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Expression of Caveolin-1 in Hepatic Cells Increases Oxidized LDL Uptake and Preserves the Expression of Lipoprotein Receptors

To Quyen Truong, Mathieu R. Brodeur, Louise Falstrault, David Rhainds, and Louise Brissette*

Département des Sciences Biologiques, Université du Québec à Montréal, Montréal, Québec, Canada H3C 3P8

ABSTRACT

Oxidized LDL (OxLDL) that are positively associated with the risk of developing cardiovascular diseases are ligands of scavenger receptor-class B type I (SR-BI) and cluster of differentiation-36 (CD36) which can be found in caveolae. The contribution of these receptors in human hepatic cell is however unknown. The HepG2 cell, a human hepatic parenchymal cell model, expresses these receptors and is characterized by a very low level of caveolin-1. Our aim was to define the contribution of human CD36, SR-BI, and caveolin-1 in the metabolism of OxLDL in HepG2 cells and conversely the effects of OxLDL on the levels/localization of these receptors. By comparing mildly (M)- and heavily (H)-OxLDL metabolism between control HepG2 cells and HepG2 cells overexpressing CD36, SR-BI, or caveolin-1, we found that (1) CD36 increases M- and H-OxLDL-protein uptake; (2) SR-BI drives M-OxLDL through a degradation pathway at the expense of the cholesterol ester (CE) selective uptake pathway; (3) caveolin-1 increases M- and H-OxLDL-protein uptake and decreases CE selective uptake from M-OxLDL. Also, incubation with M- or H-OxLDL decreases the levels of SR-BI and LDL-receptor in control HepG2 cells which can be overcome by caveolin-1 expression. In addition, OxLDL move CD36 from low to high buoyant density membrane fractions, as well as caveolin-1 in cells overexpressing this protein. Thus, hepatic caveolin-1 expression has significant effects on OxLDL metabolism and on lipoprotein receptor levels. J. Cell. Biochem. 108: 906–915, 2009.

KEY WORDS: LDL; SR-BI; CD36; CAVEOLIN-1; HEPATIC; SELECTIVE UPTAKE

It is well established that high levels of plasma cholesterol associated with LDL increase the risk of developing atherosclerosis and that the LDL-receptors (LDLr) are responsible for a major part of LDL particles metabolism [Castelli et al., 1977]. LDL can undergo oxidative modification. Oxidized (Ox) LDL were found associated with endothelial and macrophage cells and are known to be deleterious. It is also suggested that OxLDL may exist in human blood, based on the finding of their epitopes [Itabe et al., 1996]. Moreover, a positive correlation was found between the incidence of cardiovascular diseases and OxLDL epitopes [Holvoet, 2004]. It is therefore accepted that mildly OxLDL (M-OxLDL) are less deleterious than heavily OxLDL (H-OxLDL). It is also known that M-OxLDL can interact with LDLr [Berliner et al., 1990] while H-OxLDL cannot. In 1991, it was shown that when injected in rats H-OxLDL disappear faster than LDL and are taken up mainly by the liver through nonparenchymal cells [Van Berkel et al., 1991]. Few years later in vivo studies by Ling et al. [1997] showed that although the scavenger receptor-class A type I/II (SR-A) plays a significant role in H-OxLDL uptake in macrophages, it is not significantly involved with the uptake by the liver of injected OxLDL. At the same time,

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To Quyen Truong's present address is Université du Québec, Institut National de la Recherche Scientifique, Institut Armand-Frappier, Laval, Québec, Canada H7V 1B7.

Mathieu R. Brodeur's present address is Faculté de Pharmacie, Université de Montréal, Montréal, Québec, Canada H3C 3J7.

David Rhainds's present address is Genizon BioSciences, Ville Saint-Laurent, Québec, Canada H4T 2C7.

*Correspondence to: Louise Brissette, Département des Sciences Biologiques, Université du Québec à Montréal, C.P. 8888, Succursale Centre-ville, Montréal, Que., Canada H3C 3P8. E-mail: brissette.louise@uqam.ca

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Endemann et al. [1993] have revealed the importance of macrophage cluster of differentiation-36 (CD36) as an OxLDL receptor in vitro. Nozaki et al. [1995] showed that macrophages lacking CD36 degrade two times less H-OxLDL than normal macrophages. H-OxLDL were also shown to bind to the scavenger receptor-class B type I (SR-BI) [Acton et al., 1994], a receptor belonging to the same family as CD36. Moreover, chinese hamster ovary (CHO) cells overexpressing SR-BI [Gillotte-Taylor et al., 2001] were demonstrated to exhibit greater H-OxLDL degradation than control cells. Being interested in defining the implication of CD36 and SR-BI in OxLDL metabolism in vivo, we have recently analyzed their clearances in wild-type and CD36 or SR-BI knock-out (KO) mice. We found [Luangrath et al., 2008] that CD36, and not SR-BI, is responsible for a significant part of both M- and H-OxLDL clearances. However, the implication (capacity) of hepatic human SR-BI and CD36 was never addressed.

Differently from the global lipoprotein particle uptake and degradation pathways just described, lipoproteins can also be subjected to cholesteryl esters (CE) selective uptake, a process that implies the extraction of CE from the lipoprotein without concomitant degradation of its apolipoproteins. Thus, after being subjected to such a pathway, lipoproteins return to the circulation depleted in their CE. SR-BI is very well recognized for its ability to selectively take up CE from HDL [Acton et al., 1996] and LDL [Rhainds et al., 2003] but we know little on CE-selective uptake from OxLDL. Using HepG2 cells, a human parenchymal cell model, our group was the first to report evidence of a CE-selective uptake pathway with modified LDL (oxidized and acetylated LDL) [Rhainds et al., 1999]. We have also shown [Bourret et al., 2006] by in situ experimentations that both mouse parenchymal and nonparenchymal liver cells selectively take CE from M- and H-OxLDL. However, we could only determine a role of SR-BI for H-OxLDL in nonparenchymal cells. Using primary cultures of parenchymal hepatic cells from wild-type and CD36 KO mice, we recently showed [Luangrath et al., 2008] that murine CD36 is involved in H- but not in M-OxLDL-CE selective uptake. Thus, our two studies in mice revealed a significant contribution of SR-BI in H-OxLDL-CE selective uptake in hepatic nonparenchymal cells and of CD36 in parenchymal cells. Interestingly, one of the differences between hepatic nonparenchymal and parenchymal cells is that the former express much more caveolin-1 than the latter [Malerød et al., 2002]. Caveolin-1 is the marker protein of caveolae that are membrane microdomains rich in cholesterol and glycosphingolipids. Earlier, the activity of SR-BI was suggested to be linked to its localization in caveolae and as few others [Graf et al., 1999; Frank et al., 2001; Wang et al., 2003], we have investigated the effect of caveolin-1 on SR-BI activity. We found that caveolin-1 expression in HepG2 cells increases the degradation of LDL, HDL-CE selective uptake, and decreases LDL-CE selective uptake [Truong et al., 2006]. However, the possible importance of caveolae in the elimination of OxLDL through global lipoprotein particle uptake or by CE-selective uptake by hepatic cells was never addressed. The issue is important in order to explain at least partially why a major part of OxLDL metabolism occurs in nonparenchymal cells.

Our first aim was to define the importance of human hepatic CD36 and SR-BI in OxLDL metabolism and to describe the effect of caveolin-1 in this metabolism. Both M- and H-OxLDL were studied in order to define if the extent of oxidation has an impact. To this end, we compared M- and H-OxLDL metabolism between control HepG2 cells and HepG2 cells overexpressing CD36, SR-BI, or caveolin-1. Moreover as it is known that OxLDL influence endothelial smooth muscle cell viability [Pirillo et al., 1997] and expression of lipoprotein receptors in macrophages [Yoshida et al., 1998; Han et al., 2001], our second aim was to define the impact of the two types of OxLDL on cell viability and on CD36, SR-BI and LDLr expression levels in HepG2 cells. Cells overexpressing caveolin-1 was also studied in order to determine if caveolin-1 expression aggravates or attenuates these effects.

MATERIALS AND METHODS

MATERIALS

Human plasma was obtained from Bioreclamation (Hicksville, NY). The human hepatoma cell line HepG2 was obtained from ATCC (Rockville, MD). Minimal essential medium (MEM) and G418 were from Life Technologies (Burlington, Ontario), and Zeocin from Invitrogen (Burlington, Ontario). Fetal Clone I was purchased from HyClone (Logan, UT) while bovine serum albumin (BSA) (fraction V) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrasodium bromide (MTT) were obtained from Sigma–Aldrich (Mississauga, Ontario). [^{125}I] (as sodium iodide, 100 mCi/ml), and [^{3}H]-cholesteryl oleate (30–60 Ci/mmol) were bought from ICN Biomedical (Montréal, Québec) and Amersham (Oakville, Ontario), respectively. BLT-1 was from Chembridge Corporation (San Diego, CA). Antibodies and their sources were as follows: rabbit IgG anti-SR-BI and anti-CD36 were from Novus Biologicals (Littleton, CO); rabbit IgG anti-caveolin-1 (polyclonal antibody-pAb C13630) was from BD-Transduction Laboratories (Mississauga, Ontario) and rabbit IgG anti-LDLr from Research Diagnostics, Inc. (Flanders, NJ).

CELL CULTURE

HepG2 cells were grown in MEM supplemented with 10% fetal clone I, penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (4 mM). Medium was changed every 2–3 days and cells were propagated every 7 days. Three days prior to the assay, 4.5×10^5 cells were seeded in 3.8 cm^2 culture dishes (12-well dish).

PREPARATION OF STABLE HepG2 CELLS OVEREXPRESSING CD36

The full length CD36 cDNA was recovered by *Eco*RI digestion of the pUC18 vector (ATCC). The cDNA was subcloned in the eucaryotic expression vector pZeoSV (Invitrogen). HepG2 cells at 80% confluence were stably transfected with this vector using the standard calcium phosphate method. Zeocin-resistant cells were selected with 800 μg/ml of Zeocin for 3–4 weeks and clones were isolated, propagated, and maintained with 500 μg/ml of Zeocin.

PREPARATION AND RADIOLABELING OF LIPOPROTEINS

Human LDL (density 1.025–1.063 g/ml) were isolated from plasma as described in Rhainds et al. [1999]. H-OxLDL were prepared as in Lougheed and Steinbrecher [1996]. H-OxLDL typically resulted in a 2.8-fold increase in the electrophoretic mobility relative to native LDL on 0.5% agarose/barbital gels. M-OxLDL were obtained by

incubation under similar conditions but for 4 h and show a 1.5-fold increase in the electrophoretic mobility relative to native LDL. For extensive characterization of these two types of OxLDL particles see Luangrath et al. [2008]. Briefly, there are 33- and 341-fold more OxLDL epitopes in M- and H-OxLDL than in LDL, respectively. LDL and OxLDL were labeled with iodine-125 and [^{3}H]-cholesteryl oleoyl ether (CEt) as described previously [Luangrath et al., 2008] and none of the labeling procedures affects the migration on agarose/barbital gel particles, or the binding activity, or the lipid hydroperoxides levels of the labeled lipoproteins compared to their unlabeled counterpart. Thus, unlabeled and labeled lipoproteins do not significantly differ from each other.

CELL ASSOCIATION AND DEGRADATION ASSAYS

Cells were washed twice with 1 ml PBS and incubated with ^{125}I- and ^{3}H-CE-OxLDL (20 μg of protein/ml) for 4 h at 37°C in a total volume of 250 μl containing 125 μl of MEM [2×] plus 4% BSA, pH 7.4 (total association). Nonspecific association was determined in the presence of 2 mg of protein/ml of nonlabeled OxLDL. At the end of the incubation, the medium was recovered and precipitated with trichloroacetic acid (TCA) at a final concentration of 12% and degradation was estimated in the TCA-soluble fraction, as described in Rhainds et al. [1999]. For cell association data, cells were solubilized in 1.5 ml of 0.1 N NaOH. Associated protein was quantified with a Cobra II counter (Canberra-Packard) and associated CE was counted with a beta-counter (Wallach-Fisher). Associations of ^{125}I- and ^{3}H-CE-OxLDL were expressed in μg of protein/mg cell protein. Selective uptake was calculated by subtracting the protein association from the CE association. The NaOH fractions were also assayed for protein content to normalize for cell protein. Such incubation with HepG2 cells does not modify the oxidative status of M- or H-OxLDL (data not shown).

MTT ACTIVITY

MTT activity was determined in 96-well plates by microtiter tetrazolium assay after incubation of LDL, M-, or H-OxLDL for 24 or 48 h in MEM supplemented with 2% human serum deficient in lipoproteins (LPDS). Briefly, MTT reagent was added to the medium at a final concentration of 0.05 mg/ml. Four hours later, formazan crystals generated by cellular reduction of the MTT reagent were dissolved in dimethyl sulfoxide (DMSO) for 30 min at 37°C and the absorbance was determined at 575 nm. Results are expressed as the percentage of MTT values of treated versus control conditions.

EXPOSURE OF CELLS TO LDL, M-, AND H-OxLDL AND ANALYSIS OF LEVELS AND SUBCELLULAR LOCALIZATION OF RECEPTORS

Cells were grown to 85% confluence and then treated with LDL, M-, or H-OxLDL (0–1,000 μg/ml protein) for 24 h in 0.2% BSA. After treatment, cells were either used for Western blotting or cell fractionation. For Western blotting, cells were washed twice with cold PBS and then scraped and lysed in ice-cold lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, and protease inhibitor cocktail). Lysate was microcentrifugated for 25 min at 4°C and supernatant was assessed for cell proteins. Proteins (50–100 μg) were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by Western blotting to nitrocellulose membrane. For membrane protein fractionation by discontinuous sucrose gradient, the protocol of Song et al. [1996] was followed. Briefly, cells from three 75 cm^2 flasks were washed, scraped, and suspended in 2 ml of 500 mM Na_2CO_3, pH 11. The cells were maintained on ice and disrupted by 20 strokes of a 5 ml tight-fitting glass Dounce homogenizer, three 10-s bursts with a Polytron homogenizer (Brinkman) and three 20-s sonication bursts at 50% maximum power (Branson Sonifier 250). The homogenate was rapidly mixed with 2 ml of 85% sucrose in morpholinoethanesulfonic acid (MES)-buffered saline (MBS), pH 6.5 in a 12 ml ultracentrifuge tube. The top was layered with 4 ml of 35% sucrose in MBS plus 250 mM Na_2CO_3 and 4 ml of 5% sucrose in MBS–Na_2CO_3. Gradients were spun at 190,000*g* for 18 h at 4°C in an SW41 rotor (Beckman). Twelve 1 ml fractions were collected from the top. Proteins of fractions 1–12 were precipitated with 21% TCA, suspended in 300 μl 0.2 N NaOH plus 1% SDS, run on SDS–PAGE and subjected to Western blotting. The membranes were blocked with 5% dry milk for 90 min. Primary antibodies such as anti-SR-BI (1:5,000), anti-LDLr (1:500), anti-CD36 (1:1,000), or anti-caveolin-1 (1:5,000) were diluted in 20 mM Tris, pH 7.6, 137 mM NaCl, 0.1% Tween-20, and incubated with the nitrocellulose membranes for 2 h at 37°C. The membranes were washed three times for 5 min with the same buffer and incubated with the second antibody (goat anti-rabbit IgG conjugated to horseradish peroxidase). The membranes were washed and the bands visualized using enhanced chemiluminescence. For regular Western blotting such as presented in Figure 5, actin was immunodetected and used as a loading control.

OTHER METHODS

Protein content was determined by the method of Lowry et al. [1951] using BSA as standard. Two-tailed Student's paired *t*-tests (Figs. 1–3) or ANOVA tests (Fig. 4) were used to obtain statistical comparison of the data. Differences were considered significant at $P < 0.05$.

RESULTS

To define the potential roles of CD36 and SR-BI in OxLDL metabolism in HepG2 cells, cells overexpressing either CD36 or SR-BI were used. Stable transformants of HepG2 cells expressing SR-BI at 3.1-fold ($P < 0.005$; N = 3) the normal level [Rhainds et al., 2004] were already available and will be referred as SR-BI+. For this study, we have generated HepG2 cells stably overexpressing CD36. Cellular clones were obtained and we have chosen for this study a clone expressing 2.7-fold ($P < 0.0001$; N = 3) the normal level of CD36 (identified as CD36+) in order to have a similar magnitude of overexpression in SR-BI or CD36. We have also verified the effect of overexpressing one receptor on the cell level of the other and found no difference (data not shown). Figure 1A shows that SR-BI overexpression increases M-OxLDL–protein association in average by 125%, while CD36 overexpression leads to a 62% increase. Similar results were obtained for the association of H-OxLDL–protein. CE-association levels (Fig. 1B) also increased but less than for protein associations. Lipoprotein–protein and –CE cell associations reflect binding to the cell surface and internalization. Such

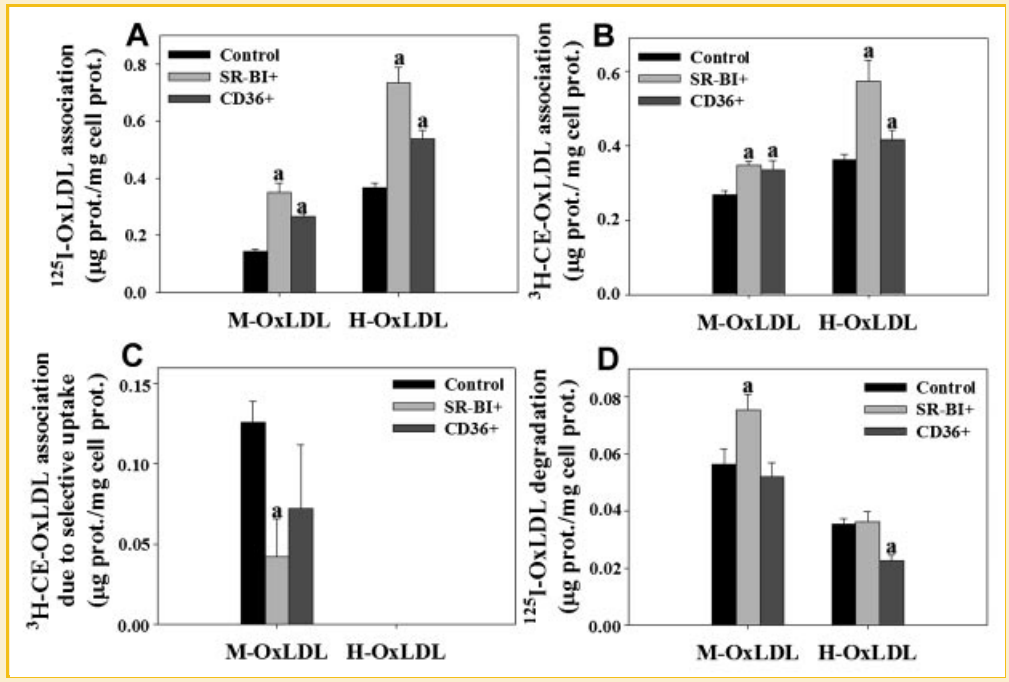

Fig. 1. ^{125}I–OxLDL association (panel A), ^{3}H–CE–OxLDL association (panel B), ^{3}H–CE–OxLDL association due to cholesteryl ester selective uptake (panel C), and ^{125}I–OxLDL degradation (panel D) in control, SR-BI+ and CD36+ HepG2 cells. Cells were incubated with either radiolabeled M- or H-OxLDL (20 μg of protein/ml) for 4 h at 37°C in 12-well plates. Nonspecific association of radioactive lipoproteins was determined by the addition of unlabeled OxLDL at 1.5 mg of protein/ml and subtracted from total association. Results are shown as the mean ± SEM of four experiments conducted each in duplicate. [a]Statistically different ($P < 0.05$) from the result obtained with control HepG2 cells.

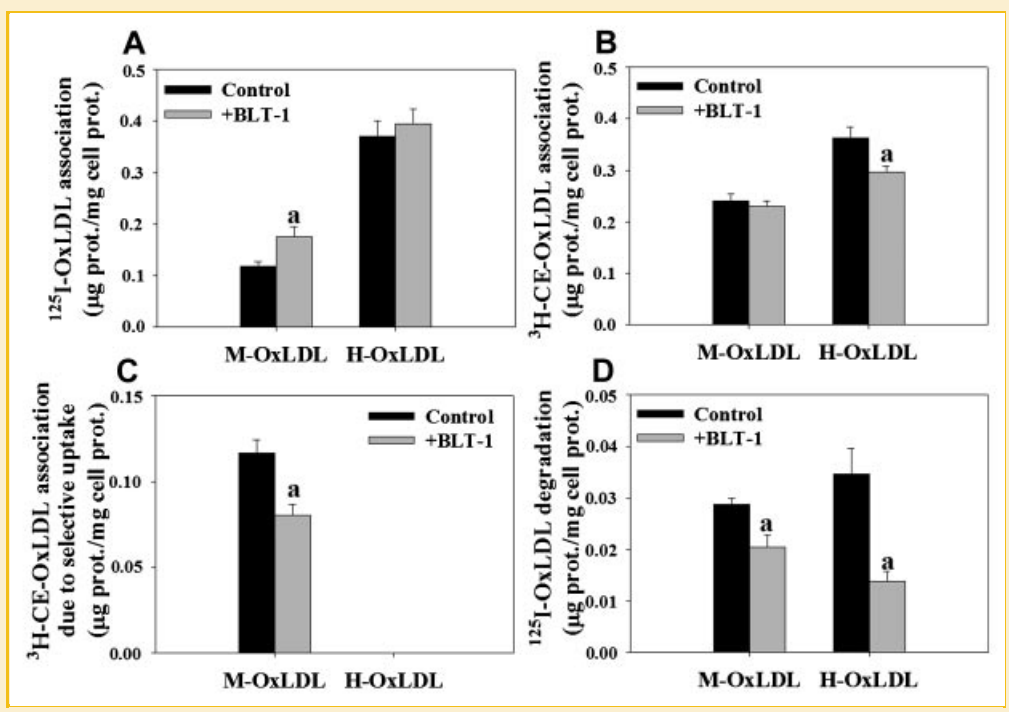

Fig. 2. ^{125}I–OxLDL association (panel A), ^{3}H–CE–OxLDL association (panel B), ^{3}H–CE–OxLDL association due to cholesteryl ester selective uptake (panel C), and ^{125}I–OxLDL degradation (panel D) in HepG2 cells treated or not with BLT-1. Cells were treated or not with 20 μM of BLT-1 for 45 min prior and during the assay and were incubated with either radiolabeled M- or H-OxLDL (20 μg of protein/ml) for 4 h at 37°C in 12-well plates. Nonspecific association of radioactive lipoproteins was determined by the addition of unlabeled OxLDL at 1.5 mg of protein/ml and subtracted from total association. Results are shown as the mean ± SEM of four experiments conducted each in duplicate. [a]Statistically different ($P < 0.05$) from the result obtained without BLT-1.

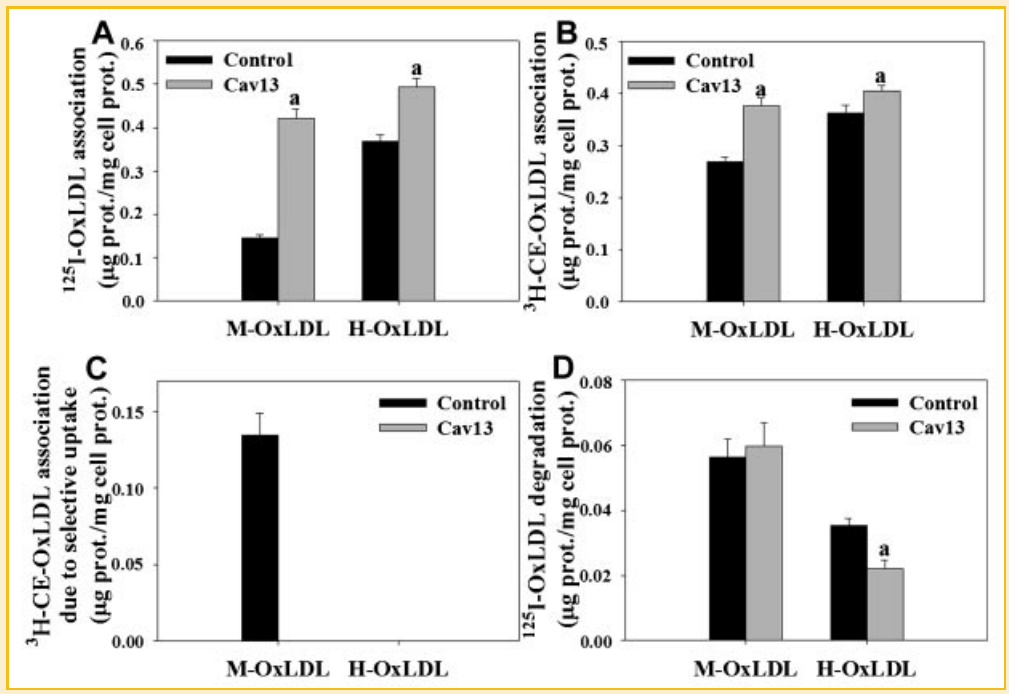

Fig. 3. ^{125}I–OxLDL association (panel A), ^{3}H–CE–OxLDL association (panel B), ^{3}H–CE–OxLDL association due to cholesteryl ester selective uptake (panel C), and ^{125}I–OxLDL degradation (panel D) in control HepG2 cells and Cav13 cells. Cells were incubated with either radiolabeled M- or H-OxLDL (20 μg of protein/ml) for 4 h at 37°C in 12-well plates. Nonspecific association of radioactive lipoproteins was determined by the addition of unlabeled OxLDL at 1.5 mg of protein/ml and subtracted from total association. Results are shown as the mean ± SEM of four experiments conducted each in duplicate. [a]Statistically different ($P < 0.05$) from the result obtained with control HepG2 cells.

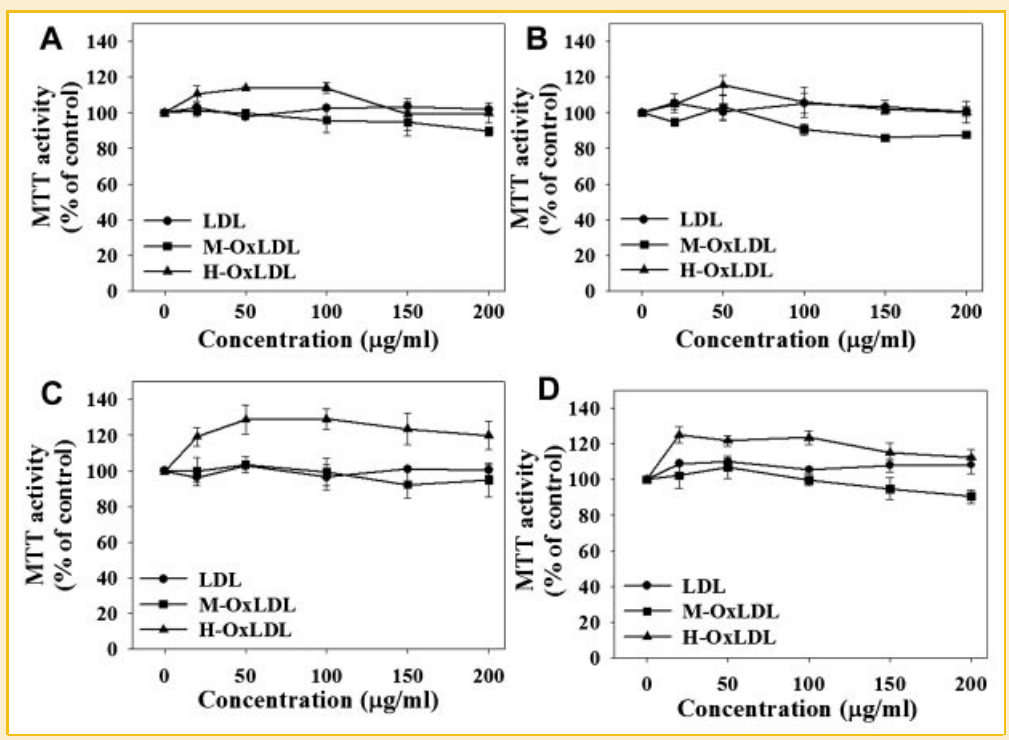

Fig. 4. Effect of LDL and OxLDL on HepG2 cells (panels A and B) and Cav13 cells (panels C and D) MTT activity. HepG2 cells were incubated with increasing concentration (0–200 μg of protein/ml) of LDL, M- and H-OxLDL during 24 (panel A) or 48 h (panel B) in MEM supplemented with 2% LPDS. Cav13 cells were treated similarly (panels C and D). Cell viability was determined by MTT assay and is expressed as the mean ± SEM of three experiments conducted each in quadruplicate. In panel B, statistical differences were found between LDL and M-OxLDL at 150 μg/ml; in panel C between LDL and H-OxLDL at 50–150 μg/ml and in panel D at 20–150 μg/ml.

internalization may either lead to complete degradation of the lipoprotein or the selective delivery of CE from the lipoprotein. As shown in Figure 1C, CE selective uptake is only detectable with M-OxLDL. Increasing the level of CD36 in the cells has no significant effect on M-OxLDL-CE selective uptake, while surprisingly SR-BI overexpression decreases this activity by 66%. The analysis of degradation data revealed that M-OxLDL are more efficiently degraded than H-OxLDL by all cell types. Also, SR-BI+ cells show 32% higher activity of degradation of M-OxLDL than control cells (Fig. 1D) which was not observed with CD36+ cells. This suggests that SR-BI overexpression drives M-OxLDL through a degradation pathway at the expense of the selective uptake pathway. Intriguingly, CD36+ cells show a 33% decrease in H-OxLDL degradation.

Being surprised that SR-BI does not increase CE selective uptake when overexpressed in HepG2 cells, we addressed the effect of BLT-1 an inhibitor of SR-BI CE selective uptake activity. BLT-1 decreases M-OxLDL-CE selective uptake by 32% in normal HepG2 cells (Fig. 2), mainly by increasing protein association (Fig. 2A) as previously shown with HDL [Nieland et al., 2002]. A 29% decrease in M-OxLDL degradation was also detected (Fig. 2D); suggesting that CE selective uptake restriction on M-OxLDL reduces the ability of HepG2 cells to degrade these particles. In contrast, BLT-1 has no effect on H-OxLDL–protein association (Fig. 2A) and decreases CE association by 19% (Fig. 2B) and degradation by 62% (Fig. 2D). However as for the study conducted within Figure 1, no CE selective uptake is detectable for these more oxidized particles (Fig. 2C). Thus, this other strategy points to SR-BI as a mediator of CE selective uptake from M-OxLDL in control HepG2 cells.

To define the effect of caveolin-1 on OxLDL metabolism, comparisons were made between control HepG2 cells and our HepG2 cell line overexpressing caveolin-1, named Cav13 [Truong et al., 2006]. This stable transformant was obtained with an expression vector pRc/CMV containing the full length of human caveolin-1 cDNA. Compared to normal HepG2 cells, these cells show higher BSA uptake and cholesterol efflux, two indicators of functional caveolae. Figure 3A shows that caveolin-1 expression increases the association of M- and H-OxLDL–protein by 166% and 35%, respectively. Similarly, CE associations (Fig. 3B) are higher in Cav13 cells but the increases are less. Panels C and D show that caveolin-1 expression decreases H-OxLDL degradation by 39%, while it abolishes CE selective uptake from M-OxLDL. Moreover, as for CD36 and SR-BI overexpressions, caveolin-1 overexpression does not induce H-OxLDL-CE selective uptake.

No information is yet available on the effects of OxLDL on hepatic cell viability and receptor levels. We therefore conducted these analyses. Moreover, we investigated if caveolin-1 expression aggravates or attenuates these effects. To determine if OxLDL have an impact on cell viability, HepG2 and Cav13 cells were incubated for 24 and 48 h with either LDL, M-, or H-OxLDL (0–200 μg/ml) and MTT assays were conducted. Figure 4A,C shows that LDL and M-OxLDL had no significant effect on the MTT activity of HepG2 and Cav13 cells after 24 h. It takes 48 h and a concentration greater than 100 μg/ml of M-OxLDL to detect a lower MTT activity (Fig. 4B,D). This reduction is, however, limited to less than 12% and occurred only in HepG2 cells. Consequently, the presence of M-OxLDL has little impact on HepG2 or Cav13 cell viability. Differently, H-OxLDL increases MTT activity in these cells, reaching 12–28% increases at 50 μg/ml. In HepG2 cells, this increase is not maintained when concentrations greater than 100 μg/ml H-OxLDL are used. Interestingly, this elevation in MTT activity is greater in Cav13 than in HepG2 cells and at concentrations greater than 100 μg/ml, the higher level of MTT activity is sustained longer in Cav13 than in HepG2 cells.

HepG2 and Cav13 cells were also treated with LDL, M- or H-OxLDL (0–1 mg/ml) for 24 h and their levels of SR-BI, CD36, LDLr, and caveolin-1 were monitored. Figure 5 (panel A) shows a decrease in SR-BI levels when control cells had been treated with M-, H-OxLDL, and particularly with LDL. In these same cells, we also report a diminution of LDLr (panel B) when cells were treated with any LDL subtype, but especially with M- and H-OxLDL. In Cav13 cells, our results indicate that caveolin-1 protects against a reduction in SR-BI (panel A) when treated with any LDL type and also in LDLr (panel B) when treated with M- and H-OxLDL but not LDL. Furthermore, the exposure of HepG2 and Cav13 cells to any of the three LDL types has minimal effect on the expression of CD36 (panel C). Finally, Figure 5 reveals that M- and H-OxLDL, but barely LDL, increase the expression of caveolin-1 (panel D). As this type of experiment reveals the total cell receptor levels, we have also investigated if exposure to LDL or OxLDL affects the membrane

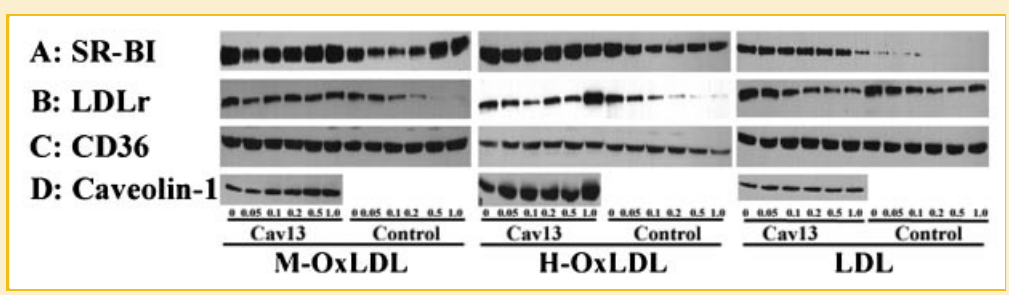

Fig. 5. Expression of SR-BI, LDLr, CD36, and caveolin-1 treated with different concentration (0–1 mg/ml) of LDL, and M-OxLDL and H-OxLDL in control and Cav13 cells. Cells were solubilized as described in Materials and Methods Section and 100 μg of protein were subjected to 10% SDS–PAGE. SR-BI (panel A), LDLr (panel B), CD36 (panel C), and caveolin-1 (panel D) were immuno-detected after transfer to nitrocellulose. As a loading control, the actin level was also measured. The images are representative from one of three independent experiments.

localization of the receptors. Thus, cells were pre-incubated with 100 μg/ml of LDL or OxLDL for 24 h, the HepG2 and Cav13 cell membrane components were fractionated by sucrose gradients in the presence of carbonate and the 12 fractions were analyzed for the occurrence of SR-BI, LDLr, and CD36. As in Rhaindsetal. [2004] we found that in HepG2 cells, SR-BI and LDLr are in low buoyant density fractions (fractions 4 and 5) often described as rafts but we could not detect any displacement of these receptors to other compartments when cells had been pre-treated with LDL or OxLDL (data not shown). However, as shown in Figure 6 (panels A–C), a pre-incubation with M- and H-OxLDL displaces a significant fraction of CD36 (the monomeric 82 kDa protein form as well as the dimeric form) from low to higher buoyant densities, with a greater magnitude for H-OxLDL. LDL had no significant effect (data not shown). This suggests that OxLDL translocate CD36 to intracellular membranes thus reducing the available plasma membrane CD36 for binding activity. The same effect was observed with Cav13 cells, indicating that caveolin-1 expression does not impede or adds to CD36 displacement due to OxLDL (data not shown). However as shown in Figure 6 (panels D–F), the two types of OxLDL particles also induce a displacement of caveolin-1 to higher density fractions in Cav13 cells. Thus, OxLDL reduce the level of expression of SR-BI and LDLr and displace CD36 and caveolin-1 to high-density membrane fractions.

DISCUSSION

Our protocol to oxidize LDL leads to M- and H-OxLDL particles in which approx. 40% and 80% of the three major CE (cholesteryl linoleate, oleate, and palmitate) are oxidized, respectively [Brown et al., 1996]. We chose to follow the selective uptake of unoxidized CE as they constitute a significant fraction of both types of OxLDL and allowed us to address the effects of the oxidized CE content on unoxidized CE selective uptake from the particles. Thus LDL were oxidized to generate either M- or H-OxLDL, which were then labeled. Accordingly, our CE-selective uptake data relate to the fraction of unoxidized CE contained in OxLDL. Our study reveals that M-OxLDL are subjected to CE selective uptake in HepG2 cells, but not H-OxLDL. This is in agreement with our clearance studies in the mouse [Luangrath et al., 2008] where we have attributed the lack of CE selective uptake activity on H-OxLDL to the much faster clearance of H- than M-OxLDL. Accordingly, Figure 1 shows that the association of H-OxLDL–protein with HepG2 cells is 2.4-fold higher than for M-OxLDL. Moreover, we also found that the kinetic of H-OxLDL–protein association is faster than that of M-OxLDL-protein (data not shown). Thus, we propose that the efficient H-OxLDL cell internalization prevents CE selective uptake. Alternatively, it is possible that the more important fraction of oxidized CE in H-OxLDL prevents unoxidized CE selective uptake either by keeping unoxidized CE in the H-OxLDL particles or by being favored by the selective uptake pathway. The latter option is more likely since Fluiter et al. [1999] have shown that the selective uptake of oxidized CE from HDL by the liver is 2.3-fold higher than for unoxidized CE.

We also found that M-OxLDL are more efficiently degraded than H-OxLDL. Several studies with macrophages have reported accumulation of free cholesterol in lysosomes after incubation of cells with OxLDL [Maor and Aviram, 1994; Maor et al., 1995]. Other studies have also shown that OxLDL are resistant to lysosomal enzymes and that OxLDL inactivate lysosomal acid hydrolases [Lougheed et al., 1991; Hoppe et al., 1994]. These observations are therefore in accordance with our results (Fig. 1D). This possible accumulation of H-OxLDL in lysosomes suggested a loss of cell viability as shown by us in osteoblasts [Brodeur et al., 2008], however this was not the case (Fig. 4). Indeed, our results show that HepG2 and Cav13 cell viability is not affected by LDL or M-OxLDL while low concentrations of H-OxLDL induce a superior viability.

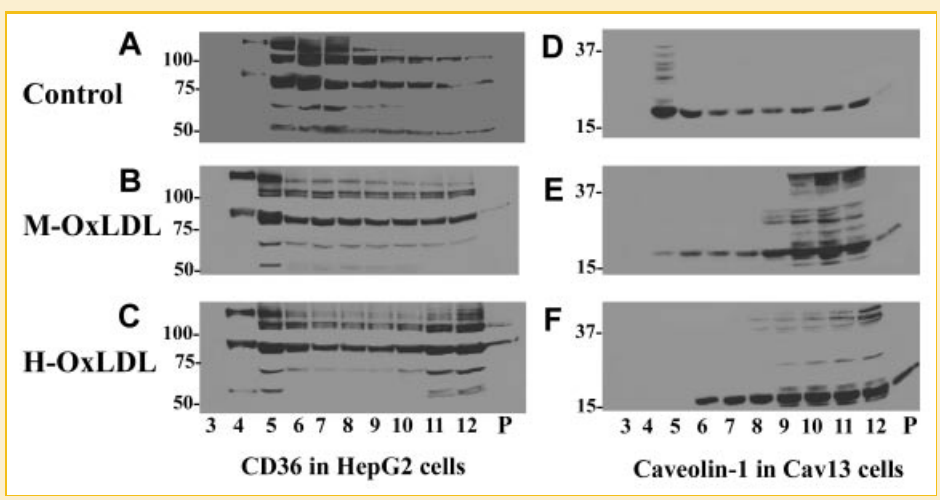

Fig. 6. CD36 and caveolin-1 detection in cell membranes fractionated by discontinuous sucrose gradient. HepG2 and Cav13 cells were pre-incubated for 24 h in the absence (panels A and D) or presence of 100 μg/ml of either M- (panels B and E) or H-OxLDL (panels C and F) and cell proteins were fractionated with detergent-free sodium carbonate discontinuous 5–40% sucrose gradients. The 12 gradient fractions were concentrated with TCA and 50 μl sample of each fraction as well as the pellet (P) were loaded on 10% reducing SDS–PAGE. The expression of different proteins was detected after transfer to nitrocellulose. The images are representative of two independent experiments.

Thus, hepatic cells, or at least HepG2 cells, are resistant to the deleterious effects that OxLDL have on the viability of other cells which can be due to inherent properties of hepatic cells.

Our data (Fig. 1) revealed that SR-BI or CD36 overexpression increases M- and H-OxLDL–protein association, which was expected since OxLDL are known ligands of both receptors [Endemann et al., 1993; Acton et al., 1994]. SR-BI overexpression also decreases M-OxLDL-CE selective uptake and increases degradation. This suggests that SR-BI overexpression drives M-OxLDL through a degradation pathway at the expense of the CE selective uptake pathway. This effect on degradation was indeed shown in COS cells overexpressing SR-BI [Gillotte-Taylor et al., 2001]. However our results obtained with BLT-1 in control HepG2 cells (Fig. 2) points to SR-BI as a mediator of CE selective uptake from M-OxLDL and as a consequence, this receptor would lead to M-OxLDL degradation as well as CE selective uptake. CD36 overexpression has no significant effect on M-OxLDL-CE selective uptake, in accordance with our work conducted in vivo in the mouse [Luangrath et al., 2008]. However, to our great surprise CD36 overexpression in HepG2 cells has no effect on M-OxLDL degradation and moreover decreases H-OxLDL degradation, which is in contradiction with our findings acquired in the mouse revealing that CD36 is responsible for a very significant part of OxLDL clearance. Disparity may be attributed to species differences. Alternatively, it could be that CD36 overexpression in HepG2 cells by increasing OxLDL internalization leads to a greater accumulation in lysosomes and thereby inhibits OxLDL degradation. Overall, these data indicate that in HepG2 cells, SR-BI and CD36 participates in M- and H-OxLDL association, while only SR-BI plays a significant role in M-OxLDL–CE selective uptake and degradation.

The issue of CD36 localization in HepG2 cells deserves attention. CD36 was suggested to require caveolin-1 to be localized at the plasma membrane of mouse embryonic fibroblasts [Ring et al., 2006]. This is in contradiction with our demonstration that although HepG2 cells do not express a detectable level of caveolin-1, a large proportion of CD36 in HepG2 cells is in low density fractions reminiscent of rafts and caveolae (Fig. 6). Moreover, expressing caveolin-1 in these cells does not significantly change this pattern of localization (data not shown). Thus, we rather suggest that the requirement for having CD36 at the plasma membrane is the presence of rafts, at least in HepG2 cells. CD36 translocation is well known in muscle cells where contraction or insulin administration results in its displacement from intracellular stores to the sarcolemma [Bonen et al., 2000]. Interestingly, our results suggest that exposition to OxLDL translocates CD36 to intracellular membranes of HepG2 cells and both CD36 and caveolin-1 in Cav13 cells (Fig. 6). At first sight, it appears that CD36 molecules follow the pool of caveolin-1 and leave low-density fractions when this pool is targeted to another site, in accordance with the conclusion of the study of Ring et al. [2006]. However, we would have to speculate that a small, undetectable, quantity of caveolin-1 in normal HepG2 cells is sufficient to keep CD36 in low-density fractions. Thus, other investigations are required to fully understand CD36 localization and translocation. Nonetheless, we are the first to suggest from our results that OxLDL moves CD36 to intracellular membranes and if this is true, then OxLDL retard their own capture by reducing the pool of CD36 at the hepatic cell surface, aggravating their atherogenic behavior.

A series of experiments was meant to define the effect of an exposure to M- or H-OxLDL on HepG2 cell SR-BI, CD36, and LDLr levels, in order to derive information on the physiological consequences of sustained levels of OxLDL in circulation. A diminution of the LDLr level was detectable when cells were treated with M- and H-OxLDL and less when they were incubated with LDL. The downregulation of the LDLr by LDL has been known for a long time [Brown and Goldstein, 1975], however, to our knowledge it is the first time LDLr levels are studied as a function of a pretreatment with OxLDL. Reductions in SR-BI levels were also observed. LDL was shown to down regulate the expression of SR-BI in rat hepatic cells [Fluiter et al., 1998], while the effect of OxLDL on SR-BI level is in accordance with results of Han et al. [2001] obtained with macrophages. It is, however, the first time that such an effect is reported on hepatic cells. It is tempting to speculate that OxLDL appearing in the blood circulation reduce hepatic LDLr levels and in turn lessen LDL uptake and by doing so increase LDL-cholesterol levels. Moreover by reducing SR-BI levels, OxLDL may impede reverse cholesterol transport by attenuating hepatic HDL-CE selective uptake. Thus our data suggest that apart from their primary deleterious consequences on the vascular wall [Galle et al., 2006], by their effects on key receptors, OxLDL trigger another harmful consequence on atherosclerosis. Although interesting, this possibility requires that, in certain conditions, oxidative stress is maintained.

As reviewed in Frank and Lisanti [2004], caveolin-1 has been suggested to play an important role in the regulation of plasma lipoprotein metabolism. Our data demonstrating that expressing caveolin in human parenchymal HepG2 cells increases M- and H-OxLDL association may explain at least partially why a major part of OxLDL metabolism occurs in the liver through nonparenchymal cells that are known to express greater levels of caveolin than parenchymal cells. Moreover a recent study [Frank et al., 2008] has concluded that caveolin-1 is atherogenic. In this present study, we looked at the effect of hepatic caveolin-1 expression on the metabolism of atherogenic OxLDL. Interestingly, we found three consequences of hepatic caveolin-1 expression that do not support an atherogenic role for caveolin-1 in the liver. Firstly, caveolin-1 expression significantly increases M- and H-OxLDL association with HepG2 cells, suggesting that caveolin-1 expression prevents the movement of these atherogenic particles to endothelial vascular cells. Secondly, it improves viability for cells exposed to H-OxLDL which may relate to the known association of caveolin-1 with cyclins [Nagajyothi et al., 2006]. Thirdly, it prevents the diminution of SR-BI and LDLr levels by M- or H-OxLDL. Thus, although caveolin-1 expression appears to play a pro-atherogenic role in the organism [Frank et al., 2008], its specific overexpression in the liver could be beneficial for the efficient removal of circulating OxLDL and prevention of atherosclerosis development.

In conclusion, our major findings are that SR-BI overexpression drives M-OxLDL through a degradation pathway at the expense of the CE selective uptake pathway, that OxLDL induce the translocation of CD36 to membranes of higher densities and that the expression of caveolin-1 in HepG2 cells increases the association of

both M- and H-OxLDL, decreases CE-selective uptake from M-OxLDL and stabilizes SR-BI when exposed to all three subtypes of LDL and the LDLr when treated with M- and H-OxLDL.

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