

Failure of the Intracellular Itinerary of β Very Low Density Lipoproteins to Augment Cholesterol Esterification in Macrophages from Watanabe Heritable Hyperlipidemic Rabbits*

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β very low density lipoproteins (β -VLDL) interact with mouse peritoneal macrophages via specific receptors leading to pronounced stimulation of cholesterol esterification. The present study has defined an alternative pathway for the processing of β -VLDL in alveolar macrophages from Watanabe heritable hyperlipidemic (WHHL) rabbits. Macrophages from either New Zealand (NZ) or WHHL rabbits degraded ^{125}I - β -VLDL to an equivalent extent. Degradation was competed to a similar extent in both cell types by either excess unlabeled β -VLDL or low density lipoprotein, indicative of a specific receptor involvement. Accumulation of intracellular degradation products of β -VLDL labeled with the residualizing label, dilactitol- ^{125}I -tyramine, was similar in both cell types demonstrating that degradation was not due to secreted proteolytic enzymes. β -VLDL promoted the incorporation of [^3H]oleate into cholesteryl-[^3H]oleate and increased the cellular mass of cholesterol in NZ macrophages. In contrast, β -VLDL did not augment cholesteryl-[^3H]oleate deposition in WHHL macrophages. This lack of cholesterol esterification occurred despite equivalent acyl-CoA:cholesterol acyltransferase activity in microsomal fractions of both cell types, and similar augmentations in cholesteryl-[^3H]oleate during incubation with phospholipase C-treated LDL. Incubation of WHHL macrophages with β -VLDL increased cellular cholesterol mass, although the response was attenuated compared to NZ cells. To determine whether these disparities in cholesterol esterification were related to the catabolic fate of β -VLDL-derived cholesterol esters, [^3H]cholesteryl oleate was exchanged into the core of β -VLDL and incubated with macrophages in medium containing [^{14}C]oleate. NZ macrophages accumulated both [^3H]cholesterol and [^3H]cholesteryl-[^{14}C]oleate after 5 h, indicating hydrolysis and re-esterification of cholesterol esters. In contrast, WHHL macrophages only accumulated [^3H]cholesterol esters, suggesting uptake of cholesterol esters without subsequent hydrolysis. These data demonstrate that WHHL macrophages possess a pathway for the intracellular processing of β -

VLDL that permits internalization of the particle without stimulation of cholesterol esterification.

Foam cells of macrophage origin are abundant in all phases of the atherogenic process (1). The formation of foam cells in atherosclerotic lesions *in vivo* is assumed to be the result of unregulated delivery of lipoproteins leading to engorgement of cholesterol esters. In accordance with this hypothesized mechanism, Goldstein *et al.* (2) demonstrated that cholesterol ester engorgement can be simulated *in vitro* during incubation of acetylated LDL (AcLDL)¹ with mouse peritoneal macrophages (MPM). In addition to AcLDL, increased cholesterol esterification can also be promoted in MPM by several lipoprotein fractions, including β very low density lipoproteins (β -VLDL; 3), *in vitro* modified forms of LDL such as, malondialdehyde- (4), and 4-hydroxynonenal-conjugated LDL (5), and modified forms of LDL present *in vivo* (6, 7).

The intracellular processing of AcLDL by MPM occurs by a pathway in which particles are recognized by a specific cellular receptor and transported via endosomes to lysosomes. Within lysosomes, apolipoproteins are degraded to their constituent amino acids, and cholesterol esters are hydrolyzed to liberate unesterified cholesterol. The unesterified cholesterol generated is released into the cytosol and induces the activity of acyl-CoA:cholesterol acyltransferase (ACAT), resulting in re-esterification of cholesterol, primarily as cholesteryl oleate (8). Cholesterol esters accumulated by this mechanism are in a dynamic state with a constant cycle of hydrolysis and re-esterification (9).

β -VLDL from several species interacts avidly with macrophages via a specific receptor and leads to profound augmentation of ACAT activity in this cell type (10, 11). This receptor appears to be a mutant form of the LDL receptor (12-14) that recognizes apoE on β -VLDL (15). The specific mutation of the macrophage receptor for β -VLDL has not been characterized, although radioiodinated β -VLDL is recognized and degraded by alveolar macrophages from Watanabe heritable hyperlipidemic (WHHL) rabbits (16). WHHL rabbits are an animal model of familial hypercholesterolemia due to a deficiency of functional LDL receptors (17). While there has been extensive characterization of cellular interactions of β -VLDL with macrophages to define the nature of the ligand and receptors, the intracellular processing route has not been fully

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¹ The abbreviations used are: β -VLDL, β very low density lipoproteins; LDL, low density lipoproteins; AcLDL, acetylated low density lipoprotein; WHHL, Watanabe heritable hyperlipidemic; MPM, mouse peritoneal macrophages; ACAT, acyl-CoA:cholesterol acyltransferase; HMG-CoA, hydroxymethyl glutaryl-CoA; DLT, dilactitol tyramine; PLC, phospholipase C.

elucidated. In fact, the recent evidence of Tabas *et al.* (18) demonstrated that intracellular processing in macrophages for β -VLDL and LDL differed. The present study defined the intracellular processing of β -VLDL with macrophages derived from both NZ and WHHL rabbits.

EXPERIMENTAL PROCEDURES

Materials—Chloroquine, phospholipase C, and fatty acid-free albumin were obtained from Sigma. 5- α -Cholestane was obtained from Alltech Associates (Deerfield, IL). Pravastatin was a gift from the Squibb Institute for Medical Research (Princeton, NJ). Na¹²⁵I (carrier-free), [³H]oleate, [¹⁴C]oleate, [¹⁴C]oleyl-CoA, [¹⁴C]acetate, and [³H]cholesteryl oleate were obtained from Amersham Corp. Newborn calf serum was obtained from JR Scientific (Woodland, CA). Tissue culture plates were obtained from Falcon (Becton Dickinson Labware, Lincoln Park, NJ).

Animals—NZ rabbits were obtained from a local supplier (Boswell Rabbit Farm, Pacific, MO). NZ rabbits used for the harvest of alveolar macrophages were maintained on a normal laboratory diet (Ralston Purina, St. Louis, MO). Rabbits used for the harvest of β -VLDL were maintained on a diet enriched in 2% (w/w) cholesterol (Purina Test Diets, Richmond, IN) for 30–60 days. WHHL rabbits were maintained under aseptic condition and supplied with water and standard laboratory rabbit diet *ad libitum*. At the time of study, WHHL rabbits were between 6 and 16 months old. All procedures that involved animals had the prior approval of the Washington University Animal Studies Committee.

Lipoproteins—Blood was obtained from marginal ear veins of either cholesterol-fed NZ or WHHL rabbits into syringes containing EDTA (1.5 mg/ml) for the harvest of β -VLDL and LDL, respectively. Plasma was harvested and lipoproteins were separated by ultracentrifugation. β -VLDL from cholesterol-fed NZ rabbits was separated by ultracentrifugation at a density of 1.006 g/ml at 1.89×10^5 g·min at 10 °C in a Beckman L8–55 ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA) with the use of a Ti-50.3 rotor. β -VLDL was purified further by re-centrifugation in EDTA-saline (sodium chloride (0.15 M), EDTA (1 mM, pH 8.2) under the same conditions. LDL was isolated by ultracentrifugation at the density cuts of 1.019–1.063 g/ml. Phospholipase C treatment of LDL was performed as described by Suits *et al.* (19). Isolated lipoproteins were dialyzed against \approx 100 volumes of EDTA-saline, with three changes of dialysis fluid over 24 h. Protein concentrations of isolated lipoprotein fractions were determined by the method of Lowry *et al.* (20) using bovine serum albumin as a standard.

Radiolabeling of Lipoproteins—Lipoproteins were directly radioiodinated with Iodo-Beads (Pierce Chemical Co.). Radiolabeled lipoproteins were separated from unbound ¹²⁵I by passing the reaction mixture through a column (10 \times 100 mm) containing Superose 12 prep grade (Pharmacia LKB Biotechnology Inc.) followed by dialysis overnight. Precipitation of protein-bound ¹²⁵I in trichloroacetic acid (10% (v/v)) exceeded 89%. Greater than 94% of ¹²⁵I was coupled to the separated protein moiety. Specific radioactivities of lipoproteins ranged from 65 to 134 cpm/ng protein. In later experiments, β -VLDL was radioiodinated via conjugation to the residualizing label, dilactitol-¹²⁵I-tyramine (*I-DLT). DLT was synthesized, radioiodinated, and conjugated to lipoproteins as described previously (21, 22)

[³H]cholesteryl oleate was incorporated into β -VLDL as described by Hough and Zilversmit (23). Liposomes were formed by the incubation of [³H]cholesteryl oleate in carrier cholesteryl oleate with egg phosphatidylcholine diluted in ethanol in a molar ratio of 1:5. The mixture was dried thoroughly under a stream of N₂. Phosphate buffer (50 mM, pH 7.4) was added to the dried lipids and vortexed vigorously for 10 min, followed by sonication for 1 h in a water bath at 55 °C. This procedure resulted in a totally cleared solution. Liposomes were incubated with plasma from cholesterol-fed NZ rabbits for 18 h at 37 °C in the presence of EDTA (0.4 M). The mass of liposome-phospholipid added did not exceed 7% of phospholipids present in plasma. After the incubation, β -VLDL was isolated from plasma by ultracentrifugation as described above. A common criterion for the absence of liposomes in the isolated β -VLDL is the lack of lipid staining at the origin after electrophoresis on agarose gels. Indeed, lipid staining at the origin was found to be minimal following the incubation of [³H]cholesteryl oleate-containing liposomes with plasma and the subsequent ultracentrifugal isolation of the density fraction of less than 1.006 g/ml. However, slicing of agarose gels at 0.5-mm intervals with subsequent counting of sections demonstrated

that approximately 36% of the total ³H remained at the origin, even though lipid staining was not clearly evident at this position. Thus, to isolate the [³H]cholesteryl oleate-labeled β -VLDL portion, the entire fraction isolated at a density of less than 1.006 g/ml was ultracentrifuged at 1.05×10^7 g·min. After this procedure, >98% of all radioactivity migrated electrophoretically in the β -position.

Harvesting of Macrophages—Pentobarbital-anesthetized rabbits of either sex were exsanguinated via abdominal aortae as described previously (24). Saline containing 5 units/ml heparin was introduced into the alveoli through tracheal cannulas. Lungs were lavaged five times with washes of 50 ml. Cells were plated in Dulbecco's minimum essential medium containing newborn calf serum (20% (v/v)) at a density of $3\text{--}5 \times 10^6$ cells/35-mm well. Metabolism of the cells was characterized the day after harvest.

Cellular Metabolism of Lipoproteins—Degradation of the apolipoprotein moiety of directly radioiodinated lipoproteins was determined by the method of Goldstein and Brown (24). Degradation of apolipoproteins radiolabeled with the residualizing protein label, *I-DLT, was determined as described by Daugherty *et al.* (25). Deposition of cholesteryl-[³H]oleate and [³H]cholesterol esters was determined by the method of Brown *et al.* (26). The mass of cellular cholesterol was determined with gas chromatography using the method described by Ishikawa *et al.* (27). These methods have been described in detail previously (23). In selected experiments, the viability of macrophages was examined and exceeded 90%, based on trypan-blue exclusion.

Determination of Microsomal ACAT Activity—Microsomal fractions from macrophages were isolated by centrifugation as described by Helgerud *et al.* (28). A substrate solution containing [1-¹⁴C]oleyl-CoA (10 nmol of specific radioactivity 5.5 mCi/mmol), fatty acid-free bovine serum albumin (10 nmol), and phosphate buffer (500 μ l) was mixed and incubated at 37 °C. The reaction was initiated by the addition of microsomal fraction (100 μ g of protein) and terminated after 10 min by extracting lipids by the method of Bligh and Dyer (29). Extracts were spotted on silica plates and developed in petroleum ether/ethyl ether/glacial acetic acid (168:30:2 (v/v)). Areas corresponding to cholesteryl-[¹⁴C]oleate were visualized by exposure to iodide vapor and scraped into vials, followed by the addition of Opti-fluor scintillation fluid (United Technologies, Downers Grove, IL). Results are expressed as picomoles of cholesteryl-[¹⁴C]oleate formed/milligram of microsomal protein/minute.

Statistical Analyses—Statistical comparisons between two groups of data were made with Student's *t* test (two-tailed) performed with Stats+ software (Statsoft, Tulsa, OK). A probability value of greater than 0.05 was considered indicative of a statistically significant difference. Values are presented as means, with standard error of means where applicable.

RESULTS

Apolipoprotein Recognition and Degradation—Incubation of ¹²⁵I-labeled β -VLDL with alveolar macrophages from both NZ and WHHL rabbits produced a concentration-dependent increase in degradation of the radioiodinated apolipoprotein moiety (Fig. 1). The degradation of β -VLDL by WHHL macrophages at a concentration of 10 μ g of protein/ml was 0.85 ± 0.14 μ g protein/g cell protein/5 h, which is consistent with that observed by other authors for macrophages derived from WHHL rabbits (30).

To demonstrate whether this interaction was related to a specific receptor on the cultured macrophages, degradation assays were performed in the presence of increasing concentrations of competing lipoproteins. Unlabeled β -VLDL was very effective in competing for the degradation of ¹²⁵I- β -VLDL in macrophages from either NZ or WHHL rabbits (Fig. 2). Maximal inhibition of degradation was achieved at 100 μ g of protein/ml; however, complete competition was not achieved with cells of either origin. LDL was also effective in competing for the degradation of ¹²⁵I- β -VLDL, again with a maximal effect occurring at 100 μ g of protein/ml. However, the maximum inhibition achieved, which was similar in the two cell types, was only approximately 60%. The results of these experiments are consistent with the majority of the recognition of ¹²⁵I- β -VLDL occurring via a receptor mechanism in macrophages from NZ and WHHL rabbits.

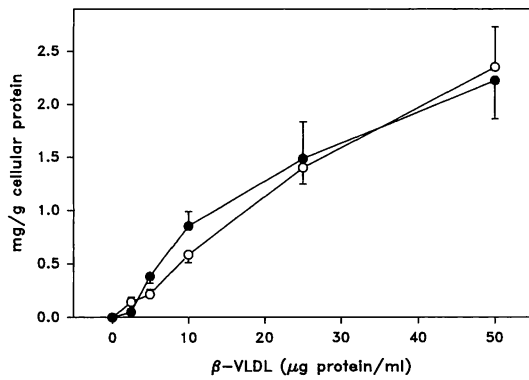


FIG. 1. Degradation of ^{125}I -labeled β -VLDL by macrophages derived from NZ (closed circles) or WHHL (open circles) rabbits. ^{125}I - β -VLDL was incubated with cells at the indicated concentrations for 5 h, prior to the quantification of aqueous trichloroacetic acid-soluble radioactivity in the media. Degradation was quantified in a minimum of duplicate wells/experiment and in at least two separate experiments. Points represent data from a minimum of five individual wells, and bars represent the standard error of the mean.

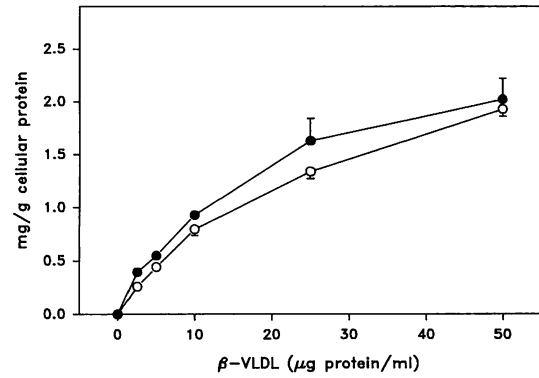


FIG. 3. Degradation of ^{125}I -DLT labeled β -VLDL by macrophages derived from NZ (closed circles) or WHHL (open circles) rabbits. ^{125}I -DLT- β -VLDL was incubated with cells at the indicated concentrations for 5 h, prior to the quantification of trichloroacetic acid-soluble fractions of total accumulated radioactivity present in washed cells. Triplicate wells were quantified in two separate experiments. Thus, points represent the means of six observations, and bars represent the standard error of means.

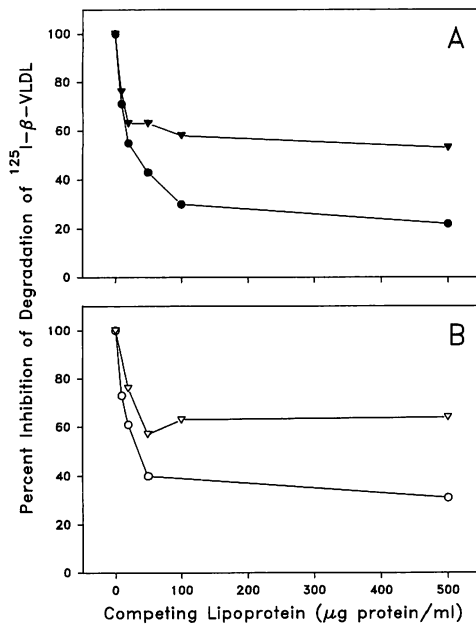


FIG. 2. Competition for degradation of ^{125}I - β -VLDL by increasing concentrations of unlabeled β -VLDL (circles) and LDL (triangles) in macrophages from NZ (A) and WHHL (B) rabbits. ^{125}I - β -VLDL was incubated at a concentration of $5 \mu\text{g}$ of protein/ml with the indicated concentrations of either unlabeled β -VLDL or LDL (ranging from 0 to $500 \mu\text{g}$ of protein/ml). Points represent the means of three observations.

Since macrophages can actively secrete a number of proteolytic enzymes (31) there was a possibility that the degradation observed was an artifact of extracellular processes. To determine whether degradation was primarily an intra- or extracellular phenomena, cells were incubated with β -VLDL conjugated to the residualizing label, $^*\text{I}$ -DLT. Using residualizing labels, the extent of intracellular degradation is determined by the presence of trapped trichloroacetic acid-soluble degradation products in solubilized cells rather than in the medium (21). Using this assay procedure, there was a similar accumulation of degradation products in both cell types (Fig. 3), indicative of the intracellular location of apolipoprotein hydrolysis.

To probe whether intracellular degradation of ^{125}I - β -VLDL occurred in analogous organelles in both cell types, degrada-

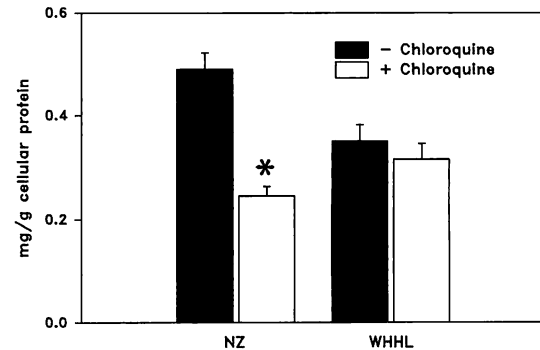


FIG. 4. Inhibition of the degradation of ^{125}I -labeled β -VLDL by chloroquine. Macrophages from NZ and WHHL rabbits were incubated with ^{125}I -labeled β -VLDL at a concentration of $10 \mu\text{g}$ of protein/ml. Chloroquine was present in the specified wells at a concentration of 1 mM which was placed with the cells at the same time as the incubation with ^{125}I -labeled β -VLDL was initiated. Histograms represent the means of four observations \pm standard errors of the means. * Denotes statistical difference produced during chloroquine treatment at the $p < 0.05$ level. Chloroquine significantly reduced the degradation of ^{125}I -labeled β -VLDL but was ineffective in WHHL macrophages.

tion was determined in the presence of the lysosomal inhibitor chloroquine. Chloroquine is an effective inhibitor of the cathepsins present within lysosomes but is ineffective toward cathepsin D present in endosomes (32). Chloroquine (10^{-3} M) reduced the degradation of ^{125}I - β -VLDL by approximately 50% ($p > 0.05$) in macrophages from NZ rabbits (Fig. 4), without producing any overt toxicity as judged by the general appearance of the cells at the end of the experiment and by the protein concentrations in the wells. However, chloroquine was ineffective in significantly modifying the degradation of ^{125}I - β -VLDL by WHHL-derived macrophages.

Intracellular Cholesterol Metabolism—Metabolism of the cholesterol and cholesterol ester moieties of β -VLDL was studied initially by determining the deposition of cholesteryl- ^3H oleate. Concomitant incubation of β -VLDL and ^3H oleate with macrophages from NZ rabbits resulted in a concentration-dependent increase in intracellular cholesteryl- ^3H oleate deposition (Fig. 5). In dramatic contrast to the results obtained in NZ-derived cells, incubation of β -VLDL and ^3H oleate with macrophages from WHHL rabbits produced no significant stimulation of cholesteryl- ^3H oleate deposition at any concentration examined. Stimulation of

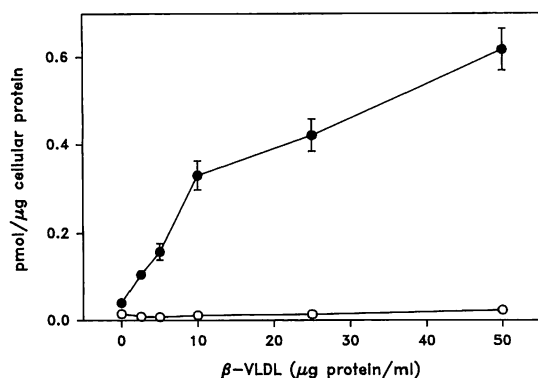


FIG. 5. Effects of increasing concentrations of β -VLDL on the incorporation of [^3H]oleate into cholesteryl-[^3H]oleate by macrophages derived from NZ (closed circles) or WHHL (open circles) rabbits. Cells were incubated with media containing [^3H]oleate and β -VLDL at the indicated concentrations for 5 h. Cholesteryl-[^3H]oleate deposition was quantified as described by Brown *et al.* (26) in a minimum of duplicate wells/experiment in at least two separate experiments. Points represent data from a minimum of five individual wells, and bars represent the standard error of the mean.

cholesteryl-[^3H]oleate deposition was similar in macrophages from NZ fed either a normal or cholesterol-enriched diet (data not shown) indicating the hypercholesterolemic state of WHHL rabbits did not affect the response to β -VLDL. In neither cell type could the deposition of cholesteryl-[^3H]oleate be stimulated by sterol depletion during incubation with an inhibitor of 3-hydroxymethyl-3-glutaryl-CoA (HMG-CoA) reductase, pravastatin (33), at concentrations that totally abolished *de novo* cholesterol biosynthesis in this cell type (10^{-5} M; data not shown). A possible explanation for the lack of intracellular deposition of cholesteryl-[^3H]oleate could have been a lack or decreased activity of ACAT within macrophages from WHHL rabbits. Therefore, microsomes were prepared from macrophages of both NZ and WHHL rabbits. Despite the total lack of cholesteryl-[^3H]oleate deposition in intact macrophages from WHHL rabbit, ACAT activity was not significantly different in microsomal fractions (2.68 ± 0.42 versus 2.81 ± 0.28 pmol/mg protein/min for NZ and WHHL cells, respectively).

Further evidence that ACAT was functional in both cell types was provided in intact cells by incubation with phospholipase C-treated LDL (PLC-LDL). Incubation of macrophages with PLC-LDL has been previously shown to stimulate cholesterol esterification (18). In both cell types, PLC-LDL augmented cholesteryl-[^3H]oleate deposition (Fig. 6). In contrast to the stimulation of ACAT activity by β -VLDL in cells from NZ rabbits, the stimulation produced by PLC-LDL was linear. Also, the PLC-LDL stimulation of ACAT activity was much less potent than for β -VLDL, even when results were normalized to the cholesterol content of the incubation media. Both malondialdehyde-treated LDL (4) and copper-oxidized-treated LDL (34) failed to influence cholesterol esterification in either cell type.

Despite the total inability of β -VLDL to stimulate cholesteryl-[^3H]oleate deposition and the highly attenuated response to *de novo* cholesterol biosynthesis increases in the cellular content of total cholesterol occurred in both cell types after incubation with β -VLDL. Incubation of β -VLDL with macrophages from NZ rabbits resulted in a concentration-dependent increase in cellular cholesterol that was approximately 50% greater than that in macrophages from WHHL rabbits (Fig. 7).

The discrepancy between the total lack of augmented cholesteryl-[^3H]oleate deposition in macrophages derived from

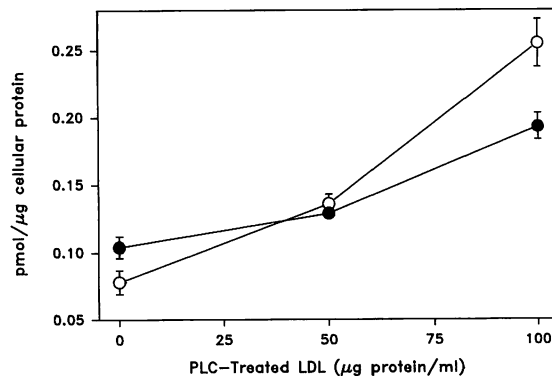


FIG. 6. Effects of PLC-LDL on cholesteryl-[^3H]oleate deposition in NZ (closed circles) and WHHL (open circles) macrophages. PLC-LDL was produced as described by Suits *et al.* (18) and incubated with cells at the indicated concentrations for 5 h prior to the determination of cellular cholesteryl-[^3H]oleate. Points represent data from four individual wells and bars the standard error of means. PLC-LDL increased cholesteryl-[^3H]oleate deposition in both cell types but the response was linear and much attenuated compared to that observed during incubation with β -VLDL.

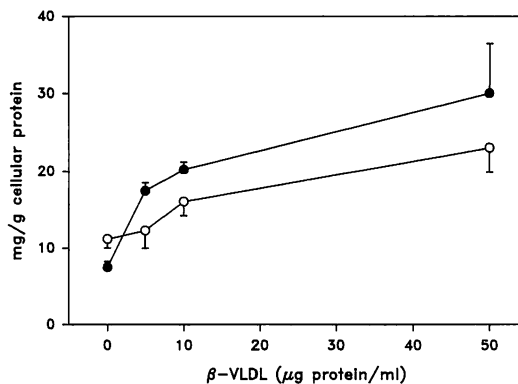


FIG. 7. Effects of β -VLDL on the intracellular cholesterol masses of macrophages derived from NZ (closed circles) or WHHL (open circles) rabbits. Lipoproteins were incubated with cells at the indicated concentrations for 5 h. Observations were recorded in at least two separate experiments in which a minimum of two wells was quantified/concentration of β -VLDL. Points represent a minimum of five observations, and bars represent the standard errors of the mean.

WHHL rabbits and the mass measurements of cellular cholesterol could be explained by delivery of the cholesterol esters of β -VLDL without hydrolysis and subsequent re-esterification. To examine this possibility, β -VLDL was radiolabeled with [^3H]cholesteryl-oleate *via* an exchange with a liposome complex and incubated with cells in medium containing [^{14}C]oleate complexed to albumin. After a 5-h incubation, the cells were washed, sterols were extracted, and cholesterol and cholesterol esters were resolved by thin layer chromatography. Macrophages from NZ rabbits accumulated [^3H]cholesterol, indicating hydrolysis of the delivered cholesteryl-[^3H]oleate (Fig. 8A). In addition, cholesterol esters extracted after the 5-h incubation contained both ^3H and ^{14}C , indicating that re-esterification also occurred in this cell type. Very little [^3H]cholesterol was present in WHHL macrophages, indicating a lack of hydrolysis of the delivered cholesterol esters (Fig. 8B). Macrophages from WHHL rabbits accumulated cholesterol esters (Fig. 8C), but ^{14}C was not present in significant amounts at any of the concentrations examined. Thus, while cholesteryl-[^3H]oleate entered the WHHL macrophages, these data demonstrate that cholesterol esters are not effectively hydrolyzed on delivery to the intracellular compartment.

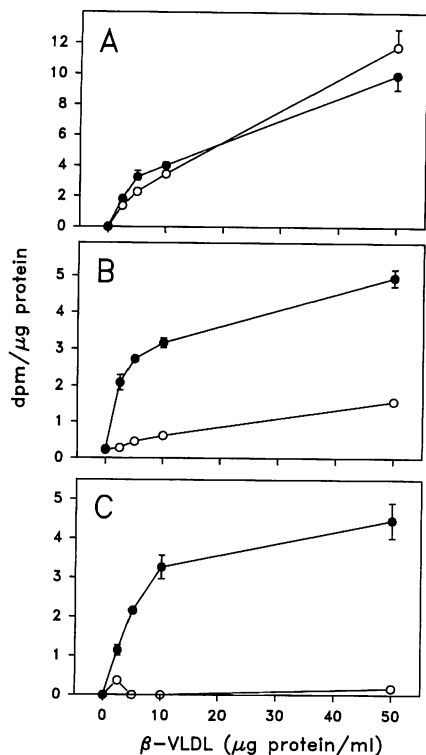


FIG. 8. Hydrolysis and re-esterification of cholesterol esters in macrophages. Intracellular deposition of [^3H]cholesterol esters, [^3H]cholesterol, and cholesteryl- ^{14}C oleate were determined after incubation with medium containing [^{14}C]oleate complexed to bovine serum albumin and β -VLDL radiolabeled with [^3H]cholesteryl oleate. [^3H]cholesteryl oleate-labeled β -VLDL was incubated with macrophages for 5 h at the indicated concentrations. Sterols were extracted from cells and resolved by thin layer chromatography. Radioactivity was quantified as follows. A, ^3H of cholesterol esters representing both delivered and re-esterified material; B, ^{14}C of oleate in cholesterol esters indicating newly delivered cholesterol esters that had undergone hydrolysis and re-esterification; C, ^3H in unesterified cholesterol indicating newly delivered cholesterol esters that had undergone hydrolysis. Data for NZ macrophages are represented by the solid circles and WHHL macrophages by the open circles. Points represent the mean of four observations, and bars represent the standard error of the mean.

Evidence that the accumulated cholesterol esters in WHHL macrophages were present in intracellular compartments rather than on cell surface structures was provided qualitatively by Oil Red O staining of cells after incubation with β -VLDL. β -VLDL incubations with NZ macrophages promoted the appearance of many punctate lipid-stained areas. Incubations of β -VLDL with WHHL macrophages produced less overall staining as would be predicted from the cholesterol mass measurements, but the staining was present in a punctate form indicating an intracellular loci (data not shown).

DISCUSSION

The elucidation of the intracellular processing of lipoproteins by macrophages may have important implications for the atherogenic process. At present, it is generally assumed that lipoproteins follow an intracellular itinerary that involves the interaction with specific receptors on the cell surface and that promotes invagination of the membrane at the site of coated pits with formation of primary endosomes. Endosomes then undergo a fusion process as maturation progresses, and ultimately the lipoprotein is transferred to the lysosome where it is catabolized (35–37). The present study defined a processing route of β -VLDL in macrophages from WHHL rabbits

that results in the degradation of the apolipoprotein moiety but fails to stimulate cholesterol esterification since the newly delivered cholesterol esters are not hydrolyzed.

β -VLDL is physically and metabolically heterogeneous due to its synthesis in both intestinal and hepatic tissues (25, 38–42). β -VLDL from a variety of species interact with mouse peritoneal macrophages to stimulate ACAT activity (3). The cellular interaction of β -VLDL by macrophages was originally thought to be modulated via a distinct receptor, although, recent studies have suggested that β -VLDL interacts with MPM through an unusual form of the LDL receptor (13, 14, 15). The inability of β -VLDL to stimulate ACAT in LDL receptor-deficient WHHL-derived macrophages demonstrated in the present study may support the hypothesis that cellular interactions of β -VLDL with macrophages are mediated by LDL receptors. However, the radioiodinated apolipoprotein moiety of β -VLDL was degraded in macrophages from both NZ and WHHL rabbits, suggesting that the lipoprotein is recognized equivalently by the cell types, and that inability to stimulate ACAT activity is due to disparate intracellular processing.

WHHL rabbits are a commonly used animal preparation of endogenous hypercholesterolemia that is thought to mimic the hypercholesterolemia and atherosclerosis present in humans with familial hypercholesterolemia (43, 44). Processing of LDL receptors is abnormal in cells from WHHL rabbits due to an in-frame deletion in the third cysteine-rich repeat region (17). This defect confers instability of the receptor protein resulting in premature degradation. The deletion of this region of the molecule also greatly reduces the binding capacity of LDL to the receptor protein. These combined effects virtually ablate degradation of ^{125}I -labeled LDL by cultured WHHL rabbit skin fibroblasts (45). Despite this decrease in the binding capacity, the mutant LDL receptor present in WHHL rabbits retains its ability to bind to β -VLDL as determined by Western blotting (17). In accord with this finding, ^{125}I -labeled β -VLDL was degraded to the same extent in endothelial cells (46) and alveolar macrophages (47) from WHHL rabbits as in the same cell types derived from NZ rabbits (48). Furthermore, skin fibroblasts from WHHL rabbits recognized β -VLDL with affinity equal to that in fibroblasts from normal rabbit, albeit with decreased capacity (48). Thus, there is consistent evidence that the apolipoprotein moiety of β -VLDL is recognized by a multiplicity of cell types from WHHL rabbits despite the absence of functional LDL receptors.

The presence of sterol loading decreases the cellular receptivity for β -VLDL in alveolar macrophages from WHHL rabbits and monocyte-derived macrophages from normal human subjects (30). The present study attempted to promote the deposition of cholesterol esters by inhibiting the production of endogenous cholesterol with the HMG-CoA reductase inhibitor pravastatin. It has previously been demonstrated that another HMG-CoA reductase inhibitor, lovastatin, stimulates the degradation of radioiodinated LDL by human monocyte-derived macrophages (49). However, incubation of macrophages with this drug failed to alter the extent of deposition of cholesteryl- ^3H oleate in cells from either NZ or WHHL rabbits.

Previous studies have also observed a dichotomy between the degradation of ^{125}I -labeled LDL and the ability to stimulate cholesteryl- ^3H oleate deposition in both MPM and human monocyte-derived macrophages (13, 50). However, in both of these reports, degradation of the radioiodinated lipoprotein occurred by a nonsaturable mechanism. In contrast, the degradation of ^{125}I - β -VLDL observed in the present study

by macrophages from both strains was not linear with respect to concentration. In addition, the degradation was considerably inhibited by excess unlabeled β -VLDL and partially by LDL, which is indicative of a specific receptor process.

Previous studies in macrophages have noted that lipoproteins may be processed by differing intracellular routes. For example, gold-labeled β -VLDL and AcLDL are transported differently in pigeon macrophages, although ultimately both lipoproteins were delivered to lysosomes (51). Also, recent studies have indicated that selected lipoproteins may also be catabolized in compartments other than the lysosome in cultured macrophages. Van Lenten and Fogelman (52) demonstrated that LDL and malondialdehyde-treated LDL may be degraded in the endosomal compartment in human monocyte-derived macrophages. Furthermore, Tabas *et al.* (18) have demonstrated a disparity in the intracellular distribution of fluorescently labeled LDL and β -VLDL in MPM.

The intracellular processing of lipoproteins was determined initially for LDL in cultured skin fibroblasts (53). The evidence for the lysosomal involvement includes the equivalency of the metabolism of the apolipoprotein and cholesterol ester moieties of LDL and the decrease in metabolism induced by lysosomal inhibitors such as chloroquine (54). However, the inhibition of degradation of the apolipoprotein moiety of β -VLDL by chloroquine was not demonstrable in WHHL macrophages. In accord with this finding, chloroquine has also been shown to be ineffective in inhibiting the degradation of radioiodinated LDL in hepatocytes cultured from WHHL rabbits (55). In addition to studies performed on fibroblasts, several recent reports have demonstrated that selected lipoproteins, in addition to other macromolecules such as mannosylated albumin, parathyroid hormone, and kininogens, are metabolized in organelles other than lysosomes. Thus, specific proteins may be degraded only in the endosomal compartment. In this regard, Diment and Stahl (32) have demonstrated mannosylated albumin degradation in an endosomal compartment in rabbit alveolar macrophages by cathepsin D. Although the signaling mechanism that determines endosomal *versus* lysosomal processing is unknown, it may simply depend on whether a specific protein is a substrate for proteases that are present in the endosomal compartment (56).

The inability to augment cholesterol esterification after incubation of β -VLDL with WHHL macrophages, despite delivery of cholesterol esters, could have been the consequence of (a) a lack of ACAT activity, (b) inactivity of cholesterol esterase, (c) a lack of delivery of cholesterol esters to a compartment containing cholesterol esterase, and (d) the deposition of cholesterol into inappropriate pools for the accessibility to ACAT (57). Based on the enzyme activity in microsomal fractions, deficiency of ACAT activity did not account for the lack of cholesterol esterification in WHHL macrophages. Stimulation of ACAT activity during the incubation with PLC-treated LDL demonstrates that cholesterol esters can be hydrolyzed in WHHL macrophages if the substrate is presented to the appropriate compartment. Further evidence that there is no defect in hydrolytic activity of WHHL macrophages is the promotion of cholesteryl oleate formation by AcLDL in WHHL peritoneal macrophages and to a similar extent to cells from NZ rabbits (58). Thus, the inability to stimulate ACAT appears to be due to substrate limitations, since extensive hydrolysis of radiolabeled cholesterol esters of β -VLDL could not be determined. Thus, lack of delivery of β -VLDL-associated cholesterol esters to the appropriate organelle would appear to be the most likely mechanism for the lack of cholesterol esterification.

In accord with our findings in macrophages, Wernette-

Hammond *et al.* (48) recently speculated that intracellular processing may differ in skin fibroblasts from WHHL rabbits, compared to those derived from NZ rabbits, based on the kinetics of receptor recycling which were more rapid in cells derived from the former. These authors have hypothesized that the intracellular processing of lipoproteins may differ in fibroblasts from WHHL rabbits based on the disparity between surface binding of β -VLDL and the degradation and extent of induction of cholesterol esterification. The extent to which this intracellular processing defined in the present study in WHHL rabbits *in vivo* cannot be estimated. However, $d < 1.006$ g/ml lipoproteins from WHHL rabbits resemble some characteristics of β -VLDL isolated from cholesterol-fed rabbits (59) such as the ability to augment cholesterol esterification in MPM. Thus, the present study has demonstrated that β -VLDL undergoes degradation of the apolipoprotein moiety without the expected effects on cholesterol metabolism in macrophages derived from WHHL rabbits.

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