Cholesteryl Esters of Aggregated LDL Are Internalized by Selective Uptake in Human Vascular Smooth Muscle Cells

Vicenta Llorente-Cortés, Marta Otero-Viñas, Sandra Camino-López, Paula Costales, Lina Badimon

Objective—Low-density lipoprotein (LDL) receptor-related protein (LRP1) mediates the internalization of aggregated LDL (agLDL)–LDL trapped in the arterial intima bound to proteoglycans—into human vascular smooth muscle cells (VSMC). LRP1-mediated agLDL uptake induces high-intracellular cholesteryl ester (CE) accumulation. The aim of this study was to characterize the mechanism of agLDL internalization in human VSMC.

Methods and Results—The lipidic component of LDL was labeled with [3H] and the apolipoprotein component with [125I].

We found that >90% of intracellular CE derived from agLDL uptake was not associated with apoB100 degradation but was selectively taken up from agLDL. The inhibition of LRP1 expression by small interfering RNA treatment led to a decrease of 80±0.05% in agLDL-CE selective uptake. AgLDL induced intracellular CE accumulation without a concomitant CE synthesis. Cytosolic and cytoskeletal proteins were not required for CE transport. Electron and confocal microscopy experiments indicate that CE derived from agLDL accumulated in adipophilin-stained lipid droplets that were not removable by high-density lipoprotein.

Conclusions—Taken together, these results demonstrate that LRP1 mediates the selective uptake of CE from agLDL and that CE derived from agLDL is not intracellularly processed but stored in lipid droplets in human VSMC. (Arterioscler Thromb Vasc Biol. 2006;26:117-123.)

Key Words: LRP1 ■ selective uptake ■ cholesteryl ester ■ adipophilin ■ aggregated LDL

One of the main events in the atherogenic process is the accumulation of lipids, mainly cholesteryl esters (CEs), in the subendothelial space of the arterial wall.1-3 Macrophages become foam cells through uptake of diversely modified low-density lipoprotein (LDL), whereas aggregation of LDL (agLDL) seems to be a key condition for lipid accumulation in vascular smooth muscle cells (VSMCs).4,5 We have demonstrated previously that the pattern of agLDL internalization differs from that of native LDL (nLDL) in human VSMC. Endocytosed nLDL were found in bright vesicles that were homogenously distributed in the perinuclear space, leading to an unstained cytoplasm surrounding the fluorescent vesicles. In contrast, agLDLs were found in bigger and more diffuse structures distributed throughout the cytoplasm.5 Contrarily to nLDL, agLDL was shown to be a strong inducer of intracellular CE accumulation in human VSMC.5–8 These findings are related to differences in the internalization mechanisms; whereas nLDL is taken up by the endocytic LDL receptor (LDLR), which is downregulated by intracellular cholesterol, agLDL is taken up through LDLr-related protein (LRP1).6,7 Uptake of agLDL through LRP1 allows high-intracellular CE accumulation not only because of its high capacity to bind and internalize agLDL but also because of its transcriptional upregulation by intracellular cholesterol.8 LRP1 collaborates with heparan sulfate proteoglycans (HS-PGs) to mediate the internalization of certain ligands.9,10 However, in human VSMC, we have demonstrated previously that HS-PGs have a minimal contribution to agLDL uptake and that, in the absence of LRP1, VSMCs do not internalize agLDL.11 Although the endocytic mechanism involved in LDLr-mediated nLDL catabolism is well characterized,12 the process involved in LRP1-mediated agLDL internalization is less known. LRP1 has been involved in endocytosis,13 phagocytosis,14 and selective uptake15,16 pathways in different cell types and with different ligands. Besides LRP1, other receptors and molecules, such as scavenger receptor class B, type I (SR-BI),17 lipoprotein lipase,18 hepatic lipase,19 and apolipoprotein E (apoE)16 have been involved in selective uptake. Although it has been studied predominantly with high-density lipoprotein (HDL), selective uptake with LDL-CE has been shown in steroidogenic cells, liver, fibroblasts, and murine macrophage cells.20 The objective of this work was to characterize the mechanism of agLDL internalization in human VSMC. We analyzed: (1) the role of LRP1 and HS-PGs in the process; (2) whether the capacity of agLDL to induce CE accumulation is because of the induction of ACAT esterification; (3) the involvement of lysosomal, cytoskeleton, and kinase proteins on agLDL-derived CE accumulation; and (4) whether the intracellularly accumulated CE is removable by HDL.

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From the Cardiovascular Research Center, CSIC-ICCC, Hospital de la Santa Creu i Sant Pau, Barcelona, Spain.
Correspondence to Lina Badimon, Cardiovascular Research Center, CSIC-ICCC, Hospital de la Santa Creu i Sant Pau, Barcelona, Spain. E-mail lbadimon@csic-iccc.santpau.es
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We demonstrated for the first time that: (1) intracellular CE accumulation in agLDL-exposed VSMC derives from LRP1-mediated agLDL-CE selective uptake; (2) this mechanism, as opposed to endocytic processes, does not involve the proteolytic lysosomal degradation of apoB100 and upregulation of cholesterol esterification rate; (3) different lysosomal, cytosolic, or cytoskeleton proteins fail to induce alterations in agLDL-CE intracellular accumulation, suggesting that these proteins are not strictly required for the intracellular transport of agLDL-CE; and (4) agLDL-CE accumulates in adipophilin-stained, CE-enriched lipid droplets that are not removable by HDL.

Methods
Materials and Laboratory Methods
Methods, LDL, agLDL, and HDL preparation, were performed as described previously, and details are provided in the online supplement. Real-time PCR, Western blot analysis, and free and esterified cholesterol content determination, were performed as described previously, and free and esterified cholesterol content determination, were performed as described previously, and details are provided in the online supplement (which can be accessed at http://atvb.ahajournals.org).

AgLDL Subfractionation
AgLDL was subfractionated using a Cell Sorter (EPICS ALTRA). Two different fractions were collected according to their forward scatter (FS) signal. FS signal is related to particle size. The ratio of cholesterol:protein in total, small, and large aggregates was determined by spectrophotometric methods.

VSMC Culture
Primary cultures of human VSMC were from human coronary arteries of explanted hearts at transplant operations performed at the Hospital de la Santa Creu i Sant Pau. VSMC were isolated by a modification of the explant technique as we described previously. VSMCs were used between passages 2 and 6.

To obtain LRP1-deficient cells [small interfering RNA (siRNA)-LRP1-VSMC], human VSMCs were transiently transfected with annealed siRNA (50 nmol/L). LRP1-specific sense and antisense oligodeoxynucleotides were synthesized by Ambion according to the kit instructions (SilencerTM siRNA Transfection kit; Ambion no. 1630). The cells did not take up trypan blue, and their morphology was not altered by the procedure. Extra wells were used in the experiments to test the inhibition of LRP1 mRNA and protein expression by real-time PCR and Western blot analysis, respectively. HS-PG-depleted VSMCs were obtained by treating cells with heparinase I and heparinase III (HSI&III; 4 U/mL) 2 hours before and during LDL incubation (18 hours). HSI&III treatment was highly effective to degrade HS-PG in human VSMC, as demonstrated previously. LRP1-siRNA-VSMC and HS-PG-depleted VSMCs were incubated with lipoproteins following the specific conditions of the experimental procedure.

To obtain VSMC-overexpressing LRP1, VSMCs were preincubated with insulin (Ins; 10 nmol/L, 1 hour) or with dexamethasone (Dx; 2 µmol/L, 2 hours). Then VSMCs were washed and incubated with agLDL (100 µg/mL) for 18 hours. To analyze the role of phagocytosis on agLDL uptake, VSMC were treated with cytochalasin B (1 µg/mL) for 2 hours. Then, VSMCs were washed and incubated with agLDL (100 µg/mL) for 18 hours.

Cholesterol Esterification Assay
This procedure is discussed in the online supplement.

Determination of Selective CE Uptake from Aggregated LDL
This procedure is discussed in the online supplement.

Immunocytochemistry and Electron Microscopy
These procedures were performed following previously described techniques and discussed in the online supplement.

Statistical Analysis
Data were expressed as mean±SEM. Multiple groups were compared by 1 factor ANOVA. Statistical significance was considered when P<0.05.

Results
Selective Uptake Is Involved in CE-agLDL Internalization and Is Dependent on LRP1 Expression
Two agLDL subpopulations, large and small agLDL, were collected according to their FS signal. The ratio cholesterol:protein was similar in total, small, and large agLDL fractions (Figure 1A). By incubating VSMC with [125I]-agLDL, we observed that most of the intracellular CE (>90% at any agLDL concentration) was not associated with [125I]-apoB100 internalization. Indeed, <10% of the intracellular CE would derive from apoB100-agLDL degradation (Figure 1B). Thus, VSMC seems to acquire most of the CE from agLDL by selective uptake of CE without apoB100 degradation.

The involvement of LRP1 in agLDL-CE selective uptake has been demonstrated by inhibiting and overexpressing LRP1 expression in VSMC. As shown in Figure 2A, siRNA-LRP1-VSMC showed a significant decrease (P<0.05) in LRP1 mRNA expression (78.57±0.73%), but they did not show any significant change in LDLr mRNA expression. In agreement with the effects on LRP1 mRNA expression, siRNA-LRP1 almost completely abrogated LRP1 protein expression (Figure 2B). Both Ins and Dx significantly increased LRP1 protein expression from 2.01±0.25 AU to 3.39±0.3 AU and 2.98±0.4 AU, respectively (P<0.05; Figure 2B). No effect of LRP1 siRNA, Ins, or Dx treatment was observed on α-actinin. siRNA LRP1 inhibition led to agLDL-CE selective uptake decrease from 47.7±2 µg/mg protein to 9.54±0.38 µg/mg protein. On the contrary, Ins and Dx-LRP1 upregulation led to agLDL-CE selective uptake increase from 47.7±2 µg/mg protein to 53±1.22 µg/mg protein and 55±1.11 µg/mg protein, respectively (P<0.05; Figure 2C). In agreement, agLDL-derived CE accumulation was inhibited from 138±2.98 µg/mg protein to 27.08±1.75 µg/mg protein by siRNA-LRP1 and increased to 151±0.77 µg/mg protein and 197±1.90 µg/mg protein, respectively, by Dx and Ins treatment (Figure 2D). No significant alterations on FC content were observed by any treatment.

Cytochalasin B, a phagocytosis inhibitor, increased agLDL-CE selective uptake by 1.47-fold (Figure 1, available online at http://atvb.ahajournals.org) and HS-PG depletion did not lead to significant alterations of CE selective uptake in VSMC (Figure 2C). These results demonstrate a major role for LRP1 but not for HS-PG on agLDL-CE selective uptake in human VSMCs.
Effect of nLDL and agLDL on CE Synthesis and CE Accumulation in VSMCs

VSMCs were incubated overnight with 0.2 mmol/L [14C]-oleate-albumin complex and simultaneously with increasing concentrations of nLDL and agLDL (50, 100, and 200 µg/mL). nLDL induced a moderate CE synthesis, from 0.61 ± 0.11 at 50 µg/mL to 2.18 ± 0.27 nmol/mg protein at 200 µg/mL (Figure II, available online at http://atvb.ahajournals.org). nLDL slightly increased the CE content of VSMCs (from 2.5 ± 0.05 at 50 µg/mL to 10.47 ± 0.04 µg CE/mg protein at 200 µg/mL; Figure II, available online at http://atvb.ahajournals.org). In contrast, agLDL induced a strong intracellular CE accumulation (from 41 ± 0.25 at 50 µg/mL to 100 ± 0.36 µg CE/mg protein at 200 µg/mL; Figure II) concomitantly with a moderate CE synthesis, from 0.27 ± 0.01 at 50 µg/mL to 1.50 ± 0.02 nmol/mg protein at 200 µg/mL (Figure II). Intracellular FC remained unaltered in the presence of nLDL or agLDL in agreement with previous results.5–8

Effect of Lysosomal, Cytoskeleton, and Protein Kinase Inhibitors on agLDL-Derived CE Content of VSMCs

Whereas the lysosomal inhibitor chloroquine induced a 19-fold increase in CE derived from nLDL uptake, it did not significantly increase CE derived from agLDL uptake. A myosin ATPase inhibitor, 2,3-butanedione monoxime, and a phosphatidylinositol 3-kinase inhibitor, wortmannin, increased CE accumulation from nLDL by 1.55-fold and 1.89-fold, respectively. However, these agents did not exert a significant effect on CE accumulation from agLDL uptake. A serine/threonine protein kinase inhibitor (H89) and a specific protein kinase C inhibitor (bisindolylmaleimide) strongly upregulated CE accumulation in nLDL-exposed VSMCs by 4.72-fold and 3.29-fold, respectively. However, they did not significantly alter agLDL-derived CE accumulation. Additionally, 2,3-butanedione monoxime, wortmannin, H-89, and bisindolylmaleimide slightly increased FC in nLDL-exposed VSMCs, whereas they did not exert any effect on FC content of agLDL-exposed VSMCs. A potent tyrosine kinase inhibitor (genistein) did not show any significant effect on nLDL or agLDL-derived CE. Cytochalasin B (a phagocytosis inhibitor) increased CE accumulation from nLDL and agLDL by 2.6-fold and 2.45-fold, respectively (Table).

Fluorescence microscopy experiments were carried out to visualize the pattern of internalization of either 1,1-dioctadecyl-3,3',3'-tetramethylindocarbocyanine (DiI)-nLDL or DiI-agLDL (50 µg/mL) with or without chloroquine (75 µmol/L). After removal of unbound DiI-LDL by extensive washing, the internalized DiI-LDL (during the 4-hour incubation) was observed under fluorescence microscopy. Whereas chloroquine increased DiI-nLDL fluorescence in vesicles, leading to an internalization pattern similar to that observed for agLDL internalization, chloroquine did not apparently change the pattern of agLDL internalization (Figure III, available online at http://atvb.ahajournals.org).
agLDL CE Accumulates in Lipid Droplets That Are Not Removed by HDL

Conventional thin-section electron microscopy showed agLDL entering VSMCs (Figure 3A) and forming enormous lipid-filled vacuoles (Figure 3B). As observed in Figure 3, these lipid vacuoles are surrounded by a membrane that seems to be continuous with that of endoplasmic reticulum. Confocal microscopy (Figure 4A) shows that adipophilin, considered a marker of lipid droplet formation,23,24 colocalizes with the lipid. As shown in Figure 4B, most cytoplasmic lipid vacuoles are positively marked by adipophilin. These results suggest that CE taken through selective uptake from agLDL accumulates in adipophilin-enriched lipid droplets.

To analyze the effect of HDL on agLDL-derived CE, VSMCs were incubated with nLDL or agLDL (100 μg/mL) for 18 hours. VSMCs were then exhaustively washed and incubated with increasing concentrations of HDL (50 and 100 μg protein/mL) for an additional 48 hours. HDL (100 μg/mL) decreased the CE accumulation derived from nLDL uptake from 7.86 ± 0.78 to 3.42 ± 0.26 μg CE/mg protein at 100 μg/mL of HDL. In contrast, HDL did not exert any effect on CE accumulation derived from agLDL (Table I, available online at http://atvb.ahajournals.org). These results indicate that agLDL CE is not susceptible to removal by HDL.

Discussion

AgLDL, contrarily to nLDL, is internalized through LRP1, leading to a high-intracellular cholesterol accumulation in human VSMCs.5–8 Fluorescence microscopy revealed that the agLDL internalization pattern differed from the pattern of cytoplasmic Dil-nLDL internalization in human VSMCs.5 Here, we demonstrated that agLDL, contrarily to nLDL, is not internalized as a whole particle, because agLDL CE is taken up in a dose-dependent manner without the concomitant proteolytic degradation of apoB100. Thus, CEs are selectively taken up from agLDL in human VSMCs. AgLDL CE selective uptake and agLDL CE accumulation are lower in LRP1-deficient VSMCs and higher in VSMCs overexpressing LRP1. These results clearly demonstrate the pivotal role of LRP1 on VSMC-agLDL-CE selective uptake. On the contrary, HS-PG depletion did not exert any significant effect on agLDL CE selective uptake. These results are in agree-
Effect of Several Lysosomal, Cytoskeleton, Protein Kinase, and Phagocytosis Inhibitors on Intracellular Cholesterol Derived from nLDL or agLDL Uptake in Human VSMCs

<table>
<thead>
<tr>
<th>Variable</th>
<th>nLDL FC</th>
<th>nLDL CE</th>
<th>agLDL FC</th>
<th>agLDL CE</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>16.83 ± 2.40</td>
<td>4.56 ± 0.21</td>
<td>19.14 ± 1.30</td>
<td>92.78 ± 14.27</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>14.56 ± 2.52</td>
<td>86.24 ± 5.06*</td>
<td>17.33 ± 1.83</td>
<td>110 ± 3.95</td>
</tr>
<tr>
<td>Genistein</td>
<td>15.33 ± 0.50</td>
<td>4.39 ± 0.40</td>
<td>18.11 ± 2.69</td>
<td>84.92 ± 5.96</td>
</tr>
<tr>
<td>BDM</td>
<td>20.22 ± 0.74</td>
<td>7.08 ± 1.42*</td>
<td>18.45 ± 6.20</td>
<td>82.67 ± 2.96</td>
</tr>
<tr>
<td>Wortmannin</td>
<td>26.30 ± 6.22</td>
<td>8.63 ± 0.51*</td>
<td>19.33 ± 2.14</td>
<td>92.18 ± 6.96</td>
</tr>
<tr>
<td>H-89</td>
<td>23.53 ± 6.93</td>
<td>21.51 ± 6.51*</td>
<td>19.81 ± 3.28</td>
<td>117 ± 7.96</td>
</tr>
<tr>
<td>BDII</td>
<td>20.20 ± 2.02</td>
<td>15.0 ± 7.34*</td>
<td>18.55 ± 2.55</td>
<td>97 ± 7.96</td>
</tr>
<tr>
<td>Cytochalasin B</td>
<td>21.24 ± 0.01</td>
<td>12.02 ± 0.01*</td>
<td>18.69 ± 0.32</td>
<td>228.46 ± 0.32*</td>
</tr>
</tbody>
</table>

VSMCs were incubated without or with the following inhibitors: chloroquine (75 μmol/L), genistein (170 μmol/L), 2,3-butanedione monoxime (BDM; 25 mmol/L), wortmannin (100 nmol/L), H-89 (25 μmol/L), bisindolylmaleimide (BDII; 10 μmol/L), and cytochalasin B (1 μg/mL; 45 min) in the presence of 100 μg/mL of nLDL or agDL. After 18 hours, free cholesterol (FC) and CE content were measured by TLC and expressed by micrograms of CE per milligrams of protein.

*P<0.05: vs the same lipoprotein treatment without inhibitor. Results are expressed as mean ± SEM of 3 experiments performed in duplicate.

ment with a previous study performed by our group in which, by means of biosynthetic labeling of proteoglycans with [35S]-Na2SO4 and specific degradation with HSI&III, we demonstrated that heparinase treatment disrupted the pericellular matrix of VSMCs and specifically degraded HS-PG.11 HS-PG depletion led to a dramatic decrease on agLDL uptake by fibroblasts, but it only slightly decreased agLDL-derived CE accumulation in human VSMCs. Although HS-PGs do not seem to have a significant role on agLDL uptake in VSMCs, Swarnakar et al16 described a pivotal role of PG on the selective uptake of apoE-enriched lipoproteins by LRP1 in adrenocortical cells, and other researchers25–27 described the important role of HS-PGs on the uptake of apoE-enriched lipoproteins in hepatocytes. It has been suggested that the selective uptake of certain LRP1 ligands can be favored by the relative inefficiency of LRP1 as an endocytic receptor compared with the LDLr.28 The pattern of agLDL-CE selective uptake is in agreement with the dose-dependent upregulation of LRP1 expression by agLDL that we have demonstrated previously in human VSMCs.8 Uregulation of the SR-BI receptor, involved in the selective uptake of HDL, has also been described in different hormone-stimulated models.29 The implication of LRP1 in the selective uptake of agLDL-CE in human VSMCs could be determined, at least in part, by the agLDL lipidic structure. The high-CE content in agLDL5,7 could explain why agLDL can induce intracellular CE accumulation without increasing CE synthesis.5–8 Our results demonstrate that CE accumulation not only occurs without lysosomal proteolytic apoB100 degradation, but also without lysosomal CE hydrolysis, indicating that there is no lysosomal processing. A possible loss or redistribution of apoB100 in LDL aggregates during the incubation time cannot be excluded. This mechanism and the requirement of apoB100 for the interaction of agLDL with LRP1 will be analyzed in the near future. In contrast to the inhibitory effect of cytochalasin on agLDL uptake by macrophages,14,30 it seems to have a stimulatory effect on agLDL uptake in human VSMCs. The inhibition of the phagocytic capacity does not interfere on LRP1 functionality. The lack of effect of cytochalasin B and of several lysosomal, cytoskeleton, and protein kinase inhibitors on agLDL uptake in human VSMCs indicates that the mechanism for agLDL uptake in VSMCs

![Figure 4](http://atvb.ahajournals.org/)

Colocalization of lipid droplets and adipophilin in human VSMCs. Confocal microscopy of VSMCs incubated with antibodies antiadipophilin (in green) and Dil (in red). VSMC were incubated with agLDL (100 μg/mL) for 18 hours. Cells were then exhaustively washed, fixed, and incubated with antiadipophilin antibody. During the incubation with the secondary antibody, Dil (1 μg/mL) was added to stain lipid droplets. A, Six of 16 consecutive images obtained by optical sectioning of a cell. B, Photo showing the majority of the lipid vacuoles are positive for adipophilin staining. Photomicrographs are representative of 2 experiments. Colocalization of adipophilin and lipid appears in yellow.
completely differs from that described in macrophages. \(^{30}\) Lysosomal and cytoskeleton proteins are not required for the intracellular transport of agLDL-CE. AgLDL, in contrast with nLDL, is not intracellularly processed in VSMCs, and CE is readily directed to their intracellular sites of storage. Our results agree with those from Reaven et al, \(^{31}\) who demonstrated that, in the selective uptake pathway, lipoprotein-donated CE flow through vesicles or intracellular membrane sheets and their connections. This process takes place without the involvement of cytosolic proteins and is facilitated by the higher capacity of neutral CE to diffuse through membranes compared with highly charged lipids. \(^{32,33}\) This transference of CE from the plasma membranes to intracellular membranes could also be favored by VSMC sphingomyelin enrichment induced by agLDL. \(^{34}\) Indeed, a dynamic relationship between cellular cholesterol and phospholipid content has been described. It seems that cholesterol and sphingomyelin directly interact and that this could contribute to the plasma membrane-bound flow of lipids. \(^{35,36}\) In agreement, electronic microscopy images revealed the continuity between the membrane of lipid vacuoles and the endoplasmic reticulum membrane in agreement with previous results. \(^{37}\) Electronic and confocal microscopy images show that CE selectively taken up from agLDL accumulates in large lipid vacuoles. Confocal microscopy images show the colocalization of adipophilin, a specific marker of lipid droplet formation \(^{21,22}\) and the big lipid vacuoles, indicating that these vacuoles are lipid droplet characteristics of foam cell formation. These results emphasize the capacity of agLDL to induce intracellular lipid accumulation and foam cell formation in VSMCs. Although it has been described that cholesterol efflux from VSMCs was poor compared with other cell types, \(^{38}\) HDL has been able to diminish the increase in intracellular CE content induced by nLDL but not that induced by agLDL. These results confirm the lack of processing of agLDL in VSMCs and are in agreement with previous results in macrophages describing that the stimulation of adipophilin expression by modified LDL promotes cholesterol storage and reduces cholesterol efflux. \(^{39}\) In summary, CE derived from LRP1-mediated agLDL selective uptake and stored in lipid droplets surrounded by adipophilin contributes to the transformation of human VSMCs into foam cells and may, therefore, contribute to atherosclerosis. Additional studies are needed to gain insight into the functional implications of these lipid-rich VSMCs in atherosclerosis plaque progression and its complications.

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References


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Methods

Materials

Cell culture medium and reagents were from Gibco Laboratories (Renfrewshire, UK). 1-[\textsuperscript{14}C]-oleic acid (57 mCi/mmol), iodine-125 and [1\(\alpha\), 2\(\alpha\) (n)-\textsuperscript{3}H]-cholesteryl oleyl ether (43.0 Ci/mmol) were purchased by Amersham (Buckinghamshire, UK). 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (DiI) and Prolonged Gold antifade reagent were purchased from Molecular Probes, Inc. Heparinase I (heparin lyase I, EC 4.2.2.7), heparinase III (heparin lysase III, heparitinase I, EC 4.2.2.8), insulin (Ins) (I9278), dexamethasone (Dx) (D4902) and cytochalasin B (C6762) were from Sigma Chemical Co. Bicinchoninic acid protein assay and iodogen-coated tubs were from Pierce (Rockford, Illinois). Tripure\textsuperscript{TM} Isolation Reagent was from Roche Molecular Biochemicals. Monoclonal antibodies against human LRP1 (\(\beta\)-chain, clone 8B8, RDI 61067) and polyclonal antibodies against adipophilin (GP40, RDI-PROGP40) were purchased from Research Diagnostics, Inc. Monoclonal antibodies against human \(\alpha\)-actinin were purchased from Chemicon International (Mab 1682).

LDL, agLDL and HDL preparation

Human LDL (d\(_{1.019-1.063}\) g/mL) and HDL (d\(_{1.063-1.125}\) g/mL) were obtained from pooled sera of normocholesterolemic volunteers and isolated by sequential ultracentrifugation. Thiobarbituric acid reactive substances (TBARS) were measured as an indirect evaluation of lipid peroxidation. TBARS levels were less than 1.2 mmol malondialdehyde/mg protein LDL or HDL.

LDL were labeled with DiI as previously described.\textsuperscript{5} The lipidic component of LDL was labeled with [\textsuperscript{3}H]-cholesteryl oleoyl ether. In brief, a 50-ml teflon round-bottom
flask was coated with \[^{3}\text{H}\] -cholesteryl oleolyl ether by evaporating 15 ml hexane containing 250 \(\mu\)l of \[^{3}\text{H}\] -cholesteryl oleolyl ether in toluene at 37°C under vacuum. Then, 25 ml of human plasma were added and the flask was rotated in a water bath for 30 hours at 37°C. LDL were isolated by sequential centrifugation and the specific activity was calculated (15-30 cpm/ng protein). Additionally, the apolipoprotein component of LDL was labeled with \[^{125}\text{I}\] using iodogen-coated tubes as follows: a solution of LDL diluted to 1-2 mg/ml in 0.3 M borate buffer, pH 9.0, was placed in IODOGEN-coated tubes, and 0.5 mCi of Na \[^{125}\text{I}\] was added. After incubation (15 min at 22°C with gentle agitation), the solution was transferred to a tube containing 10 \(\mu\)l of 0.1 M sodium bisulfite and dialyzed against 150 mmol/L NaCl containing 0.3 mmol/L EDTA, pH 7.4. \[^{125}\text{I}\] -nLDL or \[^{125}\text{I}\] -agLDL, have a similar specific activity (600-900 cpm/ng protein) and were used within 1 week of iodination. AgLDL (unlabeled or labeled) were prepared by vortexing LDL in PBS at room temperature as previously described.\(^5\text{-}^8\)

**Real time PCR**

Control, siRNA-LRP1-VSMC and HS-PG-depleted cells were washed with cold PBS and total RNA and protein were isolated by Tripure\textsuperscript{TM} Isolation Reagent according to the manufacturer. LRP1 mRNA levels were analyzed by real time PCR. TaqMan fluorescent real-time PCR primers and probes (6’FAM-MGB) for LRP1 and LDLr as previously described.\(^8\) Human gapdh (4326317E) was used as endogenous control.
Determination of free and esterified cholesterol content

Quiescent control VSMC, siRNA-LRP1-VSMC, and Ins, Dx and Cytochalasin B treated VSMC were incubated with agLDL (100 µg/mL) for 18 hours. In some experiments, arrested cells were preincubated with nLDL or agLDL (50 µg/ml) for 18 hours. Medium was then changed and HDL (50 or 100 µg/ml) was added for 48 hours. At the end of lipoprotein incubation period, cells were exhaustively washed, lipid extraction and TLC was performed as previously described. The spots corresponding to free cholesterol (FC) and CE were quantified by densitometry against the standard curve of cholesterol and cholesterol palmitate respectively, using a computing densitometer (Molecular Dynamics).

Cholesterol esterification assay

Arrested VSMC were incubated overnight with 0.2 mmol/L [14C]-oleate-albumin complex performed as previously described and with increasing concentrations of nLDL or agLDL (50, 100 and 200 µg/mL). Cells were then exhaustively washed and harvested into 1 mL of 0.10 mol/L NaOH for lipid extraction. Cellular lipids were extracted and separated by TLC as previously described. The incorporation of labeled oleate into cellular CE was determined by scintillation counting of the scraped CE spot by using OptiScint Hisafe (LKB).

Determination of selective CE uptake from aggregated LDL

Quiescent control VSMC, siRNA-LRP1-VSMC and HS-PG-depleted-VSMC were incubated for 18 hours with [3H]-[125I]-nLDL or [3H]-[125I]-agLDL (100 µg/mL). At the end of the incubation, cells were washed and solubilized in 0.10 mol/L NaOH to
determine cell-associated \[^3\text{H}\]-CE (CEt) and cell-associated \[^{125}\text{I}\]-apoB100. LDL apoB100 degradation was determined by measuring 10% trichoroacetic acid-soluble noniodide \[^{125}\text{I}\] in the culture medium. The relative specific activities of the \[^3\text{H}\]-\[^{125}\text{I}\]-LDL permitted to estimate the cholesterol:apoprotein ratio for nLDL and agLDL. This ratio was used to express the sum of \[^{125}\text{I}\]-degraded and \[^{125}\text{I}\]-cell associated undegraded apoB100 as CE equivalents. CE selective uptake was considered the difference between CEt and CE equivalents.

**Western blot analysis**

SDS/PAGE was run as previously described.\(^7\,\,8\) Blots were incubated with monoclonal antibodies against human LRP1 (β-chain, clone 8B8 RDI 61067, dilution 1:40). To test equal protein loading for the different samples, blots were also incubated with monoclonal antibodies against human α-actinin.

**Immunocytochemistry**

VSMC were seeded on glass bottom sterile culture dish (WPI, Ltd). To analyze LDL binding and internalization, arrested cells were prechilled to 4º C and washed with cold M199 containing 1% BSA. Binding experiments were performed as previously described.\(^5\) VSMC were incubated with 50 µg/mL of DiI-native LDL (DiI-nLDL) or DiI-aggregated LDL (DiI-agLDL) at 4ºC for 30 min. After the binding of the LDL, medium was removed and cells were incubated at 37ºC for 4 h in the absence or presence of chloroquine (75 µmol/L). Cells were then washed in M199-BSA containing 100 U heparin/ml for 15 min at 4º C with constant shaking, fixed at room temperature
for 10 min in PBS containing 3% paraformaldehyde and 2% sucrose and washed twice with PBS.

To analyze adipophilin-staining, VSMC were incubated with nLDL or agLDL for 18 hours. VSMC were then fixed and incubated with primary antibodies against adipophilin. Coverslips were then washed and incubated with secondary antibody (Jackson ImmunoResearch:106-095-006) and with 20 µl of DiI (3 mg/ml) for 1 hour in the dark to stain the lipid droplets. Images of immunostained cells were analyzed on a Leica inverted fluorescence confocal microscope (Leica TCS SP2-AOBS, Wetzlar, Germany). Images were processed with the Leica Standard Software TCS-AOBS.

**Electron microscopy**

For thin-section electron microscopy, VSMC were fixed in glutaraldehyde followed by OsO₄. The cells were dehydrated in graded concentrations of ethanol and embedded in epoxy resin for ultrathin sectioning. Sections were stained with uranyl acetate and lead citrate and observed with a Hitachi HU-600 electron microscope.
Figure legends

Figure I. Effect of cytochalasin B on agLDL CE selective uptake by human VSMC. VSMC were treated with cytochalasin B (1 µg/mL, 2 hours). Cells were then incubated with 100 µg/ml of [³H]-[¹²⁵I]-agLDL for 18 hours. At the end of this period, degraded [¹²⁵I]-apoB100, cell-associated [¹²⁵I]-apoB100 and cell-associated [³H]-CE (CEt) were analyzed as described in Methods. Bar graph showing CEt, the sum of [¹²⁵I]-degraded and [¹²⁵I]-cell associated expressed as CE equivalents using the ratio cholesterol:apoprotein of the LDL, and CE-selective uptake (the difference between CEt and CE equivalents) in control VSMC (open bars) and cytochalasin treated VSMC (black bars). CEt, CE equivalents and CE-selective uptake were expressed as µg CE per miligram cell protein and are shown as the mean of two experiments performed in duplicate (deviations <5% of the mean do not appear in the computer-originated graphs).

Figure II. Effect of nLDL and agLDL on CE synthesis and CE accumulation in VSMC. VSMC were incubated with 0.2 mmol/L [¹⁴C]-oleate-albumin complex and with increasing concentrations of nLDL or agLDL (50, 100, 200 µg/mL) for 18 hours. FC and CE were then extracted and separated by TLC. The incorporation of [¹⁴C]-oleate into cellular CE was determined by scintillation counting of the scraped band. A) Bar graphs showing the quantification of the incorporation of [¹⁴C]-oleate into cellular CE of VSMC exposed to nLDL (open bars) or agLDL (black bars). B) TLC showing the FC and CE bands corresponding to VSMC incubated with nLDL or agLDL and bar graphs showing the CE quantification of nLDL (open bars) or agLDL (black bars) exposed-VSMC. Results concerning CE synthesis are expressed as nmol/mg protein and those
concerning CE accumulation as µg cholesterol/mg protein. Both parameters are shown as mean±SEM of three independent experiments.

**Figure III. Effect of chloroquine on the binding and internalization of DiI-nLDL and DiI-agLDL by VSMC.** VSMC were incubated with DiI-nLDL (A,B) or DiI-agLDL (C,D) (50 µg/ml) for 30 min at 4ºC. Cells were then washed and incubated in absence (A,C) or presence (B,D) of chloroquine (75 µmol/L). After this period, VSMC were washed, fixed and photographed (magnification x 1000).

*Abbreviations:*

AgLDL: aggregated LDL; agLDL-CE: CE from agLDL; CE: cholesteryl esters;
Desamethasone: Dx; FC: free cholesterol; HS-PGs: Heparan sulfate proteoglycans;
Insuline: Ins; LRP1: Low-density lipoprotein receptor-related protein; nLDL: native LDL; TLC: thin layer chromatography; VSMC: vascular smooth muscle cells.
Table I. Effect of HDL on the intracellular cholesterol content derived from nLDL or agLDL uptake in human VSMC.

| HDL (µg/mL) | nLDL     | agLDL   |                  |                  |
|            | FC       | EC      | FC               | EC               |
| 0          | 31.21±2.62 | 7.86±0.78 | 31.81±1.25 | 130±10.2 |
| 50         | 30.47±1.23 | 3.75±0.34* | 32.19±0.62 | 118±7.2 |
| 100        | 33.09±1.75 | 3.42±0.26* | 30.20±1.22 | 128±8.55 |

VSMC were incubated with native LDL (nLDL) or aggregated LDL (agLDL) (100 µg/mL) for 18 hours, then VSMC were incubated with increasing concentrations of HDL (50, 100 µg/mL). After 24 hours, free cholesterol (FC) and cholesteryl ester (CE) content were measured by TLC and expressed by µg CE/mg protein. P≤0.05: * versus the same lipoprotein treatment without HDL. Results are expressed as mean±SEM of two experiments performed in triplicate.
Figure I

Cholesteryl ester content (μg/mg protein)

- Control VSMC
- Cytochalasin B VSMC

CEt Selective uptake (CEt-CEequiv)

VSMC

Cytochalasin B
nLDL  agLDL

LDL (µg/mL)  50 100 200

CE

A

B

Figure II

nLDL  agLDL

LDL (µg/mL)  50 100 200

Cholesteryl \[^{14}C\] oleate synthesis (nmol/mg protein)

Cholesteryl ester accumulation (µg/mg protein)

FC