were isolated from WT and LDLR−/− mice. Following a 4 h seeding period, liver cells were incubated with medium containing 125I-TC/[3H]CEt-WT-HDL. Uptake of 125I-TC/[3H]CEt-WT-HDL by WT hepatocytes is shown in Fig. 5. The uptake of both HDL-associated tracers increased in a dose-dependent manner, but internalization of [3H]CEt was higher compared with 125I-TC; the difference in uptake between both tracers yields selective CE uptake from HDL by WT hepatocytes. The uptake of both HDL tracers into hepatocytes isolated from LDLR−/− mice was significantly lower compared with WT cells. Accordingly, WT-HDL holo-particle uptake (125I-TC) and selective CE uptake ([3H]CEt - 125I-TC) were decreased in the LDLR-deficient hepatocytes. In contrast to uptake, no
significant differences in $^{125}$I-TC-WT-HDL binding to hepatocytes isolated from WT and LDLR$^{-/-}$ mice were detected (supplementary Fig. III), indicating that double labeled HDL does not interact directly with LDLR.

To validate our experimental model, we investigated the metabolism of $^{125}$I-TC-LDL in WT and in LDLR$^{-/-}$ mice in a manner analogous as outlined for $^{125}$I-TC/$[^{3}H]$CEt-HDL (supplementary Fig. IV). Following intravenous injection of murine $^{125}$I-TC-LDL, periodic blood samples were harvested during a 24 h period. The plasma decay of $^{125}$I-TC, representing LDL clearance, was substantially attenuated in LDLR-deficient mice compared with WT animals. As expected, the plasma-FCR for $^{125}$I-TC-LDL decreased significantly in LDLR-deficient mice compared with WT (supplementary Table III). The organ-FCRs for $^{125}$I-TC-LDL uptake by liver and adrenals were significantly reduced in LDLR$^{-/-}$ mice compared with the respective tissues with LDLR expression (WT). In summary, these results demonstrate the appropriateness of the experimental model used in the current study (7).

HDL selective CE uptake by the liver and adrenals in vivo and by hepatocytes in vitro is reduced in LDLR$^{-/-}$ mice compared with WT animals. Because SR-BI mediates the selective CE uptake from HDL, we next addressed whether a downregulation of this receptor is responsible for the decrease in HDL CE uptake detected in tissues of LDLR$^{-/-}$ rodents (4). Murine liver membranes were immunoblotted using antibodies specific for SR-BI or LDLR (Fig. 6). The signal for SR-BI protein was identical in membranes prepared from WT or LDLR$^{-/-}$ liver, suggesting

**Fig. 4.** Plasma-FCRs and liver tracer uptake rates for $^{125}$I-TC/$[^{3}H]$CEt-LDLR$^{-/-}$ HDL in WT mice or in LDLR$^{-/-}$ mice. $^{125}$I-TC/$[^{3}H]$CEt-LDLR$^{-/-}$ HDL was injected intravenously in WT or in LDLR$^{-/-}$ male mice. During the subsequent 24 h interval, blood was drawn periodically, and 24 h after tracer injection, the animals were euthanized and tissues were harvested. Plasma-FCRs and liver organ-FCRs for $^{125}$I-TC ($^{125}$I), for $[^{3}H]$CEt ($[^{3}H]$), and for selective uptake ($[^{3}H]$CEt - $^{125}$I-TC) were analyzed and calculated as outlined in Fig. 3. All values are means ± SEM of n = 5 (WT) or n = 5 (LDLR$^{-/-}$) mice. Where no error bars are shown, the SEM is on the respective line.

**Fig. 5.** Uptake of $^{125}$I-TC/$[^{3}H]$CEt-WT-HDL by hepatocytes isolated from WT or LDLR$^{-/-}$ mice. Hepatocytes were isolated from a WT or an LDLR$^{-/-}$ male mouse. These cells were incubated (37°C, 2.0 h) in medium containing $^{125}$I-TC/$[^{3}H]$CEt-WT-HDL, and the respective concentrations are given in the abscissae. Finally, cells were harvested, and apparent HDL particle uptake was analyzed as outlined in Materials and Methods. Values are means of n = 3 (WT) or n = 2 (LDLR$^{-/-}$) independent experiments; within each experiment, incubations were done in triplicates. Comparing all data from WT and LDLR$^{-/-}$ hepatocytes, P < 0.05; two (WT) and two (LDLR$^{-/-}$) independent similar experiments yielded qualitatively identical results.
that altered SR-BI protein expression did not contribute to the reduction in selective uptake of HDL CE in the LDLR$^{-/-}$ mice. As expected, no signal for the LDLR was detected in membranes isolated from LDLR$^{-/-}$ liver confirming the correct genotype. Besides SR-BI, scavenger receptor CD36 and LRP1 have been implicated in selective HDL CE uptake in rodent liver and in hepatocytes, respectively (30–32). To determine whether these receptors played a role in the diminished selective CE uptake of LDLR$^{-/-}$ mice, expression levels of CD36 and LRP1 were determined by immunoblots (Fig. 6). In liver membranes from WT or LDLR$^{-/-}$ mice, the signals for CD36 and LRP1 proteins were nearly identical. Thus, these data suggest that altered protein expression of neither SR-BI, CD36, nor LRP1 accounts for the reduced HDL CE uptake observed in the livers of LDLR$^{-/-}$ mice. Similarly, no obvious differences in SR-BI, CD36, or LRP1 signals were detected in adrenal membrane preparations isolated from WT or from LDLR$^{-/-}$ mice (supplementary Fig. V).

ABCA1 is a membrane protein that controls the rate-limiting step in HDL particle assembly by mediating the efflux of cholesterol and phospholipid from cells to lipid-free apoA-I, which forms nascent HDL particles (33). ABCA1 expressed by the liver has a substantial quantitative effect on HDL biogenesis and on HDL levels in plasma (34). In the studies presented previously, HDL selective CE uptake by tissues is reduced without concomitant increase in plasma HDL cholesterol, suggesting diminished HDL biogenesis. To test the hypothesis that a reduced hepatic expression of ABCA1 is responsible for a diminished HDL formation, the expression of ABCA1 protein was explored in liver membranes from WT or LDLR$^{-/-}$ mice (Fig. 7). The signal for ABCA1 was identical in membranes isolated from WT and LDLR$^{-/-}$ mice. This result argues against an altered HDL biogenesis in LDLR-deficient mice compared with WT, and this is true at least for the liver.

**DISCUSSION**

A major consequence of a deficiency of functional LDLR in mice is an increase in plasma LDL cholesterol (7). Distinct from this change, a small or a more substantial increase in HDL cholesterol in LDLR$^{-/-}$ mice has been reported (7, 11, 12). We found a minor increase in plasma HDL cholesterol in male LDLR$^{-/-}$ mice compared with WT littermates, although this difference was not statistically significant. The explanation for these differences in HDL levels is not clear, and different genetic backgrounds of the mice or the feeding conditions. In contrast to HDL levels, the composition of LDLR$^{-/-}$ HDL is significantly different compared with WT-HDL (i.e., HDL from LDLR-deficient mice is enriched in triglyceride and depleted in phospholipid).

Mechanisms underlying the increased plasma HDL and LDL cholesterol in LDLR$^{-/-}$ mice were explored in this study in vivo. As expected, the decay of$^{125}$I-TC-LDL in the circulation was decreased in LDLR$^{-/-}$ mice compared with WT animals, and this result is in line with an earlier investigation (7). The liver is a major organ for LDL catabolism in vivo, and adrenals internalize LDL for steroidogenesis (3, 35). As predicted, the LDLR deficiency yielded a decreased rate of$^{125}$I-TC-LDL uptake by liver and adrenal glands in this study.
isolated LDLR-deficient hepatocytes, a decrease in uptake of HDL-associated $^{125}$I-TC was shown, suggesting reduced HDL holo-particle internalization. In parallel, $^{125}$I-TC-HDL binding (4°C) to WT liver cells or to LDLR$^{-/-}$ hepatocytes was identical. This observation suggests that the reduced HDL holo-particle uptake of LDLR$^{-/-}$ hepatocytes is not due to reduced HDL binding to the cell membrane. Thus, the interaction between HDL and the plasma membrane is not necessarily followed by hepatocellular HDL uptake. Compared with these results obtained in vitro, the HDL experiments performed in vivo are presumably physiologically more relevant. In summary, an LDLR deficiency has no substantial effect on HDL holo-particle metabolism in plasma and by tissues.

These observations on HDL holo-particle metabolism are in strong contrast to the selective CE pathway. A lack of LDLR is associated with a significant decrease of selective CE uptake from HDL by liver and adrenals. This was true for WT-HDL and for LDLR$^{-/-}$ HDL, as well as for the 2 h and the 24 h metabolic studies. Quantitatively, the decrease in selective CE uptake by liver and adrenals in LDLR$^{-/-}$ mice was smaller in the case of radiolabeled LDLR$^{-/-}$ HDL compared with WT-HDL. The explanation for this difference may be the different composition of both HDL preparations; a similar result has been obtained previously (6). Consistent with these observations on HDL metabolism in vivo, in cultured murine hepatocytes with an LDLR deficiency, a significant decrease in selective HDL CE uptake is observed compared with WT liver cells. This regulation of the HDL CE selective uptake pathway in the absence of LDLR has not been established previously, and our data represent a novel finding.

SR-BI can bind and mediate selective CE uptake from both LDL and HDL (4). LDLR$^{-/-}$ mice have a substantial increase in cholesterol-rich lipoprotein particles in plasma. Therefore, the question has to be raised as to whether a competition between LDL and HDL for SR-BI-mediated selective lipid uptake in vivo explains the decreased selective CE uptake in LDLR-deficient mice. However, the experiments with murine hepatocytes in vitro are a strong argument against this possibility.

With respect to intravascular lipoprotein metabolism, it is established that some apoproteins, for instance HDL-associated apoA-I, are mobile and exchange between lipoprotein fractions (1). Concerning CE, in murine plasma no CETP activity is detectable; therefore, a lipid exchange reaction is unlikely in the circulation (36). To address the issue of an exchange of HDL-associated tracers with non-HDL lipoprotein, FPLC analysis of plasma lipoproteins after HDL tracer injection was done. In WT and LDLR$^{-/-}$ mice, no transfer of $^{125}$I-TC tracer (i.e., no apo transfer) out of the HDL fraction could be detected. In contrast, during the time course of 24 h after injection of radiolabeled LDLR$^{-/-}$ HDL, initially HDL-associated $[^{3}$H]Et tracer could be detected in FPLC fractions corresponding to non-HDL lipoproteins in LDLR$^{-/-}$ mice. Considering the CETP deficiency of mice (36), the hypothesis emerged of a resecretion of initially HDL-associated $[^{3}$H]Et by the liver, for instance in VLDL particles. In fact, an increased
secretion of apoB-containing lipoproteins by LDLR<sup>−/−</sup> hepatocytes has been reported (37). Experiments in which the clearance of VLDL was inhibited suggest that there is indeed substantial resecretion of [³H]CEt tracer by the liver, and this is pronounced in LDLR<sup>−/−</sup> mice.

The question had to be raised as to whether the recovery of the initially HDL-associated [³H]CEt tracer in non-HDL fractions in plasma yields the decreased selective CE uptake, for instance by the liver. Remarkably, during the initial period of the 24 h plasma decay experiments, no HDL lipid label is detected in a non-HDL lipoprotein fraction. To address this issue in more detail, short-term experiments over a period of 2 h demonstrated reduced selective CE removal from the HDL plasma pool and decreased selective CE uptake by the liver in LDLR<sup>−/−</sup> mice, a result that is consistent with the 24 h studies. Besides, in the less complex system of cultured murine hepatocytes, qualitatively identical results were obtained as in mice. Based on the concurrence of the in vivo and in vitro observations, it is unlikely that the decrease in the selective uptake of HDL CE that we observed in vivo is significantly modified by the resecretion of HDL tracer in non-HDL particles.

SR-BI, CD36, and LRP1 mediate the selective uptake of HDL CE by the liver (4, 6, 30–32). Considering the decrease in selective CE uptake from HDL in LDLR<sup>−/−</sup> mice, the hypothesis emerged that a downregulation of these receptors may be responsible for the decrease in selective CE uptake under conditions of an LDLR deficiency. However, the protein expression of SR-BI, CD36, and LRP1 was not significantly different in our study with LDLR<sup>−/−</sup> mice compared with WT littermates. Consistent with our results, a previous study found no difference in LRP1 expression between LDLR<sup>−/−</sup> and WT mice (7). Thus, even though downregulation of the selective HDL CE uptake pathway is observed in tissues of LDLR<sup>−/−</sup> mice, this is independent from established receptors that play a role in cellular HDL uptake. In summary, there is no evidence that regulation of SR-BI, CD36, or LRP1 is responsible for the decrease in selective CE uptake in liver or adrenals in mutant mice.

What is an explanation for the discrepancy between a downregulation of selective HDL CE uptake by tissues and a lack of regulation of receptors like SR-BI? A substantial increase in liver and adrenal cholesterol in LDLR<sup>−/−</sup> mice compared with WT animals is established (11, 12). In familial hypercholesterolemia, cholesterol synthesis is enhanced (38). Thus, an increase in tissue cholesterol is detected in LDLR-deficient organs. Morrison and coworkers (39) suggested that lipid-lipid interactions between a lipoprotein particle and a membrane play a role in the selective transfer of CE from the HDL particle to a cell. Cholesterol is an important component of membranes, and this compound may modulate membrane function substantially (40). Considering the lack of altered receptor expression in LDLR-deficient tissues, it is speculated that the mechanism of the decrease in selective HDL CE uptake may be due to an increase in plasma membrane lipid content of the respective cells.

In this study, no significant change in HDL cholesterol in LDLR<sup>−/−</sup> mice was detected when compared with WT rodents. Generally, decreased HDL catabolism as observed here results in an increase in plasma HDL cholesterol (6). How can this discrepancy in our findings be explained? The steady-state concentration of plasma HDL cholesterol is the result of HDL biogenesis and HDL catabolism (1). A quantitatively dominant organ in HDL catabolism in rodents is the liver, and HDL lipid uptake by this organ is decreased in LDLR<sup>−/−</sup> mice (5). A possible explanation for the discrepancy between the essentially unchanged HDL cholesterol in plasma and the decreased selective HDL CE uptake by tissues is that HDL synthesis is reduced in LDLR<sup>−/−</sup> mice. Hepatic ABCA1 is a key regulator of plasma HDL cholesterol (34). Therefore, the hypothesis was tested as to whether a reduced hepatic expression of ABCA1 protein mediates a decrease in cellular lipid efflux in LDLR<sup>−/−</sup> mice. However, ABCA1 protein expression was unchanged in LDLR-deficient liver membranes compared with those from WT mice. This finding suggests that a difference in cholesterol efflux and a modified HDL biogenesis between both groups of animals is unlikely.

LDLR<sup>−/−</sup> mice are a frequently used model for studies on atherosclerosis (8, 41). Usually, this increased atherosclerotic burden is attributed to the increase in plasma cholesterol contained in apoB-containing lipoproteins. However, our studies point to an additional mechanism that may be relevant for the observed susceptibility for atherosclerosis. Substantial changes in HDL metabolism are detected in the presence of an LDLR deficiency, and HDL plays a key role in reverse cholesterol transport, i.e., the flux of lipid from peripheral tissues to the liver for excretion via bile (42). Therefore, it is suggested that the LDLR modulates both LDL-mediated cholesterol delivery to cells as well as HDL-mediated reverse cholesterol transport to the liver, and both pathways may be relevant for the pathogenesis of atherosclerosis at least in mice.

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